Handbook of the Biology of Aging

7th Edition

Edited by

Edward J. Masoro and Steven N. Austad

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Foreword

Advances in science and technology in the 20th century reshaped 21st century life in industrialized nations around the world. Living conditions so improved that infant and childhood mortality were profoundly reduced and medical advances in the prevention and treatment of leading causes of death among adults, such as heart disease and cancer, further extended the lives of older individuals. As a result, in the course of a single century, the average life expectancy in developed countries nearly doubled. For the first time in human history, old age became a normative stage in life. Not only are individuals living longer on average, but populations have begun to age as a result of this increase in life expectancy along with a precipitous drop in fertility rates. Countries in the developed world are rapidly reaching the point where there will be more people over 60 than under 15. Thus, the status of older people holds ramifications for the functioning of entire societies.

Even though the near-doubling of life expectancy was a spectacular achievement, there were not concurrent advances in our ability to alleviate the disabling conditions of later life. Nor were there sociological advances to create a world as responsive to the needs of very old people as to the very young. In order to realize the enormous potential of longer life, scientists must come to a more comprehensive understanding of human aging and the social, psychological and biological factors that contribute to optimal outcomes. Along with the phenomenal advances in the genetic determinants of longevity and susceptibility to age-related diseases has come the awareness of the critical importance of environmental factors that modulate and even supersede genetic predispositions. This series provides a balanced perspective of the interacting factors that contribute to human aging.

The Handbooks of Aging series, comprised of three separate volumes, *The Handbook of the Biology of Aging*, *The Handbook of the Psychology of Aging*, and *The Handbook of Aging and the Social Sciences*, is now in its seventh edition and has provided a foundation for an understanding of the issues of aging that are relevant both to the individual and to societies at large. Because discoveries in these fields have been both rapid and broad, the series has played a uniquely important role for students and scientists. By synthesizing and updating progress, they offer state-of-the-art reviews of the most recent advances. By continually featuring new topics and involving new authors, they have pushed innovation and fostered new ideas. With the explosion of information and research on aging in recent decades, there has been a concomitant increase in the number of college and university courses and programs focused on aging and longevity. *The Handbook of Aging* series has provided knowledge bases for instruction in these continually changing fields.

Indeed, *The Handbooks* are resources for teachers and students alike, providing information for didactics and inspiration for further research. Given the breadth and depth of the material covered, they serve as both a source of the most current information and as an overview of the various fields. One of the greatest strengths of the chapters in *The Handbooks* is the synthesis afforded by authors who are at the forefront of research and thus provide expert perspectives on the issues that current define and challenge each field. The interdisciplinary nature of aging research is exemplified by the overlap in concepts in chapters ranging from basic biology to sociology.

We express our deepest thanks to the editors of the individual volumes for their incredible dedication and contributions. It is their efforts to which the excellence of the products is largely credited. We thank Drs. Edward J. Masoro and Steven N. Austad, editors of *The Handbook of the Biology of Aging*; Drs. Sherry L. Willis and K. Warner Schaie, editors of *The Handbook of the Psychology of Aging*; and Drs. Robert H. Binstock and Linda K. George, editors of *The Handbook of Aging and the Social Sciences*. We would also like to express our appreciation to Nikki Levy, our publisher at Elsevier, whose profound interest and dedication has facilitated the publication of *The Handbooks* through their many editions. And, finally, we extend our deepest gratitude to James Birren for establishing and shepherding the series through the first six editions.

Thomas A. Rando, Laura L. Carstensen
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Preface

Our understanding of the biological basis of aging, and how that biology can be manipulated to improve health and longevity, continues to advance at an accelerating pace. This volume, as previous volumes in this series, reviews and synthesizes exciting recent findings and discoveries in the field. However, in this edition, we have also included a more clinically oriented section on advances in understanding of the medical physiology of aging. The volume is primarily directed at basic researchers who wish to keep abreast of new research outside their own subdiscipline and learn about recent clinical findings as well. It is also directed at medical, behavioral, and social gerontologists who wish to learn what the basic scientists and clinicians are discovering. To ensure that the book appeals to a broad audience, we have tried to make all chapters as accessible to the general scientific reader as possible.

Although the book is loosely organized as described above, with one section on basic aging processes and another on the medical physiology of aging, inevitably these levels of analysis blend into each other. For instance, the chapters in the basic biology section on muscle, adipose tissue, and stem cells will have relevance to clinical research. Some of the most exciting recent discoveries have to do with one of the oldest observations in the biology of aging, namely that dietary restriction, which is simply reducing the amount of food eaten, can slow aging and extend life in a wide range of species. The molecular mechanisms by which such a simple intervention has such a stunning effect have eluded researchers for decades, but that is now beginning to change, as will be evident in the first chapter and several succeeding chapters. In addition, long-term experiments on dietary restriction in primates, including humans, are now beginning to bear fruit, as is noted in the chapter on that topic.

Sometimes the true picture of how specific putative mechanisms of aging, such as free radical damage, actually affect life span emerges only after assessing the results of scores of experiments using a range of investigative approaches and animal species. This will be clear after reading the chapters touching specifically on Harman's oxidative stress theory of aging and also in the chapters on conserved longevity-modulating biochemical pathways, such as the TOR, insulin-signaling, and sirtuin pathways.

Our medical physiology section contains several chapters on aging of the human brain. While numerous treatments of that topic cover *diseases* of the aging brain, these chapters deal not only with diseases but also with normal aging changes to cerebral vasculature and myelination as well as the clinical implications of those changes. Additional chapters cover how aging affects central features of human health such as insulin secretion, pulmonary and cardiac function, and our ability to maintain body weight and body temperature. This section is likely to be of interest to physicians who often observe these changes but are unaware of why they occur. Occasionally, it is the most obvious features of a phenomenon that go unnoticed, so we end with a chapter discussing the remarkably consistent difference in longevity between men and women.

As always, a book such as this requires the efforts of many people whose names do not appear in the table of contents. Consequently, we acknowledge our expert reviewers who generously gave us, as well as the authors, constructive suggestions on all the chapters. These reviewers include Gustavo Barja, James Carey, Richard Cawthon, Hae Young Chung, Karen Cullen, Simon Davis, Anthony Donato, Grigori Enikolopov, Sara Espinoza, Malene Hansen, Jeremiah Herlihy, Peter Hornsby, Ting-Ting Huang, Shin-ichiro Imai, Kazuhiro Ito, Jean-Paul Janssens, Joseph Kemnitz, Kevin Kregel, Christiaan Leeuwenburgh, Roger McCarter, Lawrence Mandarino, Richard Morimoto, James Nelson, Frédéric Picard, David Marcinek, Richard Miller, Charles Mobbs, Florian Muller, Eitaro Nakamura, Peter Rabinovitch, Shane Rea, Deborah Roach, Gary Ruvkun, Christian Sell, David Sharp, Anthony Suave, Edith Sullivan, Marc Tatar, Gary Van Zant, Antonio Vidal-Puig, Mingyi Wang, Walter Ward, Joanna Wardlow, and David Waters. We also thank the authors themselves, not only for their contributed chapters, but also for their helpful comments on one another's work.

Edward J. Masoro
Steven N. Austad

About the Editors

Edward J. Masoro

Dr. Masoro is Professor Emeritus in the Department of Physiology at the University of Texas Health Science Center at San Antonio (UTHSCSA), where from September of 1973 through May of 1991 he served as Chairman. He was the founding Director of the Aging Research and Education Center of UTHSCSA, which in 2004 became the Barshop Institute for Longevity and Aging Studies. He now serves as a member of that institute.

Dr. Masoro was the recipient of the 1989 Allied-Signal Achievement Award in Aging Research. In 1990, he received a Geriatric Leadership Academic Award from the National Institute on Aging and the Robert W. Kleemeier Award from the Gerontological Society of America. In 1991, he received a Medal of Honor from the University of Pisa for Achievements in Gerontology, and in 1993, Dr. Masoro received the Distinguished Service Award from the Association of Chairmen of Departments of Physiology. In addition, he received the 1995 Irving Wright Award of Distinction of the American Federation for Aging Research and the 1995 Glenn Foundation Award. He served as President of the Gerontological Society of America from 1994 to 1995, as Chairman of the Aging Review Committee of the National Institute on Aging (NIA), and as Chairman of the Board of Scientific Counselors of the NIA.

Dr. Masoro has held faculty positions at Queen's University (Canada), Tufts University School of Medicine, the University of Washington, and the Medical College of Pennsylvania. Since 1975, Dr. Masoro's research has focused on the influence of food restriction on aging. He has served or is serving in an editorial role for 10 journals and from January 1992 through December 1995, he was the editor of the *Journals of Gerontology: Biological Sciences*.

Steven N. Austad

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Dr. Austad was the recipient of the 2003 Robert W. Kleemeier Award from the Gerontological Society of America. He is also a Fellow of the Gerontological Society of America and a past Chair of the Biological Sciences Section of that organization. He received the Phi Kappa Phi/University of Idaho Alumni Association's Distinguished Faculty Award and the Fifth Nathan A. Shock Award, and he shared the Geron Corporation–Samuel Goldstein Distinguished Publication Award with former graduate student John P. Phelan. Previously, he served on the Science Advisory Board of National Public Radio. He is currently an Associate Editor of the *Journals of Gerontology: Biological Sciences* and a Supervising Editor of *Aging Cell* and Section Editor of the *Neurobiology of Aging*. His trade book, *Why We Age* (1997), has been translated into eight languages. He frequently writes and lectures to the general public on topics related to the biology of aging and ethical issues associated with medically extending life.

Drs. Masoro and Austad previously co-edited the 5th (2001) and 6th (2006) editions of the *Handbook of the Biology of Aging*.

Chapter | 1 |

The Genetic Network of Life-Span Extension by Dietary Restriction

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CHAPTER CONTENTS

Introduction 3
The DR Network in *C. elegans* 4
DR Regimens in *C. elegans* 4
DR Pathways in *C. elegans* 5
Criteria 5
Energy Sensors: Insulin–PI3K, SIR2, AMPK, TOR 9
Transcriptional Regulators: FoxO/*daf-16*, FoxA/*pha-4*, NFE2/*skn-1*, HIF-1, HSF-1,CBP-1 10
Other Genes Important for DR: Mitochondrial Genes, Autophagy Genes 12
DR Nodes Conserved in Other Species 12
Yeast DR: SIR2 and TOR 12
Fly DR: SIR2, TOR, and FoxO 12
DR in Mammals: SIR2, TOR, Insulin–FoxO, NFE2 13
Why are there Differences between DR Regimens? 14
Type of Nutrients 14
Temporal Effects 15
Tissue Specificity 15
Non-DR Parameters 16
DR Mimetics 16
Conclusions 17
References 17

INTRODUCTION

Restriction of nutrients without malnutrition extends life span in a wide range of species (Masoro, 2005). Dietary restriction (DR) does not solely extend life span, it also prolongs the youthful and disease-free period of life by delaying the onset of a number of age-related pathologies, including cancer and neurodegenerative diseases (Maswood et al., 2004; Michels & Ekbom, 2004; Wang et al., 2005). While the effects of DR on longevity and disease prevention have been known for almost a century (Rous, 1914; McCay et al., 1935), the genetic mechanisms by which DR extends life span are just beginning to be deciphered (Mair & Dillin, 2008).

Given the diversity of food regimens in different species, it is not surprising that the DR regimens used in the laboratory vary significantly between species. More surprisingly, there exist radically different methods of implementing DR within the same species (Table 1.1) (Dilova et al., 2007; Greer et al., 2007; Piper & Partridge, 2007; Mair & Dillin, 2008). Whether different methods of DR always alter total caloric content is not entirely clear in invertebrates. Thus, caloric restriction may be one of several ways by which dietary manipulations extend life span. The existence of various DR paradigms raises the important question of whether different DR regimens evoke a single universal "DR pathway" or whether they elicit independent pathways that act in a "DR network," with different hubs of the network synergizing with one another. In this chapter, we focus on the genetic pathways that mediate longevity induced by various DR regimens in *Caenorhabditis elegans* and provide evidence that these pathways are relatively independent, but interact to form a DR network. We also highlight the most conserved nodes of the DR network throughout species. Finally, we provide possible explanations as to why distinct DR regimens trigger different genetic pathways to promote longevity and discuss how these different pathways might be harnessed to mimic DR.

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Table 1.1 Twelve methods of dietary restriction in *C. elegans*

METHOD	MEDIUM	FOOD SOURCE	AGE INITIATED	FERTILITY	% INCREASE[a]	REFERENCES[b]
Axenic	Synthetic liquid	Defined chemical broth	Larval day 4 (L4)	Decrease	50–150%	[1–5]
CDLM	Synthetic liquid	Defined chemical broth	Birth	Decrease	88%	[6]
BDR	Liquid	Live *E. coli* (antibiotics)	Day 2 of adulthood	Decrease	32–101%	[5,7–11]
LDR	Liquid + solid	Live *E. coli* (antibiotics)	L4/young adult	Decrease	28%	[12]
DP	Solid	Live *E. coli*	Birth	Increase	25–33%	[10,13]
sDR	Solid	Live or dead *E. coli*	Day 4 of adulthood	Decrease	18–35%	[10,14,15]
sDR(H)	Solid	Live *E. coli*	Day 2 of adulthood	Decrease	13–18%	[16]
msDR	Solid	Live *E. coli* (antibiotics)	Day 1 of adulthood	Decrease	35–47%	[17]
sDR(C)	Solid	Live *E. coli*	Day 5 of adulthood	NT	23–28%	[11]
IF	Solid	Live *E. coli*	Day 2 of adulthood	NT	30–57%	[16]
BD/DD	Solid	No *E. coli*	Day 2 of adulthood	NT	42–50%	[18–22]
eat-2 mutation	Solid	Live *E. coli*	Birth	Decrease	0–57%	[3,5,9–11,15,17–19,22–32]

[a] Different laboratories calculate life span starting at different ages (birth vs young adult), which affects the total % increase.

[b] [1] Vanfleteren & Braeckman, 1999; [2] Houthoofd et al., 2002; [3] Houthoofd et al., 2002; [4] Houthoofd et al., 2003; [5] Zhang et al., 2009; [6] Szewczyk et al., 2006; [7] Klass, 1977; [8] Johnson, 1990; [9] Panowski et al., 2007; [10] Greer & Brunet, 2009; [11] Carrano et al., 2009; [12] Bishop & Guarente, 2007; [13] Hosono et al., 1989; [14] Greer et al., 2007; [15] Park et al., 2009a; [16] Honjoh et al., 2009; [17] Chen et al., 2009; [18] T. L. Kaeberlein et al., 2006; [19] Lee et al., 2006; [20] Steinkraus et al., 2008; [21] Smith et al., 2008; [22] Mehta et al., 2009; [23] Lakowski & Hekimi, 1998; [24] Wang & Tissenbaum, 2006; [25] Curtis et al., 2006; [26] Henderson et al., 2006; [27] Hansen et al., 2005; [28] Hansen et al., 2007; [29] Hansen et al., 2008; [30] Iser & Wolkow, 2007; [31] Jia & Levine, 2007; [32] Hsu et al., 2003.

THE DR NETWORK IN *C. ELEGANS*

DR Regimens in *C. elegans*

C. elegans normally live in the soil and feed on bacteria, for example, those present on rotten fruits. In the laboratory, worms are traditionally grown on a thin film of *Escherichia coli* bacteria spread on solid agar plates. Twelve DR regimens have been developed in *C. elegans,* and they all extend life span, albeit to different degrees (Table 1.1).

One of the most commonly used methods to mimic DR is a genetic mutation (*eat-2*) that reduces the pharyngeal pumping rate of the worms, thereby leading to reduced nutrient consumption (Avery, 1993; Lakowski & Hekimi, 1998) (Table 1.1). In addition to this genetic way of inducing DR, there are four DR methods in which the source of nutrient is altered in liquid media (Table 1.1). While liquid cultures are not the typical conditions for growing worms, they allow an easier manipulation of the nutrients. The most frequently used liquid method of DR, devised by Klass in 1977, consists in simply diluting *E. coli* bacteria in liquid cultures and has been termed bacterial DR (BDR) (Klass, 1977; Houthoofd et al., 2003; Panowski et al., 2007). Importantly, the worms do not compensate for the dilution in bacteria by eating more in this DR regimen (Mair et al., 2009). This BDR method has been further refined to include both a solid support and a liquid dilution of bacteria, and we will term this DR regimen LDR, for liquid DR, in this review (Bishop & Guarente, 2007). DR-like phenotypes can also be induced by two chemically defined liquid media: the axenic medium and the chemically defined liquid medium (CDLM) (Vanfleteren & Braeckman, 1999; Houthoofd et al., 2002; Szewczyk et al., 2006).

Seven methods of DR have been implemented on solid agarose-containing plates—the more traditional way of growing *C. elegans* in the laboratory (Table 1.1). In an initial method devised in the 1980s, the reduction of bacteria is obtained by the dilution of peptone in the agarose (Hosono et al., 1989). However, peptone dilution leads to an increase in worm fertility, which contrasts with the conserved ability of DR to decrease reproduction, raising the possibility that peptone dilution is not a bona fide DR regimen. Later, another method of DR on agarose plates, termed solid DR (sDR), was developed (Greer et al., 2007). In this method, worms are exposed to serial dilutions of feeding bacteria on agarose plates at day 4 of adulthood, which corresponds to the very end of the reproductive period in the worms (Greer et al., 2007; Greer & Brunet, 2009; Park et al., 2009a). sDR does not cause the worms to eat more to compensate for the lack of food (Greer et al., 2007). Note that sDR is provided in the absence of 5-fluoro-2′-deoxyuridine (FUdR), a drug often used in worm life-span assays to facilitate adult worm counting by inhibiting progeny production. Three variants of sDR have been implemented. In the first of these, termed modified sDR (msDR), bacteria are also serially diluted on plates, but restriction is initiated at day 1 of adulthood and FUdR is used to inhibit progeny production (Chen et al., 2009). In the second variation of sDR, which we will term sDR(H) in this review, restriction is initiated at day 2 of adulthood, and FUdR is also used (Honjoh et al., 2009). In the third variation of sDR, which we will term sDR(C) in this review, restriction is initiated at day 5 of adulthood and FUdR is also used (Carrano et al., 2009). Importantly, the total absence of bacteria on plates (bacterial deprivation, BD, or dietary deprivation, DD) in the presence of FUdR also extends life span (T. L. Kaeberlein et al., 2006; Lee et al., 2006). BD/DD has also been shown to extend the life span of wild-derived *C. elegans* strains, as well as *C. remanei* strains (Sutphin & Kaeberlein, 2008). Finally, the most recently devised method to induce DR in worms involves feeding worms only once every 2 days in the presence of FUdR and has been termed intermittent fasting (IF) (Honjoh et al., 2009).

It is important to note that in all DR methods—with the exception of sDR—worms are treated with FUdR, a compound that inhibits DNA synthesis and can even extend life span in some cases (Mitchell et al., 1979). FUdR is used because it makes counting worms easier by inhibiting the production of new progeny. However, progeny production is an energy-costly biological process that can be linked with life span, so altering this process by adding FUdR may affect the response of somatic cells to DR. On the other hand, FUdR could also help reveal a longevity phenotype in specific cases (Shaw et al., 2007), because FUdR limits matricide (the hatching of worms inside the mother), which is a confounding factor for worm life span (Mitchell et al., 1979). In addition FUdR slows the growth of bacteria on the agar plates (Bertani & Levy, 1964) and may thereby alter the food concentration for worms. Thus, there is a complex interaction between FUdR, life span, and bacteria, which should be taken into account when results are compared across various methods of DR.

DR Pathways in *C. elegans*

Studies using a variety of DR regimens in *C. elegans* have uncovered a growing number of genes that mediate the beneficial effects of DR on life span (Table 1.2, Figure 1.1). These DR genes encode proteins that act as energy sensors, transcriptional regulators, mitochondrial components, and autophagy proteins. We will discuss each of these in turn using a series of defined criteria.

Criteria

In presenting results from many different groups, it is important to consider the terminology. In this review, we will write that a DR method is *dependent* (D in Table 1.2) on gene X or that gene X is *necessary* for longevity induced by a DR method when the ablation of gene X completely blocks the effects of dietary restriction on life span. We will write that a DR method is *partially dependent* (PD in Table 1.2) on gene X or that gene X is *partially necessary* for longevity induced by a DR method to indicate that inhibition or ablation of the gene partially, but significantly, blocks the protective effects of dietary restriction. Note that some groups have termed this second category "not dependent" (or "not entirely dependent") on gene X (Mair & Dillin, 2008). Finally, we will write that a method is *independent* (I in Table 1.2) of gene X or that gene X is *not necessary* for longevity induced by a DR method when the inhibition or ablation of the gene has no significant effect on the protective effects of dietary restriction.

Second, it is important to highlight that a gene could be *necessary* for DR but not *mediate* DR. An example would be a gene that is important for the general health of the organism such that when the gene is abolished, DR can no longer exert its beneficial effect on life span. Thus, we will use the term *mediate* whenever a gene fits a series of criteria: (1) there is evidence that the gene or the gene product is

Table 1.2 Genes necessary for life-span extension by the various DR regimens in *C. elegans*

	METHOD											
	eat-2	**sDR**	**IF**	**BDR**	**DD**	**AM**	**msDR**	**LDR**	**DP**	**sDR(H)**	**sDR(C)**	**CDLM**
daf-16	I[1]	D[4,18]	PD[19]	PD/I[4,6,11,20]	I[21,22]	I[20]	I[14]	I[26]	D[4]	ND	ND	ND
daf-2	D/I?[1,2]	I[18]	PD[19]	I[20]	I[21,22]	I[20]	ND	I[26]	ND	ND	ND	ND
aak-2	I[3,4]	D[4,18]	I[19]	PD/I[4,27]	ND	ND	ND	ND	D[4]	ND	ND	ND
skn-1	D[5]	I[4]	I[19]	ND	ND	ND	ND	D[26]	ND	ND	ND	ND
pha-4	D/I?[4,6]	I[4]	I[19]	D[6]	ND	ND	ND	ND	ND	ND	ND	ND
hsf-1	I[7]	I[4]	PD[19]	D[11]	D[23]	ND	ND	ND	ND	ND	ND	ND
sir-2.1	D/I?[8,9]	I[4]	I[19]	I[27]	I[21,22]	ND	ND	ND	ND	ND	ND	ND
clk-1	D[1]	D[4]	PD[19]	ND	ND	ND	ND	ND	ND	ND	ND	ND
wwp-1	D[10]	ND	ND	PD[10]	ND	ND	ND	ND	ND	ND	D[10]	ND
cbp-1[a]	D[11]	ND	ND	PD[11]	ND	D[11]	ND	ND	ND	ND	ND	ND
let-363[a]	D/I?[9,12]	ND	PD[19]	ND	ND	ND	ND	ND	ND	D[19]	ND	ND
eat-2	—	I[4]	ND	ND	D[21,22]	I[25]	ND	ND	ND	ND	ND	ND
hif-1	I[13]	ND	ND	ND	I[24]	ND	PD[14]	ND	ND	ND	ND	ND
rheb-1[a]	ND	ND	D[19]	ND	ND	ND	ND	ND	ND	D[19]	ND	ND
egl-9	D[14]	ND	ND	ND	ND	ND	PD[14]	ND	ND	ND	ND	ND
cup-4	Db[5]	PD[5]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
nlp-7	Db[5]	PD[5]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

bec-1[a]	D[15,16]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
vps-34[a]	D[16]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
atg-7[a]	D[15]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ubc-18[a]	D[10]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
sams-1[a]	D[17]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
rab-10[a]	D[17]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
drr-1[a]	D[17]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
drr-2[a]	D[17]	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
ire-1	ND	ND	ND	ND	ND	ND	PD[14]	ND	ND	ND	ND	ND
hsp-12.6	ND	ND	PD[19]	ND	ND	ND	ND	ND	ND	ND	ND	ND
dve-1[a]	ND	ND	ND	Db[11]	ND	ND	ND	ND	ND	ND	ND	ND
age-1	ND	ND	ND	I[28]	ND	ND	ND	ND	ND	ND	ND	ND
aak-1	ND	ND	ND	I[27]	ND	ND	ND	ND	ND	ND	ND	ND
sir-2.3	ND	ND	ND	I[27]	ND	ND	ND	ND	ND	ND	ND	ND

Summary of the genes that have been tested for longevity by specific DR regimens. D, dependent; PD, partially dependent; I, independent; ND, not determined. Bold, confirmed null mutants. ?, conflicting reports in the literature. The DR methods are *eat-2,* (Lakowski & Hekimi, 1998); sDR, solid dietary restriction (Greer et al., 2007); IF, intermittent fasting (Honjoh et al., 2009); BDR, bacterial dietary restriction in liquid (Klass, 1977); BD/DD, bacterial deprivation or dietary deprivation (T. L. Kaeberlein et al., 2006; Lee et al., 2006); AM, axenic medium (Vanfleteren & Braeckman, 1999); msDR, modified sDR (Chen et al., 2009); LDR, liquid dietary restriction (Bishop & Guarente, 2007); DP, dilution of peptone (Hosono et al., 1989); sDR(H), a modified version of sDR (Honjoh et al. 2009); sDR(C), a modified version of sDR (Carrano et al., 2009); CDLM, chemically defined liquid medium (Szewczyk et al., 2006). [1] Lakowski & Hekimi, 1998; [2] Iser & Wolkow, 2007; [3] Curtis et al., 2006; [4] Greer & Brunet, 2009; [5] Park et al., 2009; [6] Panowski et al., 2007; [7] Hsu et al., 2003; [8] Wang & Tissenbaum, 2006; [9] Hansen et al., 2007; [10] Carrano et al., 2009; [11] Zhang et al., 2009; [12] Henderson et al., 2006; [13] Mehta et al., 2009; [14] Chen et al., 2009; [15] Jia & Levine, 2007; [16] Hansen et al., 2008; [17] Hansen et al., 2005; [18] Greer et al., 2007; [19] Honjoh et al., 2009; [20] Houthoofd et al., 2003; [21] T. L. Kaeberlein et al., 2006; [22] Lee et al., 2006; [23] Steinkraus et al., 2008; [24] Mehta et al., 2009; [25] Houthoofd et al., 2002; [26] Bishop & Guarente, 2007; [27] Mair et al., 2009; [28] Johnson et al., 1990.

[a] RNAi, making results more difficult to interpret.

[b] Mutation or knockdown of the gene leads to a reduction in life span in response to DR that is similar to that under ad libitum conditions.

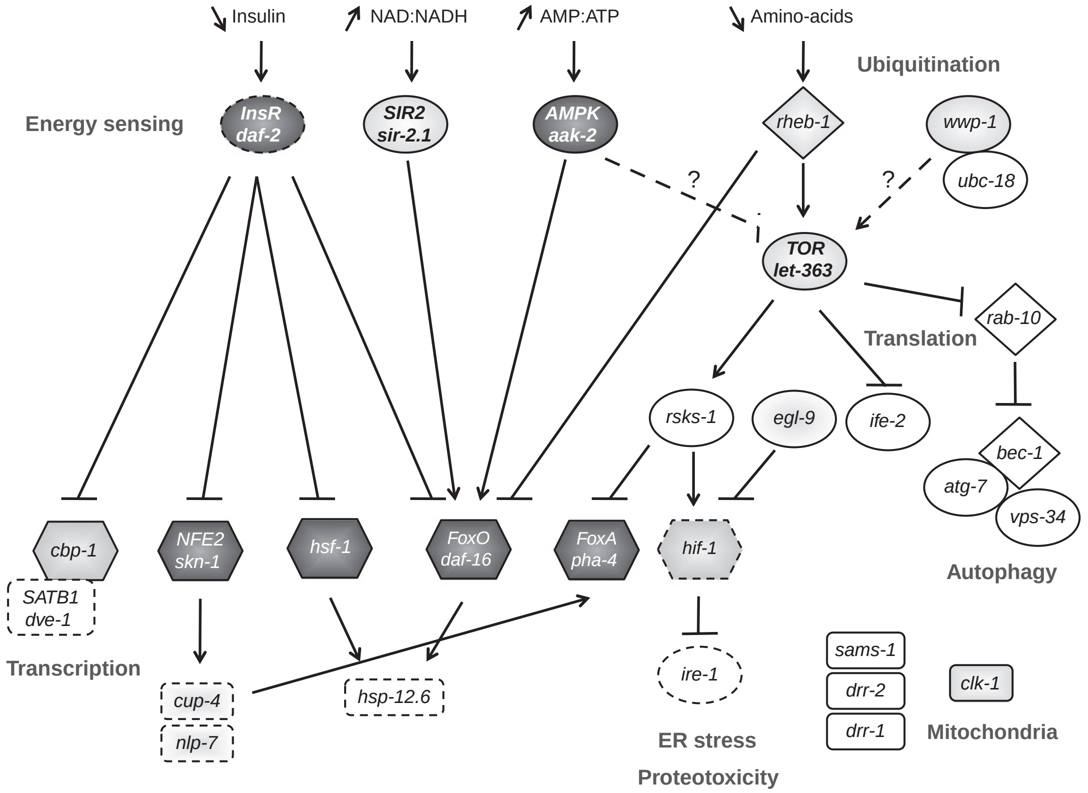
Insulin
NAD:NADH
AMP:ATP
Amino-acids
Ubiquitination
Energy sensing
InsR
daf-2
SIR2
sir-2.1
AMPK
aak-2
rheb-1
wwp-1
ubc-18
?
?
TOR
let-363
rab-10
Translation
rsks-1
egl-9
ife-2
bec-1
atg-7
vps-34
cbp-1
NFE2
skn-1
hsf-1
FoxO
daf-16
FoxA
pha-4
hif-1
Autophagy
SATB1
dve-1
Transcription
cup-4
nlp-7
hsp-12.6
ire-1
sams-1
drr-2
drr-1
clk-1
ER stress
Mitochondria
Proteotoxicity

Figure 1.1 *C. elegans* dietary restriction network. Circles, enzymes; hexagons, transcriptional regulators; diamonds, small G proteins; rectangles, other proteins. Dark gray, genes tested in five to nine DR methods; light gray, genes tested in two to four DR methods; white, genes tested in one DR method. Dotted lines, predicted interactions or pathways that have been shown in other organisms; dotted shapes, genes that are partially necessary in all methods tested or that have a similar effects on life span under DR and under ad libitum conditions when mutated or knocked down. Note that some results are controversial (details are included in Table 1.2).

regulated by DR itself; (2) the gene is at least partially necessary for the extension of life span (i.e., there is a significant difference in the way mutations in the gene affect life span under ad libitum conditions versus DR conditions in the same experiment); and (3) the ablation/inhibition of the gene specifically affects longevity in response to DR, but not in response to other longevity extension pathways.

Third, it is crucial to note that establishing the requirement of a gene in longevity by DR is difficult outside of the clear-cut case of a genetic null mutant (Gems et al., 2002). Hypomorph mutants, RNAi knockdown, or ectopic expression of a gene may all lead to faulty interpretations. The case of RNAi knockdown is further confounded by the fact that RNAi does not knock down genes uniformly in all cells in the worms—for example, neurons are quite resistant to the effect of RNAi in worms (Timmons et al., 2001). If DR still further extends the life span of a hypomorph mutant, or in the case of RNAi knockdown or of overexpression of gene X, which would normally suggest that this gene is not required for DR, this gene could in fact still be necessary for DR if it were completely abolished or overexpressed to the maximal amount. We indicate, in the text and in the figures, when hypomorph mutants, RNAi, or overexpressors have been used to draw conclusions on the implications of specific genes in the DR response.

Finally, examining the implication of a gene in DR using only two conditions (ad libitum and DR) may be misleading, as mutating a gene may only displace the optimum concentration at which DR is reached (Clancy et al., 2002; Mair et al., 2009). Methods that involve dilution of bacteria, such as BDR and sDR, allow the study of a gene mutation over a graded dilution of bacteria. To examine the specificity of the interaction between a gene and DR, it is also important to test if the effects of a specific knockdown or genetic mutation on longevity under DR conditions versus ad libitum conditions are statistically different (Gems et al., 2002). The interaction between food concentration and genotype can be assessed statistically by using proportional hazards regression tests (Tatar, 2007).

Energy Sensors: Insulin–PI3K, SIR2, AMPK, TOR

Insulin levels are potently regulated by nutrients, raising the possibility that the insulin signaling pathway—a well-known regulator of aging (Kenyon, 2005)—may play a role in mediating DR. While insulin levels are indeed decreased by DR in mammals (Ramsey et al., 2000), it is not clear whether insulin-like peptides or the activity of the insulin signaling pathway is decreased by DR in *C. elegans.* FoxO/*daf-16* nuclear translocation, which is a consequence of the inactivation of the insulin signaling pathway, does not appear to be affected by many DR methods (Henderson & Johnson, 2001; Houthoofd et al., 2003; Greer et al., 2007), although starvation and IF both trigger FoxO/*daf-16* nuclear translocation (Henderson & Johnson, 2001; Honjoh et al., 2009). Mutants of the insulin receptor *daf-2* or of *age-1*, which encodes the *C. elegans* ortholog of the catalytic subunit of PI3K, still display an extension of life span in response to BDR that is equivalent to that of the wild type (WT) (Johnson et al., 1990; Houthoofd et al., 2003). Such a result suggests that *daf-2* and *age-1* are not necessary for BDR to extend life span. Similarly, *daf-2* mutants still display extension of life span to the same extent as WT in response to sDR (Greer et al., 2007), *eat-2* (Lakowski & Hekimi, 1998), and BD/DD (T. L. Kaeberlein et al., 2006; Lee et al., 2006). Axenic medium and a modified form of BDR, termed LDR in this review, also extend the life span of *daf-2* worms significantly more than that of WT worms (Houthoofd et al., 2003; Bishop & Guarente, 2007). Consistent with a limited involvement of the insulin signaling pathway in life-span extension by DR, the life span of FoxO/*daf-16* null mutant worms is still increased by many methods of DR (Lakowski & Hekimi, 1998; Houthoofd et al., 2003; T. L. Kaeberlein et al., 2006; Lee et al., 2006; Bishop & Guarente, 2007; Chen et al., 2009; Zhang et al., 2009). However, interestingly, FoxO mutants no longer show life-span extension in response to sDR (Greer et al., 2007; Greer & Brunet, 2009) and have a decreased ability to show life-span extension in response to BDR (Houthoofd et al., 2003; Panowski et al., 2007; Greer & Brunet, 2009). In addition, because the *daf-2* and the *age-1* mutants are hypomorph mutants, it is possible that the DR and insulin pathways interact more than was initially thought. Intriguingly, the life span of one allele of *daf-2*, *daf-2(e1368)*, was not at all extended by an alternate *eat-2* mutation, suggesting that life-span extension due to *eat-2* may also involve the insulin pathway under some conditions (Iser & Wolkow, 2007). In addition, the life span of *daf-2* mutants is only mildly increased by IF (10.2% life-span extension) compared to the dramatic effect IF has on WT worms (56.6% life-span extension) (Honjoh et al., 2009). Taken together, these data indicate that the insulin pathway probably plays some role in the response to DR, even though this pathway does not seem to be the major mediator of longevity by DR.

SIR-2.1 is a NAD-dependent protein deacetylase of the Sirtuin family and has been proposed to sense the metabolic state of a cell, in part via changes in the NAD:NADH ratio (Guarente, 2005). Whether SIR2 activity or levels are affected by DR methods in worms has never been tested. The *sir-2.1* gene was found to be necessary for longevity triggered by the *eat-2* mutation, a genetic mimic of DR (Wang & Tissenbaum, 2006). However, the role of SIR-2.1 in DR-induced longevity is still controversial, as another study has shown that the *sir-2.1* gene is not necessary for longevity induced by *eat-2* (Hansen et al., 2007). The basis for this discrepancy is not known, but may be due to the fact that ad libitum conditions might already activate the endogenous SIR-2.1 protein under some circumstances, thereby masking the effect of SIR-2.1 in longevity induced by the *eat-2* mutation. Notwithstanding these differences, the involvement of SIR-2.1 in DR-induced longevity in worms is relatively limited, in that SIR-2.1 is not necessary for longevity induced by sDR, DD, BDR, and IF (T. L. Kaeberlein et al., 2006; Lee et al., 2006; Greer & Brunet, 2009; Honjoh et al., 2009; Mair et al., 2009). Thus, while SIR-2.1 may be a node of the DR network in worms, it is not a major mediator of DR-induced longevity in this organism. It is possible that the other SIR2 family members in worms may play a role in DR-induced longevity. A double mutant of *sir-2.1* and *sir-2.3* responded to BDR similar to wild-type worms (Mair et al., 2009), but *sir-2.2* and *sir-2.4* have not been tested yet for their role in life-span extension by DR.

The energy-sensing AMP-activated protein kinase (AMPK) is a protein kinase that is activated by low energy levels and by a variety of stimuli that increase the AMP:ATP ratio in cells. The AMP:ATP ratio is increased in response to a variety of DR methods in worms (Greer et al., 2007; E.G. and A.B., data not shown), suggesting that AMPK is activated by nutrient restriction in this organism. One of the two catalytic subunits of AMPK (*aak-2*) has been found to be necessary for longevity induced by sDR in worms (Greer et al., 2007; Greer & Brunet, 2009). In contrast, AMPK is not necessary at all for the longevity induced by *eat-2* (Curtis et al., 2006; Greer & Brunet, 2009) or for longevity induced by IF (Honjoh et al., 2009). Finally, AMPK/*aak-2* mutant worms still show some life-span extension in response to BDR (Greer & Brunet, 2009; Mair et al., 2009), but to a lesser extent than WT worms, at least in one study (Greer & Brunet, 2009), suggesting that AMPK is partially necessary for longevity by this liquid DR regimen. A mutant of both AMPK catalytic subunits (*aak-1*/*aak-2*) still displays life-span extension by BDR in worms (Mair et al., 2009), suggesting that AMPK catalytic activity is not absolutely essential for this method of DR to extend life span. It is possible, however, that other AMPK family members compensate for the absence of both *aak-1* and *aak-2* in worms. These studies indicate that AMPK is a node of the DR network, but that it is not the only way in which DR is mediated in worms.

TOR is a central nutrient-sensing molecule involved in the regulation of cell growth, protein translation, and autophagy (Sarbassov et al., 2005). The activity of the TOR pathway in response to DR has not been examined yet in worms, even though *eat-2* mutants have reduced protein translation levels (Hansen et al., 2007), suggesting that the TOR pathway is probably inhibited by this DR method. Reduction in TOR expression by RNAi is sufficient to extend life span (Vellai et al., 2003; Henderson et al., 2006; Hansen et al., 2007). Interestingly, the life-span extension induced by reducing TOR signaling is not further enhanced by the *eat-2* mutation, indicating that TOR is necessary for *eat-2*-induced longevity (Hansen et al., 2007), although these experiments are difficult to interpret because RNAi is used to knock down TOR. The importance of TOR in *eat-2*-induced longevity is still controversial, as another group has reported that TOR is not necessary for life-span extension by *eat-2* (Henderson et al., 2006). Intriguingly, TOR is also partially required for longevity induced by IF and for one of the three modified versions of sDR, sDR(H) (Honjoh et al., 2009). Consistent with the importance of TOR in DR, knockdown of the GTPase RHEB-1, which is known to regulate TOR activity in other species (Saucedo et al., 2003; Stocker et al., 2003; Long et al., 2005), does not display any increase in longevity in response to IF or to sDR(H) (Honjoh et al., 2009), indicating that RHEB-1 is necessary for longevity induced by these methods of DR. While the involvement of TOR and RHEB-1 in longevity induced by other methods of DR has not yet been tested, the TOR pathway appears to be important for longevity by DR.

Transcriptional Regulators: FoxO/*daf-16*, FoxA/*pha-4*, NFE2/*skn-1*, HIF-1, HSF-1, CBP-1

DR induces large-scale changes in gene expression (Lee et al., 1999), which probably reprograms cells to the new energy status. Consistent with this effect of DR on gene expression, a large number of transcriptional regulators have been shown to play a role in longevity by DR (Table 1.2, Figure 1.1).

The Forkhead transcription factor FoxO/*daf-16* is a pivotal transcriptional regulator downstream of the insulin pathway (Lin et al., 1997; Ogg et al., 1997). As mentioned above, FoxO/*daf-16* is not necessary at all for the longevity induced by *eat-2*, LDR, axenic medium, DD, or msDR (Lakowski & Hekimi, 1998; Greer & Brunet, 2009; Houthoofd et al., 2003; T. L. Kaeberlein et al., 2006; Lee et al., 2006; Bishop & Guarente, 2007; Panowski et al., 2007; Chen et al., 2009; Honjoh et al., 2009). The life-span extension of FoxO/*daf-16* by BDR is less than that of wild-type worms, indicating that FoxO/*daf-16* is partially necessary for longevity induced by BDR (Houthoofd et al., 2003; Panowski et al., 2007; Greer & Brunet, 2009). The life-span extension induced by IF is also partially dependent on FoxO/*daf-16* (Honjoh et al., 2009). FoxO/*daf-16* is necessary for longevity induced by sDR, and in this context acts downstream of AMPK/*aak-2* (Greer et al., 2007; Greer & Brunet, 2009). CDLM, a liquid medium that mimics aspects of DR, leads to the upregulation of FoxO/*daf-16* target genes (Szewczyk et al., 2006), although whether the extension of life span due to CDLM requires FoxO/*daf-16* is not known yet. Analyzing in more depth the differences in the DR regimens that lead to FoxO dependency or independency should provide information on the components of DR that render a DR regimen dependent on FoxO/*daf-16*. For example, the difference in FoxO/*daf-16* requirements between two closely related methods, msDR and sDR, could be due to the timing of DR initiation, the use of FUdR, the use of antibiotics, or the composition of the agar plates, which differ between these two methods.

The Forkhead transcription factor FoxA/*pha-4* is known to be important for pharynx development in worms (Mango et al., 1994). FoxA/*pha-4* mRNA is upregulated by the *eat-2* method of DR (Panowski et al., 2007). In addition, FoxA/*pha-4* is necessary for longevity induced by the *eat-2* mutation and by BDR (Panowski et al., 2007), although whether there is a statistically significant difference in the effect of FoxA/*pha-4* knockdown in *eat-2* mutant worms compared to wild-type worms is still unclear. FoxA/*pha-4* is specific for longevity by DR and is not necessary for life-span extension by other pathways (insulin, mitochondria) (Panowski et al., 2007). In contrast, FoxA/*pha-4* is not necessary for longevity induced by sDR and IF (Greer & Brunet, 2009; Honjoh et al., 2009), but because there are no null mutants of FoxA/pha-4, these results have to be interpreted with caution. Interestingly, FoxA/*pha-4* acts downstream of the TOR pathway (Sheaffer et al., 2008) and could mediate part of the TOR effects on DR-induced life span. Other key regulators upstream of FoxA/*pha-4* are the E3 ubiquitin ligase *wwp-1* and the E2 ubiquitin ligase *ubc-18* (Carrano et al., 2009). The E3 ubiquitin ligase *wwp-1* is also necessary for life-span extension by three methods of DR: *eat-2*, sDR(C), and BDR (Carrano et al., 2009). Whether *wwp-1* interacts with

TOR to control FoxA/*pha-4* is not known yet. These results suggest that FoxA/*pha-4* is a critical mediator of longevity by several methods of DR, even though this transcription factor may not be entirely required in some DR regimens, in particular those that may be dependent on FoxO/*daf-16*.

The NFE2 transcription factor *skn-1* is involved in the response to stress and the insulin pathway (An & Blackwell, 2003; Tullet et al., 2008). NFE2/*skn-1* is entirely necessary for life-span extension by LDR (Bishop & Guarente, 2007). The expression of SKN-1 protein increased in response to LDR, in a subset of neurons in worms (Bishop & Guarente, 2007). Knockdown of NFE2/*skn-1* by RNAi also reduces the life span of *eat-2* mutant worms (Park et al., 2009a). In contrast, NFE2/*skn-1* is not necessary at all for longevity induced by sDR or IF (Greer & Brunet, 2009; Honjoh et al., 2009), although the *skn-1* mutant is unlikely to be a null mutant, so these results have to be interpreted with caution. Knockdown of two downstream targets of NFE2/*skn-1*, *nlp-7* and *cup-4*, reduces longevity induced by the *eat-2* mutant (Park et al., 2009a,b), although the knockdown of these genes in wild-type worms also decreases life span. The expression of *npl-7* and *cup-4* mRNA is increased by sDR, and *nlp-7* and *cup-4* appear to be partially necessary for sDR-induced life-span extension (Park et al., 2009a). Interestingly, *cup-4* is necessary for the induction of *pha-4* mRNA, suggesting *cup-4* is upstream of *pha-4* (Park et al., 2009a). These findings, coupled with the observation that neither FoxA/*pha-4* nor NFE2/*skn-1* is necessary for longevity induced by sDR (Greer & Brunet, 2009), suggest that DR pathways are not linear and that the important hubs are likely to receive and evoke many branches.

The hypoxia-induced transcription factor HIF-1 (*hif-1*) is crucial for the cellular and organismal response to low oxygen (Rankin & Giaccia, 2008). Whether HIF-1 expression and/or activity is altered by DR in worms is still unclear. The life span of a negative upstream regulator of *hif-1*, the dioxygenase *egl-9*, is still extended by msDR, but not to the same extent as that of WT worms, indicating that *egl-9* is partially necessary for longevity by msDR (Chen et al., 2009). In addition *egl-9;eat-2* double mutants had a life span similar to that of *egl-9* mutants, suggesting that *egl-9* is necessary for *eat-2*-induced life-span extension (Chen et al., 2009). However, *hif-1* is not necessary for longevity induced by *eat-2* or BD (Mehta et al., 2009). The role of *hif-1* in longevity induced by other DR methods has not been tested yet. Interestingly, *hif-1* regulates life span downstream of the TOR-S6K/*rsks-1* pathway, in parallel to FoxA/*pha-4* (Chen et al., 2009), which suggests an intricate DR network with several pathways branching out from key hubs of the network, such as TOR. HIF-1 acts via the endoplasmic reticulum stress pathway to regulate life span (Chen et al., 2009). HIF-1 also appears to regulate the ability of cells to control proteotoxicity, as knockdown of two negative regulators of HIF-1, *vhl-1* and *egl-9*, enhances the resistance of *C. elegans* to polyglutamine and β-amyloid toxicity (Mehta et al., 2009). The regulation of proteotoxicity by HIF-1 could underlie some of the beneficial effects of DR in delaying age-dependent diseases. These observations suggest that HIF-1 is a node of the DR network that acts downstream of the TOR pathway to regulate life span.

The heat-shock transcription factor HSF-1 has been shown to be crucial in the cellular and organismal response to heat shock and to a number of other stress stimuli (Morimoto, 2008). HSF-1 is also important to regulate gene expression together with FoxO/*daf-16* downstream of the insulin signaling pathway (Hsu et al., 2003). It is not clear whether DR modifies HSF-1 activity directly. HSF-1 has been shown to be necessary for longevity triggered by BD, although the authors were careful to point out that the fact that a gene is necessary for a DR method to extend life span does not necessarily mean that this gene mediates longevity induced by the DR method (Steinkraus et al., 2008). HSF-1 is also necessary for BDR-induced longevity (Zhang et al., 2009). Longevity induced by IF was also partially dependent on *hsf-1* (Honjoh et al., 2009). In contrast, *hsf-1* is not necessary for sDR or *eat-2*-induced life-span extension (Hsu et al., 2003; Greer & Brunet, 2009). Thus, *hsf-1* is another intersection point of the DR network, but is unlikely to represent a central mediator of longevity by DR.

The coactivator CBP is known to bind to a large number of transcription factors, including FoxO/*daf-16* (Nasrin et al., 2000). Interestingly, CBP-1 protein levels are increased in response to BDR (Zhang et al., 2009). RNAi knockdown experiments indicated that CBP-1 is necessary for life-span extension induced by *eat-2* (Zhang et al., 2009). BDR and axenic medium still significantly extended the life span of worms in which *cbp-1* had been knocked down by RNAi, but the life-span extension in *cbp-1* knocked down worms was dramatically reduced, suggesting that *cbp-1* is partially necessary for BDR and axenic medium (Zhang et al., 2009). Mutation of SATB1/*dve-1*, a CBP-1 binding protein that recruits chromatin remodelers, reduces longevity induced by BDR, but also reduces longevity under ad libitum conditions (Zhang et al., 2009), suggesting that SATB1/*dve-1* may be important for longevity in general, but may not be specific to longevity induced by DR. The role of CBP-1 or SATB1/*dve-1* in other DR methods has not been tested yet. CBP-1 was also shown to be necessary for *daf-2*-induced life-span extension, suggesting that the role of CBP-1 in longevity is not entirely specific to DR (Zhang et al., 2009). It will be interesting to determine which transcription factors function together with CBP-1 to regulate longevity in response to a variety of DR regimens in *C. elegans.*

Other Genes Important for DR: Mitochondrial Genes, Autophagy Genes

In addition to energy sensors and transcriptional regulators, two additional categories of genes, encoding mitochondrial proteins and autophagy regulators, have been shown to mediate the extension of life span elicited by DR (Table 1.2, Figure 1.1).

CLK-1 is a mitochondrial protein involved in ubiquinone synthesis. Whether CLK-1 activity is regulated by DR is not known yet. The *clk-1* gene is required for longevity induced by the *eat-2* mutation in worms (Lakowski & Hekimi, 1998). The *clk-1* gene is also necessary for sDR-induced longevity (Greer & Brunet, 2009). However, the role of *clk-1* in other DR methods is not known yet. It is also unclear if other mitochondrial proteins are important for longevity induced by DR. Prohibitins, which are present at the inner membrane of the mitochondria and modulate mitochondria function, have been shown to be dispensable for longevity by the *eat-2* mutations, although whether the effect of prohibitin knockdown was statistically different in *eat-2* mutants versus wild-type worms was not established (Artal-Sanz & Tavernarakis, 2009).

Autophagy is a "self-eating" process that can provide nutrients in times of scarcity by degrading proteins (Cuervo, 2008a). Organelles (for example, mitochondria) that might have been damaged during aging can also be recycled by autophagy (Cuervo, 2008b). RNAi knockdown of *bec-1*, *atg-7*, and *vps-34*, three genes required for the induction of autophagy, prevents *eat-2*-induced longevity (Jia & Levine, 2007; Hansen et al., 2008). Consistent with this observation, *eat-2* mutants have elevated levels of autophagy (Hansen et al., 2008). The autophagic response to DR (*eat-2* mutation) also requires FoxA/*pha-4* (Hansen et al., 2008), suggesting that autophagy requires changes in gene expression to mediate longevity. Whether all methods of DR activate autophagy pathways remains to be established. Since TOR plays an important role in the induction of autophagy, it is possible that part of the effect of TOR on longevity by DR is due to the ability of TOR to regulate autophagy (Hansen et al., 2008).

DR NODES CONSERVED IN OTHER SPECIES

Yeast DR: SIR2 and TOR

DR can be achieved in *Saccharomyces cerevisiae* by a moderate reduction in glucose (Lin et al., 2000), a more severe reduction in glucose (Kaeberlein et al., 2004), or a reduction in amino acids while keeping glucose concentrations constant (Jiang et al., 2000). All these DR regimens extend the replicative life span of yeast. A more extreme form of DR in which yeast cells are washed in water to remove all nutrients also extends the chronological life span of yeast (Wei et al., 2008). Direct comparisons have not been made yet for each DR method, but the *SIR2* gene and the closely related homolog *HST2* have been shown to be necessary for life-span extension induced by a moderate reduction in glucose (Lin et al., 2000, 2002; Lamming et al., 2005). In contrast, *SIR2* is not necessary for longevity induced by the more severe reduction in glucose (Kaeberlein et al., 2004). The involvement of *SIR2* and *HST2* in the moderate reduction in glucose has also been disputed (M. Kaeberlein et al., 2006), suggesting that *SIR2* probably influences the DR response in yeast, but may not be completely necessary for longevity induced by DR.

Interestingly, the method of DR achieved by severe reduction in glucose requires the presence of TOR (Kaeberlein et al., 2005), indicating that TOR is necessary for DR-induced longevity in both yeast and worms. Other molecules that have been shown to mediate DR in yeast include PKA and SCH9, a protein kinase with similarities to Akt and S6K (Fabrizio et al., 2001; Kaeberlein et al., 2005; Urban et al., 2007). Consistent with the involvement of TOR in longevity elicited by DR in yeast, inhibition of protein translation by depletion of the 60S ribosome subunit extends life span by mechanisms similar to those of DR (Steffen et al., 2008). In addition, RIM15, a glucose-responsive serine/threonine kinase, which integrates TOR, PKA, and SCH9 signaling (Pedruzzi et al., 2003), was shown to be entirely necessary for both moderate and extreme DR in the chronological life-span paradigm (Wei et al., 2008). RIM15 in turn regulates three transcription factors, *GIS1*, *MSN2*, and *MSN4* (Cameroni et al., 2004), which in combination are necessary for the extension of chronological life span induced by moderate and extreme DR (Wei et al., 2008). Finally, *MSN2* and *MSN4* are both necessary for the entire beneficial effects of moderate DR on replicative life span (Medvedik et al., 2007). Studies in yeast have highlighted the role of the TOR pathway, as well as the importance of nutrient-sensing transcription factors in life-span extension by DR.

Are the other pathways identified in *C. elegans* as playing a role in longevity by DR also necessary for longevity induced by DR in yeast? SNF-1, the AMPK homolog in yeast, has been shown to regulate life span (Ashrafi et al., 2000), but has not yet been examined in longevity in response to DR. FoxO/*daf-16*, FoxA/*pha-4*, and NRF2/*skn-1* do not have orthologs in yeast, suggesting that these genes have evolved to control life span in response to DR in multicellular organisms.

Fly DR: SIR2, TOR, and FoxO

There exist a number of DR methods that extend life span of flies (Piper & Partridge, 2007; Tatar, 2007): the dilution of yeast (Chippindale et al., 1993), the restriction of both yeast and sugar components or only sugar components (Mair et al., 2005; Skorupa et al., 2008), the intermittent feeding of yeast (Partridge et al., 1987), the feeding of sugar supplemented with varying concentrations of casein to modify protein concentration (Min & Tatar, 2006b) or the replacement of sugar with various lipids to vary the lipid content of the diet (Driver & Cosopodiotis, 1979).

The histone deacetylases Rpd3 and SIR2 have been proposed to be necessary for life-span extension by reduction in both sugar and yeast components in flies (Rogina et al., 2002; Rogina & Helfand, 2004). The ortholog of the p53 tumor suppressor in *Drosophila*, Dmp53, is also necessary for life-span extension in response to DR in flies. In mammals, p53 has been reported to act downstream of Sirt1, the ortholog of SIR2 (Luo et al., 2001; Vaziri et al., 2001). A fly transgenic line expressing a dominant negative form of Dmp53 no longer showed an extended life span when exposed to lower sugar and yeast (Bauer et al., 2005), suggesting that p53 is necessary for DR-induced longevity.

Interestingly, Tsc2, which regulates the TOR pathway, is partially necessary for life-span extension in response to a reduction in yeast concentration (Kapahi et al., 2004). Furthermore, deletion of d4E-BP, a translational repressor downstream of TOR, impaired longevity triggered by dilution of yeast (Zid et al., 2009), suggesting that in *Drosophila* TOR is also necessary for longevity induced by DR. 4E-BP protein levels were increased in response to DR (Zid et al., 2009). DR leads to a decrease in overall translation, but to an increase in the translation of specific mRNAs that encode mitochondrial proteins (Zid et al., 2009). In line with these observations, several components of the mitochondrial electron transport chain were partially required for longevity induced by DR in flies (Zid et al., 2009). Thus, at least for this DR method (dilution of yeast), the TOR pathway appears to regulate fly life span via protein translation and mitochondrial activity, rather than by affecting transcription factors, as was described in yeast and worms.

The *Drosophila* FoxO transcription factor, dFoxO, is not entirely necessary for longevity induced by two DR methods: dilution of yeast (Min et al., 2008) and dilution of both sugar and yeast (Giannakou et al., 2008). However, dFoxO mutant flies have a different life span response to DR compared to WT flies, indicating that dFoxO participates to some extent in the DR benefits in flies (Giannakou et al., 2008). It is possible that dFoxO deficiency is compensated for by other nutrient-sensing transcription factors such as FoxA or that the way DR is initiated in flies primarily alters amino acids, thereby evoking the TOR pathway. The roles of FoxA, NFE2, and AMPK have not been tested yet in the DR response in flies.

Indy, a sodium dicarboxylate cotransporter (Rogina et al., 2000), was reported to be necessary for DR induced by dilution of both sugar and yeast (Wang et al., 2009). However, there are conflicting reports as to whether Indy regulates life span in flies, and the effects of Indy on fly life span appear to be strain specific (Rogina et al., 2000; Toivonen et al., 2007; Wang et al., 2009). Indy mutants and DR induced by dilution of both sugar and yeast led to increased dFoxO nuclear localization in fat-body cells (Wang et al., 2009), raising the possibility of a cross talk between Indy and the FoxO node in response to DR in flies.

Thus, longevity induced by DR in flies is dependent on the TOR pathway and may involve the modulatory action of SIR2 and FoxO. In *Drosophila*, specific nutrients have been measured in response to DR (Min & Tatar, 2006a; Skorupa et al., 2008; Wong et al., 2009), and it seems that DR elicits an imbalance in essential amino acids (Skorupa et al., 2008; Grandison et al., 2009), which may be what triggers the dependence on the TOR pathway.

DR in Mammals: SIR2, TOR, Insulin–FoxO, NFE2

Several dietary manipulations extend life span in rodents. The most frequently used is called CR (caloric restriction) and corresponds to a 40% reduction in the total amount of food (Weindruch et al., 1986). Alternating cycles of feeding and fasting (feeding every other day, EOD) also extends life span by ~25% depending on the strain and the age of initiation in mice (Goodrick et al., 1990). EOD generally reduces caloric intake substantially. In rats, EOD feeding can lead to as large as an 83% increase in life span (Goodrick et al., 1982). Interestingly, a reduction in the protein concentration while keeping other parameters constant (Goodrick, 1978), or even a reduction in the amount of methionine in the diet, also extends rat life span (Orentreich et al., 1993). The mechanisms underlying the benefits of DR might not entirely be conserved between rats and mice, as DR decreases the mortality rate in rats but appears to affect mostly the initial mortality in mice.

Deletion of Sirt1, the mammalian SIR2 ortholog, abolishes the beneficial effect of 40% reduction in food DR on behavioral activity (Chen et al., 2005). Conversely, mice that overexpress Sirt1 have the metabolic characteristics of 40% reduction in food DR mice (Bordone et al., 2007) and Sirt1 overexpression protects from several negative consequences of high-fat diets (Pfluger et al., 2008), though these mice do not live longer. In addition, resveratrol, which can activate Sirt1, although it also activates a large number of molecules including AMPK, has beneficial effects on life span, at least on obese mice

(Pearson et al., 2008a). Resveratrol triggers changes in gene expression that resemble those elicited by EOD (Pearson et al., 2008a) and by a 25% reduction in food intake (Barger et al., 2008).

The observations that a reduction in protein concentration extends life span (Goodrick, 1978) and that TOR is responsive to amino acids raise the possibility that the TOR pathway might be critical in life span induced by some DR methods in mice. Indeed, rapamycin, a potent TOR inhibitor, has been found to extend life span in mice (Harrison et al., 2009), although it is not yet clear if rapamycin acts via the same mechanism as DR to extend life span. Interestingly, the deletion of S6K, a protein kinase downstream of TOR, has been found to extend life span in mice (Selman et al., 2009). The liver of S6K mutant mice shows a gene expression profile similar to that of liver from mice with a 40% reduction in food (Selman et al., 2009). Together, these observations suggest that a TOR–S6K pathway mediates longevity by DR in mice and that the TOR pathway may be a conserved mediator of DR from yeast to mammals.

The role of AMPK in DR-induced longevity has not been tested in mammals. However, EOD and a 40% restriction in food both activate AMPK in the liver of rats (Pallottini et al., 2004). Short-term DR (60% reduction of food for 5 days) also activated AMPK in the hippocampus of mice (Dagon et al., 2005). The muscle of S6K mutant mice had a gene expression profile similar to that of mice with a 40% reduction in food, but also to that of mice treated with the AMPK activator AICAR (Selman et al., 2009). These results suggest that the TOR pathway may act via AMPK to extend life span in response to some DR regimens. Conversely, AMPK has been reported to inhibit the activity of the TOR pathway by regulating TSC2 phosphorylation (Inoki et al., 2003). These findings indicate that the TOR and AMPK pathways are highly interconnected in mammalian cells and suggest that these pathways may coordinately orchestrate longevity in response to DR in mammals.

The role of FoxO transcription factors, and more generally of the insulin-signaling pathway, has not been tested yet in DR-induced longevity in mice. However, the life span of mice that are deficient for the growth hormone receptor (GHR) is no longer extended by a 30% reduction of food (Bonkowski et al., 2006) or by EOD (Bonkowski et al., 2009), suggesting that DR and the GHR pathway function in the same pathway to extend life span. Because GHR is thought to act by regulating the circulating levels of IGF-1 and insulin, it is possible that the beneficial effects of DR on life span are dependent on an insulin–FoxO pathway in mice. Evidence indicates that a short DR regimen of 40% reduction in food prevented the growth of some tumors, but not others, in mice (Kalaany & Sabatini, 2009). Interestingly, tumor cells that were insensitive to the beneficial effects of DR on tumor growth showed alterations in the insulin-signaling pathway (Kalaany & Sabatini, 2009). Expression of FoxO1 in these cells allowed them to respond better to DR (Kalaany & Sabatini, 2009). These results suggest that FoxO transcription factors may play a conserved role in some of the beneficial effects of DR, at least on cancer, in mice.

The mammalian ortholog of NFE2/*skn-1*, Nrf2, is necessary for the anticarcinogenic effects of DR (40% reduction in food) (Pearson et al., 2008b). However, Nrf2 is not necessary for insulin sensitivity and life-span extension in response to DR (Pearson et al., 2008b). Thus, it is possible that DR engages different pathways to mediate beneficial effects on cancer and on overall life span.

WHY ARE THERE DIFFERENCES BETWEEN DR REGIMENS?

Type of Nutrients

The different genetic pathways evoked by the various DR methods may be the consequence of the fact that some nutrients may be more limiting than others depending on the DR method (Figure 1.2A). One DR method could reduce amino acid levels more prominently than lipids or carbohydrates, thereby triggering a specific signaling pathway, whereas another DR method could cause a reduction in carbohydrates, thereby eliciting another signaling pathway. For example, it is possible that sDR reduces carbohydrates, which would render it dependent on AMPK and FoxO, while other methods may reduce amino acids more readily, which would evoke the TOR pathway, a well-known amino acid-responsive protein kinase (Dann & Thomas, 2006).

Whether the beneficial effects of DR are due to a decrease in the total amount of calories or a decrease in specific nutrients, or the relative ratio of nutrients, is still unclear in many species (for review see Piper & Partridge, 2007). In rats, reduced caloric intake was shown to be the responsible dietary agent for the life extension of CR (McCay et al., 1939). A series of rat studies published in the 1980s (Yu et al., 1982; Iwasaki et al., 1988a,b; Masoro et al., 1989) extended these findings and showed that specific reduction in the intake of protein, methionine, fat, minerals, or vitamins was not involved in the life-extending action of a 40% reduction of food intake. While methionine or tryptophan restriction alone can extend life span in mice (Miller et al., 2005) and rats (Ooka et al., 1988; Orentreich et al., 1993), it probably works by causing an imbalance of essential amino acids and a distortion in protein metabolism (Masoro, 2009).

In *C. elegans*, BD was shown to still extend life span at an age at which control worms were no longer

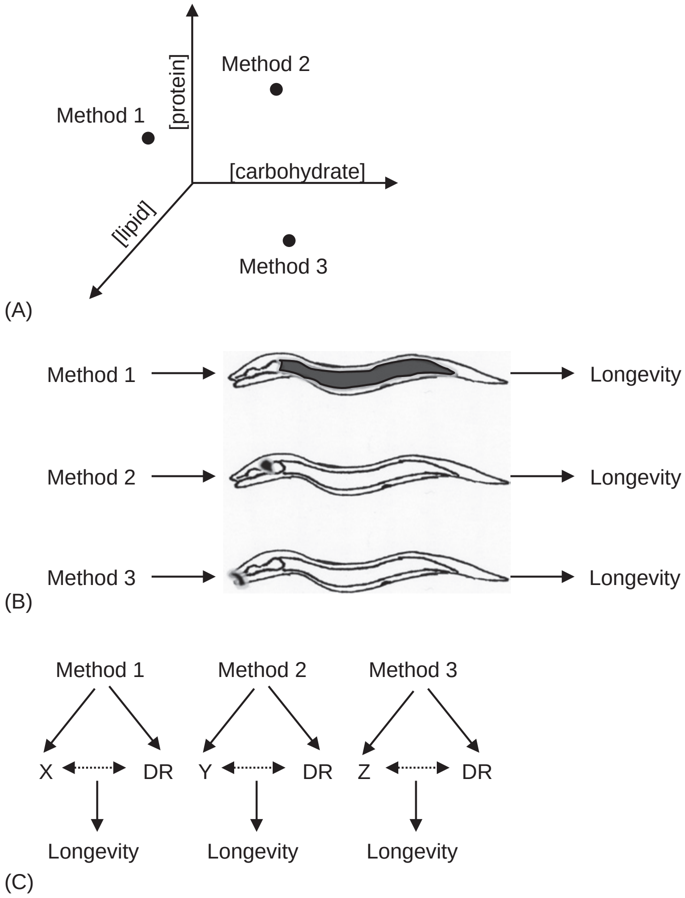

Figure 1.2 Potential mechanisms underlying the different gene requirements for longevity by various dietary restriction regimens. (A) Different methods of DR could extend longevity by reducing different sources of calories, for example, carbohydrates, amino acids, or lipids. (B) Different tissues (intestine versus neurons) in *C. elegans* could sense different methods of DR to extend longevity. (C) Different methods of DR could alter unknown parameters to affect life span and the life span effects could be due to these unknown parameters or to the interaction between these unknown parameters and the reduction of nutrients.

consuming food (Smith et al., 2008), suggesting that part of the life-span extension by BD is independent of calories. In flies, reduced olfactory function is sufficient to extend fly life span and exposure to food-derived odorants is sufficient to reduce DR-induced life-span extension (Libert & Pletcher, 2007; Libert et al., 2007), suggesting that reducing calories is not the only way in which DR is mediated. Indeed, the long life span of dietary-restricted flies could be reverted by the addition of amino acids, but not by the addition of carbohydrates, lipids, or vitamins (Grandison et al., 2009). It is possible that if a key nutrient (perhaps one that is necessary for proper reproduction) is missing from the diet, this deficiency may trigger "DR-like" beneficial effects on life span, perhaps such that the youthfulness of the organism is preserved until balanced nutritional conditions compatible with reproduction return (Skorupa et al., 2008).

Temporal Effects

DR methods could trigger different genetic mechanisms depending on the time at which DR is initiated. sDR is initiated at an almost entirely postreproductive stage, while other methods are initiated earlier (Table 1.1). However, the temporal parameter is unlikely to account for the entire difference in the genetic pathway mediating life-span extension by diverse DR methods because: (1) *eat-2* and liquid DR are initiated at different times of life, yet both methods are dependent on FoxA/*pha-4* (Panowski et al., 2007), and (2) dilution of peptone in the plates is initiated at birth, like *eat-2*, but unlike *eat-2* it engages the AMPK/*aak-2* and FoxO/*daf-16* pathway (Greer & Brunet, 2009). Nevertheless, it is possible that the interplay of different temporal and nutrient differences between DR methods results in the requirement for distinct genetic pathways.

Tissue Specificity

Different methods of DR may be sensed by distinct tissues and thereby require specific genes. For example, some forms of DR may be sensed by specific neurons (olfactory or gustatory), whereas other DR regimens may be sensed by the intestine (Figure 1.2B).

In *C. elegans*, ablation of olfactory neurons or defects in sensory cilia have been shown to extend life span (Apfeld & Kenyon, 1999; Alcedo & Kenyon, 2004). Life-span extension induced by reduction in olfaction in *C. elegans* is independent of FoxO/*daf-16*, whereas life-span extension caused by ablating the gustatory ASI neurons is dependent on FoxO/*daf-16* (Alcedo & Kenyon, 2004). Surprisingly, a modified version BDR was shown to trigger a gustatory system response, but was also shown to be independent of FoxO/*daf-16* (Bishop & Guarente, 2007). Because reduced olfactory function is sufficient to extend fly life span (Libert & Pletcher, 2007; Libert et al., 2007), it is possible that olfactory neurons may also be crucial in *Drosophila* to mediate DR. Other DR methods may be sensed via multiple different tissues, as highlighted by the finding that in worms, expression of *egl-9*, a regulator of *hif-1*, in either serotonergic neurons or muscle cells, rescued the defects of *egl-9* mutants in responding to msDR (Chen et al., 2009). Dissecting the tissues responsible for the action of specific genes in different DR methods should help in understanding the role of tissues in the response to DR.

Non-DR Parameters

The various DR methods may also trigger other non-DR signaling pathways that in turn have beneficial effects on life span (Figure 1.2C). The *E. coli* strain OP50 used as the source of food in *C. elegans* assays is mildly pathogenic under some laboratory conditions, although OP50 is not as pathogenic as many other strains of bacteria (Vanfleteren et al., 1998; Labrousse et al., 2000; Garsin et al., 2001; Garigan et al., 2002). It is possible that a portion of the DR response is due to a dilution of an *E. coli* toxin. As the insulin–FoxO signaling pathway (Garsin et al., 2003) has been implicated in the *C. elegans* immune response, this raises the possibility that methods of DR that are FoxO/*daf-16* dependent are so because of an immune response pathway that would become activated under ad libitum conditions and would subsequently be diluted under bacterial dilution conditions. However, reduction in pathogenicity is unlikely to explain entirely the effect of DR in these assays because diluting bacteria decreases fertility, whereas it would be expected to increase fertility if it were only diluting a toxin. Nevertheless, using killed bacteria or a less pathogenic source of bacteria, such as *Bacillus subtilis* (Garsin et al., 2001), for worm life span assays, as well as performing variations in nutrient content of axenic medium, could be used in all DR methods to uncouple food concentration from food pathogenicity.

It is also possible that other non-DR parameters may affect the dependency of DR methods on specific genes. For example, the way sDR is achieved (by adding diluted amounts of *E. coli* every 2 days) may induce cycles of feeding and fasting, similar to IF, that may cause this method to be FoxO/*daf-16* and AMPK/*aak-2* dependent, whereas the other methods might have a more constant bacteria dilution throughout the experiment. sDR may also induce a mild oxidative stress response that could trigger the AMPK and FoxO pathways, as these pathways have both been shown to transduce stress stimuli (Henderson & Johnson, 2001; Apfeld et al., 2004; Schulz et al., 2007; Lee et al., 2008). Conversely, the *eat-2* mutation, which affects the acetylcholine receptor, may have other effects in addition to reducing pharyngeal pumping that could interact with the food restriction pathways. In addition, liquid methods of DR may also evoke additional parameters (e.g., altered oxygen consumption; Honda et al., 1993) that may interact with reduction in nutrients to result in life-span extension in a manner that is dependent on specific genes. Identifying the parameters that are embedded in or interact with DR methods will be important to gain complete insight into the mechanisms by which DR extends life span.

DR Mimetics

Several chemical compounds have been shown to mimic the beneficial effects of DR on life span and on gene expression profiles in a variety of organisms. These DR mimetics include metformin (Dhahbi et al., 2005), oxaloacetate (Williams et al., 2009), resveratrol (Howitz et al., 2003), and rapamycin (Harrison et al., 2009). Resveratrol extends the life span of yeast (Howitz et al., 2003), worms (Wood et al., 2004; Viswanathan et al., 2005), flies (Wood et al., 2004), fish (Valenzano et al., 2006), and mice on a high-fat diet (Baur et al., 2006), although whether resveratrol promotes life span under all circumstances is still unclear (Bass et al., 2007; Pearson et al., 2008a). Metformin decreases tumor incidence and increases the life span of HER-2/neu transgenic mice (Anisimov et al., 2005). Intriguingly, a number of these chemicals have been shown to activate AMPK (Baur et al., 2006; Zang et al., 2006; Dasgupta & Milbrandt, 2007; Hardie, 2007; Hwang et al., 2007). Indeed, resveratrol and 2-deoxyglucose have been shown to require AMPK/*aak-2* to extend life span in worms (Schulz et al., 2007; Greer & Brunet, 2009). The effect of resveratrol on longevity in worms was also shown to require *sir-2.1* (Wood et al., 2004; Viswanathan et al., 2005), although these results are still being debated (Bass et al., 2007). Metformin increases *C. elegans* life span in an AMPK/*aak-2*- and NFE2/*skn-1*-dependent manner (Onken & Driscoll, 2010). The AMPK agonist AICAR was shown to induce metabolic genes and have beneficial effects on endurance (Narkar et al., 2008). It is important to note that thus far none of the DR mimetics extends life span as effectively as DR itself. This observation suggests that multiple pathways may need to be activated concomitantly to achieve optimal effects on life span and health span through chemical treatments.

CONCLUSIONS

Collectively, these studies suggest that different DR regimens evoke mostly separate pathways rather than a unique universal DR pathway. A prediction based on the relative independency of DR pathways is that different DR regimens should act additively to extend life span. Consistent with this prediction, combining sDR or axenic medium with the *eat-2* mutation leads to an even further increase in life span (Houthoofd et al., 2002; Greer & Brunet, 2009). More generally, manipulating more than one DR pathway may allow additive or even synergistic life-span and health-span benefits.

Interestingly, the genetic pathways involved in mediating the various DR methods have been shown to cross talk extensively, at least in mammalian cells. This observation indicates that even though the pathways seem relatively separate, when analyzed individually, they are not completely independent. It is possible that when one node of this network is specifically activated by one DR regimen, other nodes become dispensable. Thus, we propose that these genes form a "DR network" rather than a "DR pathway." Consistent with this possibility, a study using Gompertz mortality rate analysis has provided evidence that the pathways involved in dietary restriction, insulin signaling, and mitochondria are all linked to delay aging in *C. elegans* (Yen & Mobbs, 2009). These observations suggest that it is important to study multiple DR methods to understand the network as a whole, as a way to reveal the ensemble of players in the DR network. Understanding all the different nodes of the DR network may help identify ways to harness the full benefits of DR on life span and health span.

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Chapter 2

Role of the Somatotropic Axis in Mammalian Aging

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CHAPTER CONTENTS

Introduction 25
Body Size and Composition 26
Glucose Metabolism and Insulin Sensitivity 27
Oxidative Metabolism and Related Factors of Stress Resistance 28
Cognitive Function 30
Immune Function 32
Age-Related Pathology and Neoplasia 34
Conclusion 35
References 35

INTRODUCTION

The somatotropic hormone axis has been implicated in the regulation of aging processes and life span. This large and growing body of literature indicates that interfering with signaling at various points along this axis results in extension of life span in multiple species. Importantly, long-living mutant mice appear to benefit from a longer "health span" as well as longer life. Mechanisms of the life-span extension are numerous, as this pathway affects a multitude of physiological systems. The beneficial effects of delayed or decelerated expression of specific aging-related characteristics will be discussed in the context of the somatotropic axis.

This endocrine axis consists of the hypothalamic factors that control the secretion (growth hormone-releasing hormone (GHRH) and somatostatin) of pituitary growth hormone, growth hormone, insulin-like growth factor 1 (IGF1), and various downstream effectors. Studies of a variety of mutant rodents in addition to studies in humans have contributed to our understanding of the impact of these hormones in aging mammals. Critical information about the insulin and IGF1 systems in invertebrate species, specifically *Caenorhabditis elegans*, *Drosophila melanogaster*, and yeast, are strongly supportive and demonstrate the evolutionary significance of this growth-related pathway in aging and longevity. In flies and worms, it is thought that the activity of the insulin/IGF1 receptor (via the insulin receptor substrate/phosphoinositol 3-kinase/forkhead transcription factor pathway) plays a significant role in the control of aging (Lin et al., 1997; Ogg et al., 1997). Growth hormone (GH) is not present in flies and worms. In mammals, an additional level of complexity is present with GH, a hormone that exhibits both IGF1-dependent (somatic effects) and IGF1-independent effects (i.e., glucose regulation, lipolysis, oxidative metabolism; reviewed in Lanning & Carter-Su, 2006).

There are a large number of phenotypic indices that appear to be age sensitive in their expression. Typically, the definition of delayed or decelerated aging in an organism is based on the expression of several indices. These age-sensitive markers are diverse and cross over a number of physiological systems and include the expression levels of insulin, GH, and IGF1 signaling molecules; components of energy metabolism (body temperature, mitochondrial biogenesis); appearance of aging-related pathology (lipofuscin, amyloid, collagen cross-linking, neoplastic disease); degree of oxidative damage (protein and DNA); antioxidative defense capacity; and other factors of stress resistance, body size, and composition (fat in particular); immune function; cognitive function; and activity/mobility, among others. The expression of these various characteristics is influenced by somatotropic

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hormone levels as well as a wide range of other factors. The evidence supporting the assertion that somatotropic signaling plays a mechanistic role in aging and longevity will be reviewed. In addition, data from studies of calorie-restricted (CR) animals will be presented for comparison, as this dietary manipulation certainly delays or decelerates multiple indices of aging, many of which are thought to occur at least in part via the declines in serum GH and IGF1 that are frequently observed in CR animals (Breese et al., 1991; Ruggeri et al., 1989; Sell, 2003; Weindruch & Walford, 1982).

BODY SIZE AND COMPOSITION

A solid literature base has been amassed showing that a reduction in one of three interrelated pathways, GH, IGF-1, and insulin, results in the delayed or decelerated appearance of several age-related characteristics in mice. Endocrine mutants, the Ames and Snell dwarf mice, are phenotypically very similar animals that lack circulating GH because of different mutations affecting pituitary somatotrope development (Li et al., 1990; Sornson et al., 1996). The loss of GH's somatic effects result in animals of small size (one-third the body size of wild-type mice) but extended longevity. In fact, these animals are among the longest-lived genetic mutant mice currently studied (>50% extension in Ames mice; Brown-Borg et al., 1996; Flurkey et al., 2001). These particular mutations ($Prop1^{df}$ and $Pit1^{dw}$) affect development of other pituitary cell types (lactotropes, thyrotropes); therefore these animals also exhibit circulating prolactin and thyrotropin deficiencies. Plasma IGF1 levels depend upon stimulation of liver GH receptors by GH. Therefore, without GH stimulation, the levels of IGF1 are undetectable in dwarf mouse serum. The majority of GH's actions on growth are dependent on the downstream activity of IGF1. Thus, animals with altered GH or IGF1 secretion patterns are generally characterized by smaller body size. For example, growth hormone receptor knockout (GHRKO) mice exhibit high levels of plasma GH but lack circulating IGF1 because of the inability of GH to activate the mutated receptor and, therefore, are also small in size in comparison to wild-type mice (Coschigano et al., 2000). Smaller body size has been implicated in lifespan extension in several organisms, including dwarf mutant mice, genetically normal strains and lines of mice, genetically heterozygous populations of mice, wild-derived mice, dogs, horses, flies, and possibly humans (Conover & Bale, 2007; Eklund & Bradford, 1977; Miller et al., 2002; Harper et al., 2006; Patronek et al., 1997; Rollo, 2002; Samaras et al., 2003; Sutter, 2007; Tatar et al., 2003). CR animals, if restricted early in life, also grow at a slower rate, resulting in mature adults of smaller body size compared to ad libitum fed controls. Lower plasma growth factor (GH and IGF1) concentrations are implicated in these growth deficits in CR mice (Bartke, 2005). Mechanisms of slowed aging that may be linked to the suppression of somatotropic signaling include alterations in insulin signaling, delayed puberty, reduced metabolism, reduced incidence of cancer, and enhanced stress resistance.

The anabolic actions of GH occur principally through IGF1 and insulin stimulation of protein synthesis. However, GH has direct metabolic effects (IGF1-independent) affecting lipids (thus further regulating body composition), insulin sensitivity, and oxidative metabolism. Growth hormone induces lipolysis in human omental adipose tissue and in rat adipocytes by inducing hormone-sensitive lipase mRNA among other lipolytic factors (Fain et al., 2008; Yang et al., 2004). Thus, body composition can be significantly affected by GH status. In regard to long-living GH-deficient Ames dwarf mice, fat mass is actually lower, possibly reflecting the cross talk between the GH and the glucocorticoid actions on lipid oxidation, lipid mobilization, and lipolytic activities (Dominici et al., 2005; Heiman et al., 2003). However, these findings may differ depending on strain, age, and diet of the animals when fat mass is determined.

In contrast to the smaller body size and altered composition in GH deficiency, when GH is overexpressed (pharmacological levels) in rodents, growth is accelerated, body size is significantly increased, and life span is remarkably reduced (Bartke et al., 1999). In addition, the high levels of growth hormone lead to other physiological anomalies such as normal lactation in the absence of pregnancy, enhanced sleep-related patterns, and other metabolic disturbances (Hajdu et al., 2002; Milton et al., 1992). Several GH transgenic mice that have been created over the years, some of which overexpress the bovine (bGH) or ovine GH gene and others of which express a human GH transgene. These transgenes are driven by different promoters and in specific tissues. Importantly, some of the primary physiological outcomes of GH overexpression result from differences in the GH gene itself. Human GH has both somatogenic and strong lactogenic activities, whereas bovine and ovine GH activities are purely somatogenic (Posner, 1976; Aguilar et al., 1992a,b). Thus, data collected from the bGH transgenic animals reflect physiological and pharmacological effects of GH and IGF1 and not prolactin. The physiology of GH overexpression includes several aspects of early onset pathology or premature aging, including scoliosis, weight loss, deterioration of coat and body condition, increased astrogliosis, deficits in learning and memory, declines in neurotransmitter turnover, increased corticosterone, reduction in reproductive life span, and a reduced ability to recover from stress (reviewed in Bartke, 2003). Many of these characteristics are present in 6- to 8-month-old mice. In addition, these animals exhibit a reduced cellular replicative potential, increased indices of free radicals, and shortened life spans. The magnitude of these differences is related to the circulating level of GH (Cecim et al., 1994; Bartke, 2003).

An additional animal categorized as a GH mutant is the dwarf rat. Dwarf rats expressing an antisense GH transgene have moderately suppressed GH levels and live longer than normal rats (Shimokawa et al., 2002). However, dwarf rats (dw/dw) expressing specific and limited GH and IGF-1 (40% reduction) in adulthood do not exhibit life-span extension (Sonntag et al., 2005). Problematic is that the dw/dw mutation disturbs somatotroph differentiation, resulting in an increase in prolactin accompanying the GH deficiency, a highly unusual event that could alter life span (Tierney & Robinson, 2002). High pituitary prolactin, in this case, may compensate for the GH deficiency, as it has been shown that prolactin overexpression increases body size (Greenman et al., 1998; Byatt et al., 1993). Thus, the degree of change in various parameters related to somatotropic signaling and life span is associated with the degree of hormone deficiency, the timing of the deficiency, and the background strain, among other factors. Overall, the effects of GH and IGF1 on body size and composition are evident in these endocrine mutants and some of these somatic effects are dependent upon age.

GLUCOSE METABOLISM AND INSULIN SENSITIVITY

A significant enhancement in insulin sensitivity compared to normal wild-type or more poorly functioning individuals is a common finding in long-living mammals and is considered a marker of attenuated aging (Masternak et al., 2009). Growth hormone is considered a diabetogenic factor in that it opposes the actions of insulin. GH elevates plasma glucose concentrations by stimulating gluconeogenesis and glycogenolysis and inhibiting glucose uptake at the tissue level. It has been shown that in humans, GH decreases insulin sensitivity by elevating plasma free fatty acids via lipolysis (Salgin et al., 2009). Even physiological concentrations of GH as a consequence of overnight fasting have been shown to decrease insulin sensitivity (Bratusch-Marrain et al., 1982; Rizza et al., 1982). Studies have shown that this adverse effect of GH on insulin sensitivity is due to the cross talk between GH and insulin signaling pathways (Argentino et al., 2005). Transgenic mice that overexpress GH are hyperglycemic and hyperinsulinemic and exhibit marked reductions in life span (50% of wild type) as well as multiple measures of aging acceleration (Bartke, 2003; Dominici et al., 1999; Quaife et al., 1989). Insulin resistance in humans is a risk factor for several age-related ailments including diabetes and cardiovascular disease (Facchini et al., 2001). It was once thought that reduced circulating glucose delayed aging by decreasing the accumulation of and detrimental processes associated with glycation end products (Baynes et al., 1989; Reiser, 1998), reducing metabolism (less fuel) and the associated metabolic reactive oxygen species generation. However, more recent studies using mutant mice that overexpress GLUT4 (glucose transporter) have shown that lower glucose throughout the life span does not alter several age-related indices nor longevity (McCarter et al., 2007). A major consequence of the overall GH deficiency in dwarf animals is low levels of plasma glucose and insulin culminating in a significant enhancement in insulin sensitivity throughout the life span of Ames and Snell mice and GHRKO mice (Borg et al., 1995; Dominici et al., 2002; Hseih et al., 2002; Masternak et al., 2009). The increase in insulin sensitivity throughout life and, certainly, in old animals, is thought to contribute to the extended longevity exhibited by these dwarf animals. Stimulated insulin secretion is reduced in dwarf mice, while insulin injection causes greater suppression of plasma glucose levels compared to responses in normal mice (A. Bartke, unpublished; Dominici et al., 2002). The total number of islets of Langerhans is not altered but the islet volume is significantly reduced owing to fewer large islets in dwarf mice (Parsons et al., 1995), thus leading to lower insulin secretion rates. Low insulin and glucose levels are also found in GH-resistant GHRKO mice, “Little” mice that express a mutation in the growth hormone-releasing hormone receptor, and fat-specific insulin receptor knockout mice (FIRKO), each of which express alterations in one of the three pathways (GH, IGF1, or insulin; Bluher et al., 2003; Coschigano et al., 2000; Flurkey et al., 2001).

Caloric restriction, long known to increase life span in rodents (McCay et al., 1935; Weindruch & Walford, 1982), flies (Chapman & Partridge, 1996), and possibly primates (Colman et al., 2009; Lane et al., 1996; Rezzi et al., 2009), also reduces insulin levels in the blood. In addition, although CR tends to lower blood insulin levels, animals with life-long reductions in glucose levels (GLUT4 transgenic mice) do not exhibit differences in insulin levels. When these same mice were subjected to CR, the lower glucose levels were not found to be associated with survival, function, or tissue pathology (McCarter et al., 2007). Liver-specific IGF1-deficient mice (LID; Yakar et al., 1999) display a 75% reduction in circulating IGF1 levels. Despite normal blood glucose concentrations and clearance, the LID mice are hyperinsulinemic and exhibit increased insulin resistance due to GH hypersecretion and β-cell hyperplasia. These mice do not exhibit life-span extension possibly because the signaling of this pathway is not truly reduced (Yakar et al., 2001; Yaskar & Rosen, 2003; Yu et al., 2003). Other studies have shown that wild-derived Majuro mice are long-lived despite high glucose and poor glucose tolerance, again suggesting that glucose metabolism may not be necessary for life-span extension (Miller et al., 2002; Harper et al., 2005).

It is important to note that Ames dwarf mice subjected to calorie restriction live significantly longer than ad libitum fed dwarf mice (Bartke et al., 2001) and this extension is believed to be related to a further increase in insulin sensitivity (Bartke et al., 2007). In contrast, calorie restriction in GHRKO mice does not extend life span further (Bonkowski et al., 2006; Arum et al., 2009). Several studies conclude that the lack of a life-extending effect of CR in the GHRKO mice results from the absence of a functional GH receptor, suggesting that an intact GH signaling system is necessary for the longevity effects of CR. In agreement, insulin sensitivity is not further increased by CR in these mice (Masternak et al., 2009). Moreover, when expression patterns of insulin-related genes were compared between Ames and GHRKO mice subjected to CR, it was found that the effects of CR partially overlapped but did not duplicate the effects of Ames dwarfism or GHR deletion (Masternak et al., 2007) and the effects of CR on gene expression patterns were tissue-dependent. In a 2009 study, tissue responsiveness to insulin stimulation in CR and ad libitum fed GHRKO and wild-type mice was measured (Bonkowski et al., 2009). Significant differences in downstream insulin action were identified between genotypes and shown to be tissue-specific (muscle responses were similar but liver responses differed), a finding that is thought to contribute to the absence of CR effects on whole-animal insulin sensitivity and life span in GHRKO mice.

Additional evidence that specifically supports the role of reduced insulin signaling as an index of slow aging is derived from studies in *C. elegans* and *D. melanogaster* in which mutations in the insulin pathway have been shown to increase life span greatly (Guarente & Kenyon, 2000; Tatar et al., 2003). Human centenarians have also been shown to exhibit a major increase in insulin sensitivity compared to elderly individuals from the same population (Kojima et al., 2004; Paolisso et al., 1996; van Heemst et al., 2005). Furthermore, some reports suggest that alterations in the GH, IGF1, or insulin pathways are associated with long life in human populations (Flachsbart et al., 2009; Suh et al., 2008; Willcox et al., 2008). Therefore, there is evidence in multiple species that insulin signaling plays a role in health span and life span and that the somatotropic axis strongly influences this measure.

OXIDATIVE METABOLISM AND RELATED FACTORS OF STRESS RESISTANCE

The ability to resist stress is considered a significant feature of longevity assurance and a contributor to delayed aging in long-living mammals. The resistance to oxidative stress in particular, and the resulting deceleration in the accumulation of oxidative damage to DNA, protein, and lipids, was long thought to be a major mechanism leading to successful aging. However, many reports have shown that altering the levels of antioxidative enzymes in mice does not affect life span and has brought the free radical theory of aging into question (Lapointe & Hekimi, 2009; Perez et al., 2009). Nevertheless, a significant number of studies have explored the relationships between oxidative stress, oxidative damage, and aging at the molecular level and several investigators will probably continue to investigate these associations.

The somatotropic axis has been shown to be involved in oxidative metabolism and related factors of stress resistance. The oxidative defense capacity of an organism is determined by the sum of several counterregulatory mechanisms that serve to prevent oxidative damage, the expression of which is tissue-, organelle-, age-, and physiological status-specific. The primary endogenous enzymatic and nonenzymatic antioxidative compounds include catalase, the glutathione peroxidases (GPX) and superoxide dismutases (SOD), glutathione, and the thioredoxins. These factors work together to regulate the presence and activities of reactive oxygen species. Growth hormone is an anabolic factor that increases cellular metabolism. In many tissues, the increase in metabolic activity (oxygen consumption, glucose oxidation) leads to an increase in oxidative phosphorylation resulting in increased ROS generation as a by-product of metabolism. There is strong evidence, both in vitro and in vivo, that growth hormone downregulates the expression of several components of this defense system. In GH transgenic mice, the high plasma levels of GH are associated with increased superoxide radicals and oxidative damage to membrane lipids (lipid peroxidation; Rollo et al., 1996). Tissues from these mice exhibit significantly reduced levels of antioxidative enzymes, including manganese superoxide dismutase (SOD2), copper–zinc superoxide dismutase (SOD1), catalase, and GPX (Brown-Borg et al, 1999; Brown-Borg & Rakoczy, 2000; Hauck & Bartke, 2001). Direct and specific effects of GH and IGF1 in vitro support the in vivo data demonstrating that these two hormones downregulate the expression of antioxidative enzymes. Catalase activity and the activities and protein levels of both GPX and SOD2 were suppressed in primary hepatocyte cultures from normal, wild-type mice that were treated with somatotropic hormones (Brown-Borg et al., 2002). As mentioned previously, the expression of these enzymes is organ-specific. For example, our previous work has shown that liver, kidney, heart, and brain tissues of GH transgenic mice exhibit significant reductions in the activity, protein, and/or mRNA levels of catalase and suppression of GPX and SOD activities in liver. A 2008 study reported that GH transgenic mice display elevated GPX1 protein levels in the vascular endothelium compared to wild-type control mice, while catalase protein levels were similar between genotypes (Csiszar et al., 2008). Glutathione peroxidase has several isoforms that are expressed in specific cellular compartments; thus when GPX activity is assayed, all of the isoforms present can contribute to the activity levels measured. The GPX1 isoform, for instance, is present in both cytosolic and mitochondrial compartments and expressed predominantly in erythrocytes, kidney, and liver (Mates et al., 1999). Therefore, the downside of GH overexpression in mice is a significant suppression of oxidative defenses and multiple indices of physiological decline associated with accelerated or premature aging including early reproductive senescence and shortened reproductive life span, glomerulonephritis, glomerulosclerosis, and an early onset of and increased tumor incidence compared to normal animals (and other indices listed previously; Cecim et al., 1994; Lachmansingh & Rollo, 1994; Meliska et al., 1997; Miller et al., 1995; Naar et al., 1991; Pendergrass et al., 1993; Steger, 1994; Steger et al., 1993). I believe that this loss of integrity in multiple systems results in the reported 50% reduction in life span (average 12 months at death; Steger et al., 1993). In comparison, IGF1 transgenic mice do not experience such severe pathological alterations in the kidney, for instance, suggesting that GH is probably the main effector (Doi et al., 1988).

The upside of the GH story in mice is that in GH deficiency, a very different picture emerges. The antioxidative defense capacity of Ames and Snell dwarf mice, which lack peripheral somatotropic action (GH and IGF1 deficient), is significantly enhanced. The reports of this enhancement vary somewhat as the identical measures of defense have not been performed simultaneously in each mutant. This enhancement in antioxidative defense also pertains to reports in the GHRKO, IGF1R$^{+/-}$, and FIRKO mice mentioned previously (Hauck et al., 2002; Hauck & Bartke, 2001; Holzenberger et al., 2003; Bluher et al., 2003). Ames mice exhibit elevated catalase, GPX, and SOD levels in kidney, liver, heart, and hypothalamic tissues across multiple ages (Brown-Borg et al., 1999; Brown-Borg & Rakoczy, 2000; Hauck & Bartke, 2000; H. M. Brown-Borg, unpublished data). GPX activity in Ames dwarf muscle tissue is preserved after acute and chronic exercise, while that in wild-type mice declines with age (Romanick et al., 2004). Convincingly, when GH is administered to dwarf mice, catalase, GPX, and SOD proteins and activities decline significantly in both young and middle-aged mice (Brown-Borg & Rakoczy, 2003). Thiol-containing proteins involved in defense (glutathione, metallothionein) and pathways responsible for the generation of cysteine (methionine) are also highly upregulated in Ames mice (Brown-Borg et al., 2001; Brown-Borg, 2009; Brown-Borg & Rakoczy, 2005; Uthus & Brown-Borg, 2003, 2006).

Several reports involving GH mutant mice support and extend the enhanced defense accounts reported above. First, liver mitochondrial H_2O_2 production is lower in Ames mice (Brown-Borg et al., 2001). This reduction of ROS concomitant with elevated antioxidants has resulted in multiple reports demonstrating lower oxidative nuclear DNA, mitochondrial DNA, protein, and lipid damage in several tissues (Bokov et al., 2009; Brown-Borg et al., 2001; Choksi et al., 2007; Sanz et al., 2002). Additional in vivo and direct functional evidence is provided in persuasive studies showing that dwarf mice are more resistant to the free radical-generating compounds paraquat and diquat and the cardiac stressor dobutamine (shifts nitroso-redox balance toward increased reactive species), compared to wild-type mice (Bartke et al., 2000; Bokov et al., 2009; Mattar & Haffor, 2009). Growth hormone receptor knockout mice do not exhibit a similar enhancement of antioxidative defense, suggesting that intact GH signaling is key (Hauck et al., 2002), whereas IGF1R$^{+/-}$ mice appear to respond favorably to paraquat challenge in comparison to wild-type mice with intact IGF1 receptor signaling (Holzenberger et al., 2003).

Enhanced resistance to multiple other stressors is also observed in GH mutant animals. A series of valuable studies conducted using cultures of primary fibroblasts in Ames, Snell, and GHRKO mice showed enhanced resistance to a variety of stressors including H_2O_2, the heavy metal cadmium, heat stress, UV light, and paraquat (Murakami et al., 2003; Salmon et al., 2005). Heat-shock proteins, metal chelators (metallothionein—also an antioxidant), and enzymes involved in protein repair (methionine sulfoxide reductase) are also elevated in dwarf mice (Brown-Borg & Rakoczy, 2005), lending credence to the overall suggestion that numerous defense systems are upregulated. Several gene expression studies also lend strong support to the evidence demonstrating enhanced defense against oxidants and other stressors (Al-Regaiey et al., 2005; Amador-Noguez et al., 2004; Boylston et al., 2006; Swindell, 2007; Tsuchiya et al., 2004). These reports show that multiple gene families are altered in GH mutant mice and include both phase I and phase II xenobiotic metabolism enzymes involved in detoxification and heat-shock proteins, among others. Finally, reduced GH and IGF1 signaling is one of the major mechanisms underlying the resistance to cancer development after administration of chemical carcinogens, the reduced growth of transplanted tumors, and the delay and reduced severity of spontaneous tumor incidence in dwarf mice (Bielschowsky & Bielschowsky, 1961; Conover & Bale, 2007; Flurkey et al., 2001; Ikeno et al., 2003, 2009; Rennels et al., 1965).

In calorie-restricted rodents, very similar evidence demonstrating elevated resistance to other stressors and reduced indices of neoplastic growth have been reported and reviewed in the context of GH and IGF1 signaling (Berryman et al., 2008; Kalaany & Sabatini, 2009). The reports are more mixed regarding enhanced oxidative defense in calorie-restricted rodents. Calorie restriction studies in humans and nonhuman primates also indicate that insulin sensitivity and metabolism are altered in a "healthy" direction (Colman, 2008; Colman et al., 2008, 2009; Heilbronn et al., 2006; Redman et al., 2009; Rezzi et al., 2009). Taken together, reduced somatotropic signaling is not only positively correlated with but also may directly affect the capacity of an organism to resist various types of stress. I believe that this overall upregulation in multiple defense mechanisms serves as a major contributor to the life-span extension observed in these mutant mice. Looking at decades of oxidative stress studies, in comparison, suggests that increasing the capacity of only one aspect of stress resistance (antioxidative enzymes) is less likely to affect overall longevity in an organism. The significance of a coordinated upregulation of resistance to several different stressors leading to longevity assurance has been proposed by several laboratories (Brown-Borg, 2007; Jazwinski, 1996; Martin et al., 1996; Piper & Bartke, 2008; Rollo, 2002). The evidence to date suggests that the somatotropic axis is involved in regulating multiple defense systems, perhaps in a coordinated manner that, in turn, affects aging processes and life span.

COGNITIVE FUNCTION

Declines in brain function, including both cognitive and motor activities, are considered a characteristic of aging. This deterioration results in changes in learning, memory, balance, and coordination. Age-related declines in behavioral activity have been reported in several strains of mice and are thus considered an index of aging (Dean et al., 1981; Gold et al., 1981; Kinney et al., 2001; Weinert & Waterhouse, 1999). It has been shown that GH, IGF1, and their respective receptors are produced in the brain at various sites other than the pituitary (Araujo et al., 1989; Breese et al., 1991; Lesniak et al., 1988; Zhai et al., 1994). IGF1 is also known to cross the blood–brain barrier and bind to IGF1 receptors that are expressed throughout the brain but concentrated at specific sites including the hippocampus (Reinhardt & Bondy, 1994). Somatotropic signaling influences components of central nervous system function. Animals that overexpress GH, characterized by high plasma levels of both GH and IGF1, exhibit impaired learning and memory and significant decreases in behavioral activities (Meliska et al., 1997; Rollo, 1999). Young normal mice (6 months of age) displayed more spontaneous locomotor activity (both ambulations and stereotypies) compared to young GH transgenic (6 months of age) and old normal mice (25 months of age; Meliska et al., 1997). In inhibitory avoidance learning tasks (both acquisition and retention), there were no differences between young GH transgenic mice and old normal mice, both of which differed significantly from young, normal mice. In some tasks, the young transgenic animals performed more poorly than old normal mice. These cognitive differences were apparent prior to the appearance of physical decline, suggesting that the differences were not due to deterioration of body condition. Premature aging of the CNS may be the underlying mechanism of the impaired learning and memory in these mice. Decreased central catecholamine metabolism and increased onset of significant astrogliosis are apparent at much younger ages in GH transgenic mice compared to normal mice (Miller et al., 1995; Steger et al., 1993). In addition, the age-related increase in plasma corticosteroid is advanced and more pronounced in mice that overexpress GH compared to wild-type mice and stress-induced elevations in plasma corticosteroids persist longer in these mice, resembling age-related dysregulation of glucocorticoid feedback (Miller et al., 1995).

A 2009 study examined the effect of short-term GH replacement on cognitive function in hypophysectomized rats (Kwak et al., 2009). In a Morris water maze assay, rats treated with GH exhibited shorter escape latencies on the third trial day compared to saline-treated rats, suggesting increased spatial memory acquisition. However, by days 4 and 5, GH-treated rats were no different from controls, indicating no long-term benefit of GH therapy. Insulin-like growth factor 1 can signal via IGF1 receptors, insulin receptors, and insulin/IGF hybrid receptors (Jones & Clemmons, 1995; Soos et al., 1993; Treadway et al., 1992). Thus, related evidence in animals with a deficiency in insulin receptor substrate signaling ($IRS1^{-/-}$) is also pertinent. These IRS1-deficient mice displayed better motor control, coordination, and balance at 450 days of age compared to controls, suggesting that behavior and cognition were intact in animals with defective insulin signaling (Selman et al., 2008). In addition, IGF1 gene-ablated mice were shown to exhibit brain growth retardation, reduced total brain size, impaired neuronal somatic and dendritic growth, a reduction in granule cell numbers in the hippocampus, plus fewer oligodendrocytes and lower neuronal density in the olfactory bulb (Cheng et al., 2003). Taken together, these data suggest that levels of GH, IGF1, and insulin in the brain are important factors in development and maintenance of function.

Growth hormone-deficient Ames dwarf mice do not exhibit the age-related declines in cognitive function (including memory) and behavior observed in wild-type mice (Kinney et al., 2001a,b; Kinney-Forshee et al., 2004). Similarly, GHRKO mice show no decline in cognitive function, while age-matched wild-type mice appear significantly impaired (Kinney et al., 2001). These studies suggest that the absence of peripheral GH signaling maintains or enhances memory retention compared to normal, wild-type mice. Both Ames and GHRKO mice exhibit delays in the age-related decline in locomotor activities (open-field testing) compared to their respective wild-type mice (Kinney et al., 2001a,b; Kinney-Forshee et al., 2004). In addition, Ames mice do not exhibit an age-related decline in behavioral activity as assessed by ambulations and stereotypies. The open-field tests also serve as a measure of emotionality in response to a novel environment. Both old and young GHRKO and wild-type mice did not differ in emotionality, confirming earlier studies using the elevated plus maze (Kinney et al., 2001a). No differences in emotionality were observed in Ames mice, while young normal mice appeared more fearful (anxious) of the novel environment. When GHRKO and Ames mice were tested using an inhibitory avoidance learning and memory paradigm, old Ames and GHRKO mice performed better than age-matched wild-type mice on the retention test (and were not different from young mice). In addition, old and young GHRKO mice performed similarly when spatial learning and memory were tested using a Morris water maze. Importantly, old wild-type mice performed poorly in comparison to young and old GHRKO mice. Together, these reports strongly suggest that cognitive and behavioral aging is delayed in GH mutant mice and that reduced peripheral GH signaling plays a substantial role. New evidence in support of this conclusion demonstrates that Ames mice exhibit enhanced acquisition and short-term spatial memory using a dry-land Barnes maze and a T-maze (Sharma et al., in press).

Other laboratories have addressed the contributions of GH and IGF1 to cognition and aging from a different perspective, by examining replacement strategies in aged rodents. Old rats (30 months) exhibit reduced brain microvascular density that is highly correlated with the 30–40% decline in brain IGF1 levels that occur with aging (Sonntag et al., 1997, 2000). This reduction in vascular density results in decreased cerebral blood flow (Sonntag et al., 2000). In these same animals, the age-related decline in spatial and reference memory was reversed with GH-releasing hormone or IGF1 treatment. In addition, the administration of an IGF1 antagonist to young animals impaired performance on this task (Sonntag et al., 2000). These reports suggest that GH or IGF1 could be important in maintenance of memory in aged animals via changes in the vasculature. Treatment of aged rats with GHRH from 9 to 30 months of age prevented age-related cognitive decline (Thornton et al., 2000), while IGF1 delivered by intracerebroventricular infusion for 28 days attenuated age-related deficits in memory (Markowska et al., 1998). Other studies indicate that short-term treatment with IGF1 or GHRH leads to changes in NMDA receptors, increased glucose utilization, and increased neurogenesis, each of which may contribute to improved learning and memory in aged animals (Le Greves et al., 2002; Lichtenwalner et al., 2001; Lynch et al., 2001; Sonntag et al., 2005).

These apparent differences between reports showing that GH mutant mice exhibit enhanced learning and memory and those showing that GH or IGF1 replacement improves cognitive function can be explained. First, we know that in Ames mice, neurogenesis is enhanced and hippocampal IGF1 and GH levels are elevated, both of which may lead to the maintenance of cognitive function in GH-deficient mice (Sun et al., 2005). We have evidence that neurogenesis is also significantly elevated in Ames mice compared to age-matched wild-type mice after challenge with a hippocampal oxidative stressor, kainic acid (H. M. Brown-Borg & S. Sharma, unpublished observations). Increased resistance to β-amyloid toxicity has been reported in the hippocampus of Ames mice (Schrag et al., 2008). Thus, the enhanced neurogenesis and neuroprotected environment in Ames animals appears to delay the declines in both locomotor and cognitive activities that are typically observed in aging wild-type mice. These observed differences in neuronal numbers under basal conditions as well as after a targeted challenge have not been evaluated in other long-living GH mutants but these studies are certainly warranted. Second, most of the other studies focused on aged rats, some of which were GH deficient only as adults. The GH mutant mouse data are based on lifelong reductions in peripheral GH signaling and, at least in the Ames mouse, higher IGF1 and GH in the central nervous system. Thus, the effects of GH and IGF1 are not as different as they first appear. We also have preliminary data showing that NMDA receptor subtypes are increased in Ames dwarf hippocampal neurons, a potential explanation related to their enhanced performance on these tasks (H. M. Brown-Borg & S. Sharma, unpublished observations).

In rodents and nonhuman primates, several studies have shown that caloric restriction maintains or enhances behavioral and cognitive functioning compared to ad libitum fed control animals. The tests utilized are wide-ranging and differ in sensitivity, and other factors appear to confound interpretation within and between studies (such as physical activity, species, age at initiation of CR), thus preventing the universal conclusion that CR is always beneficial. Calorie restriction does appear to protect the hippocampus, striatum, and cortical neurons and prevents functional decline in aging animals and those modeling human neurodegenerative disease (Bruce-Keller et al., 1999; Duan, 2001; Duan & Mattson, 1999; Fontan-Lozano et al., 2007; Ingram et al., 1987, 2007; Maswood et al., 2004; Patel et al., 2005; Stewart et al., 1989; Wang et al., 2005; Weed et al., 1997). In addition, according to Colman and co-workers (2009), brain volume is preserved in calorie-restricted rhesus macaques, indicating that CR reduced age-related brain atrophy in regions that involved both motor function and aspects of executive function. Taken as a whole, the evidence suggests that modifying dietary intake with a reduction in calories is neuroprotective and may prevent aging-induced declines in brain function.

There are several reports that show a relationship between serum IGF1 levels and various cognitive measures in aged humans with results ranging from positive associations to those showing no association at all (Aleman et al., 1999, 2000; Dik et al., 2003; Landi et al., 2007; Paolisso et al., 1997; Papadakis et al., 1995). Results of cognitive function assessment in elderly humans after GH or IGF1 therapy are similar. Women over age 60 treated with IGF1 for 1 year showed no improvement in memory-related tasks, but men (mean age 75 and low serum IGF1) treated with GH for 6 months performed better on the Trails B test compared to those receiving placebo (Friedlander et al., 2001; Papadakis et al., 1996). However, this same group of GH-treated men did not show any differences from the placebo-treated individuals on the Mini-Mental Status examination. A more recent study showed that 6 months of GH replacement in elderly patients that were GH deficient slightly improved cognitive function (serial digit learning) but no differences were observed after 12 months of GH (Sathiavageeswaran et al., 2007).

Overall, there appears to be some conflicting evidence in the literature regarding the roles of GH and IGF1 in cognitive function in aging. Rodents with reduced somatotropic signaling in the periphery demonstrate significant delays in cognitive and behavioral declines with age, while animals that overexpress GH display impairments in cognitive activity. Other reports suggest that in aged rats, short-term GH and IGF1 may rescue or improve function. Determining the peripheral versus central levels of these growth factors in the various model systems may assist in sorting out the true role of somatotropic action on cognitive function. In the rodent studies, the age of the animals, the underlying GH status, the species, as well as the timing and length of GH or IGF1 replacement therapy may well contribute to some of the differences reported. In the human and nonhuman primate studies, the data are even more difficult to interpret because the studies are mostly correlative or in calorie-restricted animals in which somatotropic signaling is indirectly reduced. Cognitive and behavioral activities in mammals do change with age; however, further studies are necessary to determine the specific roles of peripheral and central GH and IGF1 in these functions.

IMMUNE FUNCTION

The numbers of immune cells and their respective activities and the response to challenge have all been used in the evaluation of immune function as a biomarker of aging in mammalian species. It is well documented that B cells and specific subsets of T cells and their respective functions decline with aging, while inflammatory cytokine expression tends to increase with age (Anisimov, 2005; Hirokawa & Utsuyama, 2002; Kiecolt-Glaser et al., 2003; Mascarucci et al., 2001; Masoro, 2005; Merry, 2000). Many reports have examined the roles of GH and IGF1 in immune function but few of these have focused on the roles of these hormones in immunity and aging. The cross talk between the neuroendocrine and the immune systems is significant and the somatotropic signaling hormones have been at the center of this discussion. The data collected in support of this relationship include GH modulation of cytolytic activity of T lymphocytes, cytokine production, B cell activities and antibody synthesis, natural killer cell activity, phagocytosis and phagocyte migration, oxidative burst, and killing capacity of neutrophils and macrophages (reviews include Kelley et al., 2007; McCusker et al., 2006; O'Connor et al., 2008; Redelman et al., 2008). Cells of the immune system produce GH and express receptors for GH and IGF1 (all lineages of hematopoietic cells; see review by Pankov, 1999). The GH receptor is a member of the cytokine receptor superfamily, emphasizing the importance of this hormone in the cross talk and common regulatory mechanisms shared between these systems (Imada & Leonard, 2000). This cross talk is possible among GH and the cytokines via the JAK–STAT pathway, which is involved in several receptor-mediated signaling processes including GH, PRL, IFN-γ, IL2, IL3, IL4, IL5, IL6, IL7, IL12, IL13, IL15, GM-CSF, and others. In the context of aging, some of the reports on immune function have addressed reproductive senescence (menopause–ovariectomy models), while others focus on the decline of somatotrope signaling and its relationship to immune function with age (Baeza et al., 2008, 2009; Dixit et al., 2007; Tresguerres et al., 2008).

Both plasma GH and thymic volume decline with increasing age in mammals. These observations led to early experiments in which administration of GH was shown to expand the number of thymocytes and thymic epithelial cells, prevent thymic involution, and improve thymic function (de Mello Coelho et al., 2002; Dobashi et al., 2001; Timsit et al., 1992; Yamada et al.,1994). Transgenic mice overexpressing GH or GHRH are characterized by thymic and splenic overgrowth and increased mitogenic responses to concanavalin A, lipopolysaccharide, and phytohemagglutinin (Dialynas et al., 1999; Hall et al., 2002). Thymulin, a peptide produced by thymic epithelial cells, is involved in numerous aspects of T cell differentiation (Bach, 1983) and is known to decline with aging (Folch et al, 1986). The production and secretion of this thymic peptide are controlled by neuroendocrine hormones, including GH, which stimulates its synthesis and release (Timsit et al., 1992; Ban et al., 1991; Goff et al., 1987). Thymulin levels were increased in GH transgenic animals and significantly decreased in GHRKO animals compared to their respective wild-type mice (Savino et al., 2003). In agreement, studies of old rats implanted with syngeneic GH_3 cells (pituitary tumor line) showed improvement of thymic cell numbers and function (Kelley et al., 1986). Work from this same laboratory (French et al., 2002) used 24-month-old rats and provided either the GH-producing tumor cells (GH_3) or recombinant human GH and found similar effects on the thymus gland plus increased hematopoietic precursor cells, extensive extramedullary hematopoiesis, and reduced adipocytes in the bone marrow. Another report also demonstrated that GH administration in 24-month-old rats reduced or even reversed the age-related changes in several key immune function parameters (to the levels of 6-month-old animals; Baeza et al., 2008). Insulin-like growth factor 1 administration to older mice increased thymic cellularity twofold after sublethal irradiation and transplant with syngeneic young bone marrow cells (Montecino-Rodriguez et al., 1998). Studies examining the immune-enhancing effects of GH secretogogues in aging have been under way in the pharmaceutical industry for years (Koo et al., 2001). The downside of GH overexpression on immune parameters includes fewer peripheral $CD8^+$ cells compared to control animals and age-matched Ames dwarf mice, along with low specific antibody responses, indicating premature aging despite the enlargement of both the thymic and the splenic tissues (Hall et al., 2002). Specific populations of T cells, including naïve $CD8^+$ cells, are known to decline with aging, while CD4 and CD8 memory cells accumulate with aging (Chen et al., 2010; Gruver et al., 2007; Hadrup et al., 2006; Herndon et al., 1997).

In contrast, animals with GH deficiency exhibit delayed splenomegaly and T cell proliferative responses, both of which are characteristic of an aging immune system (Flurkey & Harrison, 1990). Additional age-sensitive immune markers were examined in dwarf mice in a life-span report by Flurkey and co-workers (Flurkey et al., 2001). The expression of CD4 memory and CD8 memory positive cells, as well as specific subsets of these memory T cells (expressing P-glycoprotein activity), in aged dwarf mice was at levels similar to those in young normal mice, versus the high levels expressed in aged normal mice. Aging effects on T cell function were also observed to be blunted in dwarf mice compared to wild-type mice, suggesting that indeed, GH deficiency was associated with delayed immunosenescence. Esquifino and co-workers showed that the numbers of splenic and thymic lymphocytes were lower in GH-deficient Ames mice and that those numbers were normalized after extrapituitary engraftment or administration of bGH (Esquifino et al., 1991; Villanua et al., 1992). In addition, B cell antibody response to antigen challenge in Ames mice does not differ from that of wild-type mice but the numbers of T helper 2 cells were greater in these animals (Hall et al., 2002). These mice are considered mildly immunodeficient based on immune cell numbers but are not compromised functionally (Cross et al., 1992; Esquifino et al., 1996). A critical report by Cross and co-workers (Cross et al., 1992) showed that the immunocompetence differences between dwarf and wild-type mice were dependent on the time of weaning, suggesting that development of the immune system was lagging behind that of wild-type mice but eventually the immune system of dwarfs was equally responsive (by 37 days of age). Analysis of peripheral blood leukocyte gene expression profiles in Ames mice showed that the expression of numerous anti-inflammatory and anticlotting genes was suppressed (Dhahbi et al., 2007). Collectively, these studies suggest that GH plays an important role in the expression of various immune parameters but that GH deficiency does not lead to an immunocompromised state. Rather, lower inflammatory markers and delayed expression of age-sensitive immune system indices in GH-deficient mice may contribute to their delayed aging and life-span extension.

There are many similarities in immune parameters between dwarf mice and calorie-restricted mice. Caloric restriction delays development of immunity and prevents the decline in immune function that typically occurs with age, including cytokine production, T cell proliferation, cytotoxic T cell activities, cell- and antibody-mediated responses to viral challenge, and natural killer cell activities (Effros et al., 1991; Masoro, 2005; Messaoudi et al., 2006; Nikolich-Zugich & Messaoudi, 2005; Pahlavani, 2004; Ritz & Gardner, 2006; Weindruch et al., 1983, 1986; Weindruch & Walford, 1982). In an artificial model of bacterial infection (polymicrobial peritonitis induced by cecal ligation and puncture), young calorie-restricted mice died earlier than control mice and expressed lower levels of macrophage markers, suggesting a potential delay in maturation of macrophage function in these mice (Sun et al., 2001). The physiological mechanisms underlying the beneficial effects of calorie restriction on immune function are unknown but may be related to neuroendocrine factors. Definitive studies to address the hormonal control of immunity in calorie-restricted states are needed.

AGE-RELATED PATHOLOGY AND NEOPLASIA

It is logical to conclude that with growth hormone's widespread effects on components of metabolism and somatic growth as aided by IGF1, there are likely to be long-term consequences dependent on the presence or absence of hormone action. The presence of high circulating levels of this hormone in rodents is strongly associated with signs of premature and accelerated aging. Growth hormone transgenic rodents display early onset age-related kidney pathology in the form of glomerulonephritis and glomerulosclerosis (Doi et al., 1988; Quaife et al., 1989; Wanke et al., 1992). Multiple studies have shown that glomerular basement membrane thickening occurs with normal aging in rodents and primates (Dodane et al., 1991; Schaeverbeke et al., 1988; Duan & Nagata, 1993; Cusumano et al., 2002). Basement membranes of glomeruli exhibit enhanced production of extracellular matrix components in GH transgenic animals leading to early onset and progressive kidney disease. Specifically, gene expression of type IV collagen, laminin, and basement membrane heparan sulfate proteoglycan is remarkably increased in animals overexpressing GH compared to normal littermates, with lesions resembling diabetic nephropathy (Doi et al., 1991). Further work in these mice showed that early stage renal lesions were characterized by glomerular hypertrophy and mesangial expansion leading to a mean glomerular volume twice that of age-matched controls at 7 weeks of age, as well as significant podocyte dysfunction (Wanke et al., 1992). Hypophysectomized (no plasma GH) rats exhibit significant decreases in glomerular basement membrane collagen synthesis and GH replacement restores this activity (Reddi, 1985). In GH-deficient Ames mice, renal glomerular basement membrane thickness is dramatically reduced throughout the life span in comparison to wild-type mice and is thought to contribute to their delayed aging (Meyer et al., 2003). In addition, the onset of age-related pathology is reduced and the incidence and severity of nonneoplastic diseases including glomerulonephritis are significantly attenuated in dwarf mice (Ikeno et al., 2003). Furthermore, in calorie-restricted rodents, kidney disease is averted (Stern et al., 2001; Wiggins et al., 2005).

Relatively young (6–8 months of age) GH transgenic mice begin to display numerous physical signs of aging, including weight loss, scoliosis, and coat deterioration. In addition, they exhibit early onset astrogliosis, reduced hypothalamic neurotransmitter turnover, and an increase in age-related plasma corticosterone levels (Miller et al., 1995; Steger et al., 1993). As mentioned previously, they also undergo reproductive maturity and senescence at much younger ages (Bartke et al., 1994; Chandrashekar et al., 1988; Steger et al., 1993), most likely as a result of altered IGF1 levels and actions (Chandrashekar & Bartke, 2003).

Growth hormone and IGF1 are anabolic hormones that support cell proliferation and prevent apoptosis and, as such, high levels of either lead to the development of cancer. Significant mammary and liver tumors are observed in GH transgenic rodents at relatively young ages in comparison to wild-type mice (Cecim et al., 1994; A. Bartke & Y. Ikeno, unpublished data). Liver tumors in these mice are characterized by the presence of apoptotic cells, hypertrophic cells, and a large proportion of dysplastic hepatocytes (Snibson, 2002).

In contrast to GH overexpression, the absence of GH action leads to a decreased propensity to develop tumors. Dwarf mice and rats resist cancer development after administration of chemical carcinogens and exhibit a reduction in the growth of transplanted tumors (Bielschowsky & Bielschowsky, 1961; Chen et al., 1972; Ramsey et al., 2002; Rennels et al., 1965). Spontaneous tumor incidence is delayed in dwarf mice as well as the severity of the tumors and their progression (Flurkey et al., 2001; Ikeno et al., 2003; Mattison et al., 2000). Lower spontaneous DNA mutation frequencies were observed in Ames dwarf liver, kidney, and small intestine compared to wild-type, mice indicating reduced somatic mutation accumulation in specific tissues (Garcia et al., 2008). This, in turn, may explain the delayed appearance of other age-related pathologies as well as the life-span extension observed in dwarf mice (Vijg & Dolle, 2002; Garcia et al., 2008). In support of the findings in Ames and Snell dwarf mice, it was recently shown that GHRKO mice also exhibit a reduced incidence and delayed occurrence of fatal neoplastic diseases (Ikeno et al., 2009). Furthermore, LID mice exhibit circulating levels of IGF1 that are 25% of that in control mice (Liu et al., 2000). Mammary tumor development was delayed and the number of tumors was significantly lower in LID mice compared to wild type (Wu et al., 2003). In addition, in the setting of obesity, LID mice exhibited lower liver inflammatory responses and a lower incidence of hepatic metastasis after inoculation with colon carcinoma cells (Wu et al., 2010), further indicating the significant role of IGF1 in cancer risk.

Similarly, numerous studies have shown that calorie restriction is associated with reduced tumor incidence and progression, some of which have attributed this protection to lower growth factor levels (Gross & Dreyfuss, 1984; Weindruch, 1996; Weindruch et al., 1986). Elegant studies by de Cabo and co-workers show that CR increases NF-E2-related factor (Nrf2) expression that, in turn, upregulates antioxidative and carcinogen-detoxification enzymes, resulting in anticarcinogenic effects (Pearson et al., 2008). Tissues from Ames mice exhibit similar increases in Nrf2 expression (H. M. Brown-Borg, unpublished data). The PI3K pathway has also been shown to be an important determinant of the sensitivity of tumors to CR (Kalaany & Sabatini, 2009). Caloric restriction also results in significantly reduced mutation frequencies (Garcia et al., 2008), similar to those observed in dwarf mice. Additional support is derived from studies in heterozygous p53-deficient mice. Calorie restriction in p53-deficient mice significantly decreased tumor progression in comparison to ad libitum fed control mice after treatment with a bladder carcinogen (Dunn et al., 1997). As expected, serum IGF1 concentrations were decreased 24% in the calorie-restricted mice. However, when recombinant IGF1 was restored in the CR p53-deficient mice, tumor progression was enhanced. The rates of apoptosis in the preneoplastic lesions were 10 times higher in the CR-treated mice compared to the IGF1/CR-treated mice and the ad libitum fed controls, suggesting that the decreased serum IGF1 that occurs as a consequence of CR is responsible for the protective effect of CR on neoplastic progression.

The lack of circulating GH and IGF1 also contributes to delayed tail-tendon collagen cross-linking, an index of aging in extracellular macromolecules, and a reduction in osteoarthritis in knee joints of dwarf mice compared to normal control mice (Flurkey et al., 2001; Silberberg, 1972). Similarly, collagen cross-linking in skin, tail tendon, and the aorta is significantly reduced in diet-restricted mammals (Reiser, 1994; Sell et al., 1996). Taken together, multiple indices of delayed aging are observed in GH-deficient dwarf mice and rodents subjected to long-term calorie restriction.

Of significance is a study by Yuan and co-workers (2009) that evaluated aging in 31 genetically diverse inbred mouse strains. Median life spans were negatively correlated with circulating IGF1 levels at 6, 12, and 18 months of age, strongly suggesting that IGF1 level is involved in regulating longevity.

CONCLUSION

It is clear that growth hormone plays a role in the functioning of multiple organ systems and metabolic pathways. Plasma levels of GH and its main downstream effector, IGF1, contribute to alterations in body composition, glucose, and oxidative metabolism as well as cognitive and immune function and age-related pathologies. High levels of GH and IGF1 generally exert negative effects on metabolism and cause age-related pathologies, with observations of premature or accelerated aging of many organ systems and significantly reduced longevity. On the other hand, GH deficiency in rodents leads to a delayed or decelerated expression of several key age-sensitive characteristics. This slowing of age-related processes results in significant increases in longevity. Calorie restriction exerts very similar actions on age-related processes, although the magnitude of difference from controls can be different. Further studies delineating the pathways that lead to delayed expression of these markers of senescence are of great interest and could lead to significant therapeutic interventions in human aging.

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Chapter 3

Mechanisms of Mitochondrial Free Radical Production and their Relationship to the Aging Process

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CHAPTER CONTENTS

Introduction 47
Mitochondrial Generation of Free Radicals 48
Major Sites of Mitochondrial ROS Generation 48
  Complex I 48
    Complex I ROS Production 48
    Complex I Inhibitors 50
  Complex III 50
    Complex III ROS Production 50
    Complex III Inhibitors 50
  Other Sites of Mitochondrial ROS Production 51
    Complex II 51
    α-Glycerolphosphate Dehydrogenase 51
    Electron Transfer Flavoprotein–Ubiquinone Oxidoreductase (ETF–Q Oxidoreductase) 51
    Dihydrolipoamide-Containing Enzyme Complexes 52
  Physiological ROS and its Measurement in vivo 52
Mitochondrial Antioxidant Systems 52
Free Radical Theories of Aging 54
Conclusions 56
Acknowledgments 57
References 57

INTRODUCTION

Mitochondria are critical to the life and death of a cell. Their importance in such processes as metabolism, energy conversion, and apoptosis has raised them to the forefront of many physiological and pathological hypotheses. Of these hypotheses, one of the most intriguing is the free radical theory of aging, first proposed by Harman (1956). Oxygen radicals are produced during enzymatic redox chemistry in biological systems, and this theory proposes that these radicals result in oxidative damage, which is the cause of aging. In the wake of the free radical theory, many associated hypotheses have appeared; of particular interest to this report is the mitochondrial free radical theory of aging. The mitochondrion's tendency to produce reactive oxygen species (ROS) quickly elevated it to importance in the field of aging. The potential role of mitochondria in aging led to the formation of the oft-cited "vicious cycle" subhypothesis. The vicious cycle proposes that ROS generated by mitochondria damage mitochondrial DNA (mtDNA), creating mutations in the DNA that lead to compromised electron transport chain complexes, which in turn lead to greater ROS production and more damage (Hiona & Leeuwenburgh, 2007; Wei, 1998). The mitochondrial free radical theory of aging is not contingent upon the vicious cycle hypothesis, which is a separate subtheory. This chapter aims to explore mitochondrial reactive oxygen species and specifically to explore their production and neutralization. The free radical aging hypothesis cannot be properly tested without a thorough understanding of the ROS production mechanisms. Our primary focus is on the mechanisms and sites of ROS production and,

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secondarily, on how it fits into the context of aging and central tenets of the free radical aging hypotheses. To contextualize this analysis, ROS-related mitochondrial physiology and biochemistry will also be presented.

MITOCHONDRIAL GENERATION OF FREE RADICALS

Mitochondria consume oxygen while performing the critical task of energy conversion for cells. In brief, electron donors generated during the Krebs cycle funnel their electrons into NADH:Q oxidoreductase (complex I) and fatty acid beta-oxidation or succinate:Q oxidoreductase (complex II) and ETF:Q oxidoreductase of the electron transport chain. These electrons are subsequently passed downstream through the ubiquinone (Q) pool to the cytochrome bc_1 complex (complex III). They finally pass into cytochrome oxidase (complex IV) where they participate in the coordinated four-electron reduction of oxygen to water. As electrons move down the electron transport chain, protons are simultaneously pumped across the membrane from the matrix to the intermembrane space. This proton pumping generates the proton-motive force (PMF). This consists of two transmembrane components: a pH gradient and an electrical potential. A full description of mitochondrial bioenergetics is beyond the scope of this chapter, but interested readers are encouraged to read Nicholls & Ferguson (2002). In this context, we are interested in the premature reduction of oxygen that results in the formation of reactive oxygen species.

Diatomic oxygen, despite itself being a strong oxidant, is surprisingly unreactive in the classic sense. Oxygen's two unpaired electrons are segregated into different molecular orbitals and have parallel spins. For oxygen to accept two electrons, the donating species' electrons must possess antiparallel spins relative to the unpaired electrons of oxygen. The rarity of this in nature implies that oxygen prefers to accept its electrons one at a time. Therein lies the propensity for single-electron reduction of oxygen. In vivo, enzymes typically catalyze reduction of oxygen serially in the presence of transition metals such as iron or copper to generate water or hydrogen peroxide (H_2O_2). In the absence of the controlled enzymatic environment, one-electron reduction of oxygen will yield the superoxide anion ($O_2^{\bullet -}$) and two-electron reduction gives hydrogen peroxide.

Although there are at least seven potential sites of superoxide formation in mitochondria, they produce ROS predominantly at two electron transport complexes: complex I and complex III of the electron transport chain. The amount of ROS produced is frequently debated. Early work has often been erroneously interpreted to suggest that it is as much as 1–4% of O_2 consumed and it is not uncommon for papers to cite this overestimated statistic. More recent data indicate that physiologically relevant conditions will give substantially lower values, perhaps as low as 0.15% (Hansford et al., 1997; St-Pierre et al., 2002). A more thorough discussion of our understanding of sites and amounts of ROS production is presented in the following section.

MAJOR SITES OF MITOCHONDRIAL ROS GENERATION

Within the mitochondrial electron transport chain, the formation of superoxide is known to occur after the reduction of certain precursor species. The generation of a semiquinone radical (SQ) by the partial reduction of ubiquinone within specific enzymes and complexes can result in superoxide. Other redox centers, including flavins (FMN and FAD) and Fe–S centers, have also been implicated in the generation of ROS by mitochondria. Figure 3.1 shows a cartoon of the superoxide precursor species.

Complex I

Complex I ROS Production

The first complex within the electron transport chain oxidizes NADH generated during oxidation of Krebs cycle and beta-oxidation substrates and passes the electrons on to Q and into the rest of the electron transport chain. Electron transfer starts with the sequential transfer of two electrons to the flavin mononucleotide moiety (FMN). These electrons then move through several of the eight identified Fe–S clusters of the complex to the ubiquinone reduction site where they ultimately reduce quinone to hydroquinone. Mammalian complex I consists of at least 45 subunits. The crystal structure of the complex has yet to be fully solved. The details of electron transfer and proton conductance across the membrane are a matter of contention and beyond the scope of this review (Ohnishi & Nakamaru-Ogiso, 2008; Yakovlev et al., 2007; Zickermann et al., 2009). In this context, complex I is viewed as a major source of superoxide generation by mitochondria. It has been suggested to be the most significant source of physiologically relevant ROS generation (Zickermann et al., 2009; Murphy, 2009; Herrero & Barja, 1997; Kushnareva et al., 2002; Nakamura et al., 2001), although the evidence for this assertion is circumstantial.

In experiments performed on isolated mitochondria, superoxide from complex I can be generated in the forward electron flow direction (e.g., respiration driven by the NADH-generating substrates pyruvate and malate). It can also be generated by reverse electron transport, which can be driven by succinate

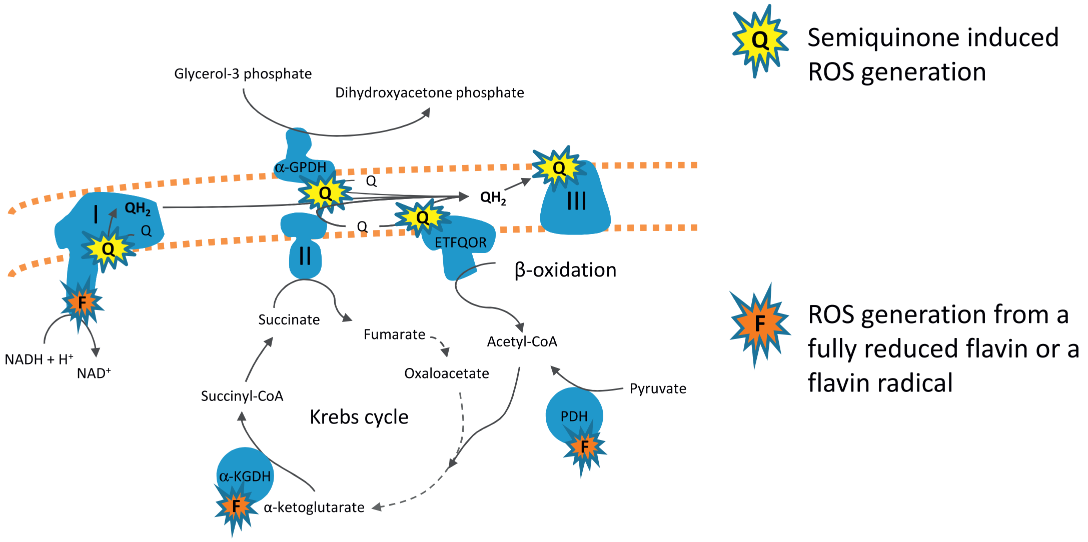

Figure 3.1 The production of superoxide in mitochondria can be instigated by a number of enzymes. We propose that superoxide arises from either a quinone or a flavin precursor species. Those enzymes that are thought to generate superoxide from a semiquinone precursor are shown with a "Q", and those that are proposed to generate radicals from a fully reduced flavin or a flavin radical are shown with an "F". Complex I, the NADH:Q oxidoreductase (I), is shown with both a flavin and a semiquinone possibility for the superoxide precursor species. There is some evidence that the Fe–S centers of complex I may form ROS (Genova et al., 2001), but this is not generally accepted and is not shown here. α-Glycerolphosphate dehydrogenase (α-GPDH), electron transfer flavoprotein–quinone oxidoreductase (ETFQOR), and cytochrome bc_1 complex (III), are shown with a semiquinone precursor species. As both α-ketoglutarate dehydrogenase (α-KGDH) and pyruvate dehydrogenase (PDH) are flavin-containing enzymes that generate superoxide, it seems likely that the flavin is the superoxide producer in these enzymes, but this has yet to be shown experimentally.

oxidation in the absence of rotenone. Superoxide formation may be from separate sites depending on the conditions, primarily the direction of electron flow (Figure 3.1). The observed native rates of superoxide in the forward direction are relatively low because the complex is running largely oxidized. However, the addition of a downstream inhibitor such as rotenone will block electron flow out of the complex and allow full reduction of the FMN. This will result in maximum superoxide production from this site (Hansford et al., 1997; Fato et al., 2009; Kussmaul & Hirst, 2006; Ohnishi et al., 2005). Some investigators have suggested a role for the Fe–S clusters in this phenomenon (Genova et al., 2001), but most consider that the site of superoxide production in the forward direction is the fully reduced flavin of the FMN moiety (Kussmaul et al., 2006).

High rates of superoxide are generated during reverse electron transport. This superoxide is thought to arise not from the flavin site but from the formation of a semiquinone in the quinone binding region of complex I (Ohnishi et al., 2005; Lambert & Brand, 2004b). This is supported by data indicating a dissociation of the superoxide production rate and the redox state of the NADH/NAD$^+$ couple (Lambert et al., 2008b). The NADH/NAD$^+$ couple is used as a reporter for the reduction state of the flavin, and if the fully reduced flavin was solely responsible for the superoxide generated during reverse electron transport, then a more reduced NADH signal should be observed under conditions of highest superoxide production. However, what is observed is a partially reduced NADH pool when ROS conditions are highest, suggesting a species other than the flavin as the source of precursor for superoxide during reverse electron transport (Lambert et al., 2008b). Also examined were the differential effects of the inhibitor diphenyleneiodonium during forward and reverse transport-mediated superoxide production from complex I (Lambert et al., 2008a). Diphenyleneiodonium (DPI) has been shown to bind to flavin groups and to complex I, and so it has been assumed to be an inhibitor of flavin-mediated complex I superoxide production (Majander et al., 1994; Ragan & Bloxham, 1977). Although DPI does inhibit superoxide production during reverse electron transfer, it does not do so by binding to the reduced flavin in complex I, but by acting at a second site, presumed to be the quinone binding site (Lambert et al., 2008a). The superoxide formation during reverse electron transfer is particularly sensitive to the ΔpH component of the PMF, implicating the proton-pumping mechanism in the

ROS formation (Lambert & Brand, 2004a). There is also a ΔpH and PMF dependence of ROS production from this site in complex I during forward electron transport (Lambert et al., 2004b).

Complex I Inhibitors

As discussed above, the use of inhibitors that target specific sites within the complex can be very useful in the laboratory. For example, inhibition of the Q binding region of complex I (IQ) has shed some light on the production of ROS. Rotenone is the most commonly used IQ inhibitor; however, myxothiazol and piericidin also fall into this broad class of inhibitors (Degli Esposti et al., 1993, 1996; Fato et al., 2009; Lambert et al., 2004b). Inhibitor specificity can be complicated when working with intact mitochondria because myxothiazol also binds tightly to and inhibits complex III (Degli Esposti et al., 1993). Most of the high rates of superoxide production found during reverse electron transport with substrates such as succinate can be abolished with the addition of IQ inhibitors, which block electron flow into the sites of superoxide production. Under conditions that can generate a high NADH/NAD$^+$, such as during forward electron transport with NADH-generating substrates, IQ inhibitors result in a marked increase in superoxide production from the flavin site. Under this same condition the PMF is abolished because protons are not pumped. However, when a PMF is generated by the addition of exogenous ATP, the ROS production under this condition is markedly increased, from site IQ (Lambert et al., 2004b). DPI, as discussed above, is not a simple flavin site inhibitor and should be used with care (Lambert et al., 2008a).

Complex III

Complex III ROS Production

The cytochrome bc_1 complex (complex III) oxidizes the ubiquinol (QH_2) produced by complex I, complex II, and other Q-linked dehydrogenases. It passes the electrons onto cytochrome *c* and cytochrome *c* oxidase for final reduction of oxygen to form water. This is accomplished by the Q cycle, originally proposed by Mitchell (1975a,b) and revised by several groups (Crofts & Berry, 1998; Crofts, 2004; Trumpower, 1976; Chobot et al., 2008; Osyczka et al., 2005). To conceptualize superoxide production from complex III properly, a mechanistic appreciation of the Q cycle is useful. Each complete turnover of the Q cycle oxidizes one QH_2 molecule from the membrane Q pool and reduces two cytochrome *c* molecules. This process can be broken into two steps, or half-turnovers of the enzyme. In the first step, a molecule of QH_2 binds in the Q_o site of the complex (so called because it is located on the outer face or cytosolic side of the protein) and delivers its electrons divergently down two paths. The first electron is sent down the high-potential chain to the Rieske Fe–S protein. It then sequentially reduces cytochrome c_1 and cytochrome *c*. The second electron is sent down the low-potential chain, which consists of two cytochrome *b* hemes. It ultimately reduces a quinone to a SQ molecule in the Q_i site (so called because of its location near the inside or matrix side of the protein). Therefore, at the end of the first half-turnover of the enzyme, one QH_2 has been oxidized to Q in the Q_o site, one quinone has been partially reduced (to a SQ) in the Q_i site, and one cytochrome *c* has been reduced. The second step, and completion of the Q cycle, requires the oxidation of a second QH_2 at the Q_o site and a repeat of step 1 above. However, in this case the electron that is sent through the low-potential cytochrome *b* chain now encounters a SQ in the Q_i site and reduces it to form a fully reduced QH_2. Therefore, at the end of step 2, a second QH_2 has been oxidized to Q in the Q_o site and the electrons have gone to reduce a second cytochrome *c* and fully reduce the SQ in the Q_i site. In total, the complete Q cycle results in the oxidation of two QH_2 molecules in the Q_o site, the passage of two electrons to cytochrome *c*, the sequential reduction of one Q to form a QH_2 in the Q_i site, and the electrogenic extrusion of the equivalent of two positive charges, with two protons disappearing on the matrix side of the membrane and four protons appearing on the cytosolic side.

Superoxide production from complex III is thought to arise from the single-electron reduction of oxygen by a SQ radical formed in the Q_o site. As described above, the rapid bifurcated electron transfer that occurs during quinol oxidation limits the SQ occupancy of the Q_o site. It thereby limits the formation of superoxide during normal Q-cycle activity. However, in the presence of the Q_i site inhibitor antimycin A, oxidation of the *b* hemes is restricted and therefore the transfer of the second electron to the *b* hemes is limited. Electrons back up on the *b* hemes, limiting the turnover of the enzyme, increasing the concentration of SQ in the Q_o site, and increasing the formation of superoxide (Turrens, 1997). This process may also occur to some extent under physiological conditions when a high membrane potential causes a back-pressure that restricts electron flow through the *b* hemes (Turrens et al., 1985).

Complex III Inhibitors

In the laboratory, antimycin A is often used as a positive control for superoxide generation, as it will very predictably lead to the production of measurable quantities of superoxide from mitochondria or isolated bc_1 complex. As mentioned above, this superoxide is thought to be generated in the Q_o site by the reaction of SQ with oxygen. Two other inhibitors are often employed in the laboratory to modulate superoxide production from complex III. Stigmatellin and myxothiazol are common representatives of two classes of Q_o site inhibitors. Although they both bind to the Q_o site and prevent quinol oxidation, they have distinct effects on mitochondrial superoxide production. Stigmatellin (and its analog 5-undecyl-6-hydroxy-4,7-dioxobenzothiazol) binds proximally in the Q_o site and competitively excludes quinol binding (Crofts, 2004). The crystal structure indicates that stigmatellin binds to the histidine residue on the Rieske Fe–S protein that hydrogen bonds to the QH_2 in the first step of QH_2 oxidation (Crofts, 2004). Stigmatellin will therefore prevent QH_2 binding and SQ formation and eliminate the superoxide production observed in the presence of antimycin A. Myxothiazol, and related MOA-type inhibitors (those that contain the characteristic sturctural β-methoxyacrylate (MOA) group) such as MOA-stilbene or mucidin, bind distal in the Q_o site in closer proximity to heme b_L (Zhang et al., 1998). This indicates that the QH_2 binding is not entirely excluded in the presence of myxothiazol. This changes the dynamics of the inhibitory action, and antimycin A-stimulated superoxide is not entirely inhibited by the presence of myxothiazol (Muller et al., 2002). Interestingly, in the absence of antimycin A, the binding domain of myxothiazol will allow some quinol oxidation to occur. It will therefore slightly increase superoxide generation on its own (Sun & Trumpower, 2003). It should be noted that inhibition of sites downstream of complex III, e.g., by using cyanide to inhibit cytochrome oxidase or by removal of cytochrome *c* during initiation of apoptosis, does not lead to an increase in superoxide from complex III. In fact, downstream inhibition will largely eliminate superoxide production because the Rieske Fe–S center becomes reduced and cannot accept the first electron from QH_2 to allow SQ generation in the Q_o site (Borek et al., 2008).

Generally, experiments designed to assess mitochondrial ROS production use respiratory chain inhibitors to isolate the specific sites. This approach has the huge advantage of allowing maximal ROS production from well-defined sites in the electron transport chain. Of course, the major limitation of this approach is the inherently nonphysiological constraints that are imposed with the addition of pharmacological inhibitors to the system. Invaluable knowledge has been gained from the use of respiratory inhibitors, but it is important to the advancement of our understanding of mitochondrial ROS to begin to assess ROS production under physiologically relevant conditions.

Other Sites of Mitochondrial ROS Production

Complex II

Complex II is a Krebs cycle enzyme that oxidizes succinate to fumarate and feeds the resulting electrons into the electron transport chain. Although succinate is often used to reduce Q via complex II, it is important to stress that complex II is not normally a substantial source of ROS production by mitochondria (St-Pierre et al., 2002; Murphy, 2009; Drose & Brandt, 2008). Instead, it is the interaction of the QH_2, generated by succinate oxidation, with complex I or III that forms superoxide. Analysis of the structure of *Escherichia coli* complex II, which is very similar to the mammalian complex, suggests that the Fe–S redox centers are physically shielded from exposure to oxygen. This may explain some of the lack of ROS production (Yankovskaya et al., 2003). Also in *E. coli*, an important protective effect is caused by electron redistribution to the *b* heme, preventing SQ formation during partial reduction of the enzyme; however, this may not be as effective in the mammalian enzyme. Fumarate reductase lacks the heme, and thereby this protective mechanism, so it is a good generator of ROS when driven in reverse as a succinate dehydrogenase (Messner & Imlay, 2002; Cecchini et al., 2002).

α-Glycerolphosphate Dehydrogenase

The mitochondrial α-glycerolphosphate dehydrogenase (m-αGPDH), a FAD linked flavoenzyme that feeds electrons into the electron transport chain at the level of Q, can form superoxide at substantial rates. For example, in fruit fly mitochondria, where m-αGPDH is highly expressed, the rate of ROS production from the enzyme is among the highest observed (Miwa et al., 2003; Miwa & Brand, 2005). Mammalian mitochondrial ROS generation by m-αGPDH is tissue-dependent (Kwong & Sohal, 1998). It is significant where this enzyme is found at high levels, such as the brain (Tretter et al., 2007) and brown adipose tissue (Drahota et al., 2002; Vrbacky et al., 2007). Radical generation during glycerol phosphate oxidation occurs in part by reverse electron transport at complex I, which is highly sensitive to small decreases in PMF and can be blocked by rotenone (Miwa et al., 2005; Tretter et al., 2007). It is unclear whether the site of radical generation by m-αGPDH is the flavin or Q binding site. The flavin binding region is located outside of the membrane (Yeh et al., 2008); therefore if the flavin were the site of superoxide production, all the observed ROS would be produced to the outside of the mitochondrion. However, ROS are released to both sides of the membrane, indicating that the flavin is not the main site of production (Miwa et al., 2005) and implicating the Q binding site instead.

Electron Transfer Flavoprotein–Ubiquinone Oxidoreductase (ETF–Q Oxidoreductase)

Many tissues rely on the oxidation of long-chain fatty acids as a major aerobic fuel source. This may be a physiologically significant but underappreciated source of ROS generation. Mitochondria oxidizing palmitoyl carnitine produce significant ROS but the site of production is not clear. The electron-transferring flavoprotein of β-oxidation accepts electrons from the reduced acyl-CoA dehydrogenase and transfers them to Q via the enzyme ETF–Q oxidoreductase, which is a candidate for ROS production (St-Pierre et al., 2002). Moreover, by reducing the Q pool and generating NADH, this pathway (β-oxidation) may also contribute to superoxide production at complex I (St-Pierre et al., 2002; Murphy, 2009).

Dihydrolipoamide-Containing Enzyme Complexes

Pyruvate and α-ketoglutarate dehydrogenase complexes (PDH and αKGDH, respectively) are both mitochondrial matrix enzyme complexes that contain a FAD-linked dihydrolipoamide dehydrogenase component. Flavins can make superoxide or H_2O_2, and the isolated complexes PDH and αKGDH can form both (Bunik & Sievers, 2002; Starkov et al., 2004). However, it has not been definitively shown that the flavin is the ROS-producing site in these enzymes. The rate of superoxide production by the isolated complexes is elevated in the presence of substrate and CoA–SH, concomitant with the absence of NAD^+, which is the terminal electron acceptor of the catalyzed reaction. In brain mitochondria, αKGDH may be one of the major sites of ROS formation (Starkov et al., 2004). Brain mitochondria from apoptosis-inducing factor-deficient harlequin mice display a reduction in complex I level and activity, but ROS production with NADH-generating substrates is unaffected (Chinta et al., 2009). The lack of correlation between the amount of complex I and the observed ROS production indicates that sites other than complex I, such as αKGDH, are significant sources of ROS in brain mitochondria.

Physiological ROS and its Measurement in vivo

It is apparent from the preceding sections that our knowledge of the sites and mechanisms of mitochondrial ROS production is based almost entirely on in vitro assays in the presence of electron transport inhibitors. For example, it is not known whether reverse electron transport occurs in cells or under other more physiologically relevant conditions. To address this issue, a more thorough understanding of the in vivo conditions, such as substrate utilization and PMF, is needed. Studies in some cell types have found that rotenone decreases cellular ROS formation. This suggests that reverse electron flow may be occurring or that complex III is insufficiently reduced to generate ROS under these conditions (Aon et al., 2003; Chandel et al., 1998; Zamzami et al., 1995). However, many other studies report that rotenone increases ROS production in cells (Barrientos & Moraes, 1999; Li et al., 2003; Nakamura et al., 2001). The conflict between these results could probably be explained by differences in cell types, substrate conditions, and measurement techniques.

Essentially there are two major hindrances to the accurate identification of sites and rates of ROS production in cells. The first is the intracellular ROS-detection methods that currently are available. The accessible and widely used fluorescent ROS-detecting probes, such as dichlorodihydrofluorescein and dihydrorhodamine, have pitfalls that lead to interpretation issues (Wardman, 2007). However, it is possible that probes such as MitoSOX and dihydroethidine will provide more reliable results for the accurate measurement of ROS in cells (Mukhopadhyay et al., 2007; Song et al., 2007). Second, the use of inhibitors raises the same issue that we encounter in experiments with isolated mitochondria. It is difficult to tease apart the origin of the ROS in a complex cellular system without the use of inhibitors, and yet inhibitors enforce nonphysiological constraints on the system. Also an issue in cells is that ROS may arise from sites other than mitochondria, such as the NADPH oxidases or cytochrome P450 enzymes (Caro & Cederbaum, 2004; Babior et al., 2002), and this complicates the interpretation of ROS observed in vivo even further. These concerns and others are an area of intense interest in the field, and the development of robust intracellular measurement techniques will no doubt lead to a greater understanding of the physiological ROS production sites and mechanisms.

MITOCHONDRIAL ANTIOXIDANT SYSTEMS

Mitochondria contain an impressive array of antioxidant enzymes, as well as small-molecule antioxidants (Beckman & Ames, 1998). These include enzymatic systems, such as superoxide dismutase (SOD) and possibly catalase, and nonenzymatic components, such as protein thiols, glutathione (GSH), and ascorbic acid. Many of the small-molecule antioxidants are maintained in their reduced form by such enzymes as glutathione reductase. The maintenance of the overall reducing environment of the cell is of crucial importance to the effectiveness of the antioxidant systems. The cellular redox environment is maintained by levels of GSH and NADPH, which are lynchpins in the antioxidant system. These systems work in concert to consume and detoxify free radicals. This section will outline the role of mitochondrial homeostasis in the

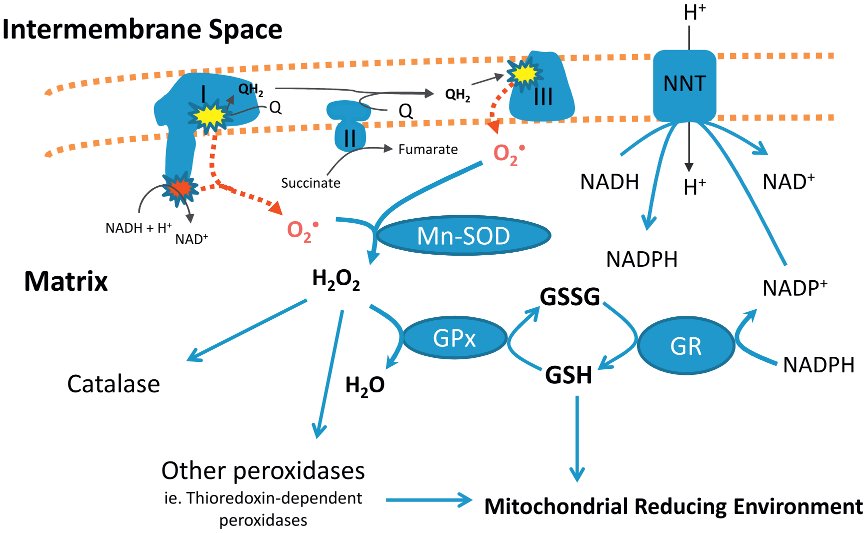

Figure 3.2 The mitochondrial antioxidant system is complex and interrelated. The potency of the reducing environment is maintained largely by the reduction potential of the NADPH/NADP$^+$ couple and the GSH/GSSG couple. The H_2O_2 generated by the dismutation of superoxide by superoxide dismutase (SOD) is neutralized by the GSH-requiring enzyme glutathione peroxidase (GPx), catalase (not present in mitochondria from many tissues), or others such as the thioredoxin-dependent peroxidases. GSH is maintained in its reduced state by the NADPH-requiring enzyme glutathione reductase (GR). In turn, the NADPH pool is kept reduced by the mitochondrial proton-motive force-dependent nicotinimide nucleotide transhydrogenase (NNT) and NADH. From this it can be said that the capacity for radical neutralization within mitochondria is contingent upon the functionality of mitochondria and the maintenance of this interrelated system.

maintenance of the antioxidant environment as well as provide an overview of the some of the key enzymatic components and their associated small molecules. Figure 3.2 outlines the interconnected nature of the mitochondrial antioxidant enzymes and small-molecule systems.

The major routes of matrix H_2O_2 consumption, the glutathione peroxidase and peroxiredoxin pathways, are intimately linked to the GSH pool, mitochondrial bioenergetics, and, more specifically, to the degree of reduction of the NADP$^+$ pool. The tripeptide glutathione (γ-L-glutamyl-L-cysteinylglycine) is an essential component of the cellular antioxidant system because of its relatively low redox potential (approximately −240 mV) and high intracellular concentration of ≈2–10 mM (Schafer & Buettner, 2001). The antioxidant function of glutathione is mediated via the redox-active thiol group that becomes oxidized when GSH reduces target molecules. The detailed chemistry of these reactions and the complex assortment of enzymes involved are reviewed elsewhere (Bindoli et al., 2008; Fourquet et al., 2008; Kalinina et al., 2008; Schafer et al., 2001). Briefly, the reduced thiol-containing intermediates (GSH and thioredoxin) donate electrons to the peroxidase enzyme systems, which then use the electrons to convert H_2O_2 into H_2O. The now oxidized thiol compounds are regenerated via the consumption of reducing equivalents from NADPH (Schafer et al., 2001). Matrix NADPH can be made by the NADP$^+$-specific form of isocitrate dehydrogenase, as well as malic enzyme when the matrix form of this enzyme is present (Vogel et al., 1999). However, the nicotinaminde nucleotide transhydrogenase, which is driven by the mitochondrial proton-motive force, maintains a very high matrix NADPH/NADP$^+$, at the cost of NADH reducing equivalents (Hoek & Rydstrom, 1988; Rydstrom, 2006). The balance achieved by this system ensures that the NADPH-dependent antioxidant systems are maintained in a state that is already primed to react to an increasing demand for oxidant neutralization. Under conditions that may lead to increased ROS production, such as a very high PMF, the NADPH/NADP$^+$ redox couple will naturally be maintained in a highly reduced state. Therefore, NADPH-linked antioxidant systems appear to be well suited to continued function as long as mitochondrial function and integrity are maintained (i.e., prior to induction of the permeability transition pore).

An important antioxidant enzyme is the superoxide dismutase. SOD exists as three isoforms in mammalian systems: SOD1 (Cu,Zn-SOD) is present in the cytoplasm and mitochondrial intermembrane space, SOD2 (Mn-SOD) is present in the mitochondrial matrix, and SOD3 is present in the extracellular matrix (Madamanchi et al., 2005). SOD enzymes dismutate two superoxide anions into hydrogen peroxide. Its matrix location, as well as its rapid reaction with superoxide, make Mn-SOD the first line of defense against mitochondrial superoxide. The concentrations of superoxide are kept very low in the matrix probably because of the high concentrations of Mn-SOD and its high activity (Murphy, 2009). A description of the genetic models that have explored the role of this enzyme is given later in this chapter.

The enzyme catalase decomposes two molecules of H_2O_2 into water and O_2. Although normally associated with peroxisomes, which can produce substantial amounts of H_2O_2 during fatty acid oxidation, a mitochondrial catalase has been identified in heart and liver (Salvi et al., 2007; Antunes et al., 2002). Although similar in function, one significant aspect that differentiates mitochondrial catalase from peroxidases is that a catalase would not require any small-molecule metabolite intermediates or cofactors to facilitate the reaction. The potential tradeoff is that although catalase has extraordinarily high catalytic turnover (K_{cat} is about $4.0 \times 10^7\ s^{-1}$), it has very low affinity for its substrate (K_m values of 25–40 mM are commonly reported) compared to peroxidases (Deisseroth & Dounce, 1970). Despite its presence in heart mitochondria, the contribution of catalase to H_2O_2 removal appears not to be significant (Antunes et al., 2002). Thus, catalase may not be as effective as the peroxidase systems at keeping H_2O_2 low enough to mitigate Fenton reaction-induced hydroxyl radical formation.

FREE RADICAL THEORIES OF AGING

The mitochondrial theory of aging predicts that normal metabolic processes cause ROS production from the electron transport chain and that these ROS cause damage to cellular components, including genetic material, membranes, and enzymes. This damage leads to a decrease in mitochondrial bioenergetic function, cellular decline, and senescence. The vicious cycle hypothesis predicts a downward spiral initiated by chronic exposure of mtDNA to ROS. In theory, increased DNA mutations will lead to synthesis of dysfunctional respiratory chain subunits and therefore to even greater ROS production. These two hypotheses are closely related but not identical; the mitochondrial free radical theory of aging implicates mitochondrially derived ROS in general cellular aging phenomena, whereas the vicious cycle is really a subhypothesis that specifically implicates mtDNA damage and subsequent ROS production caused by this damage.

The big question is: are mitochondrial ROS a major driving force in the aging process? This larger question can be, and has been, broken down into numerous testable hypotheses based on its component predictions. The purpose of this section is to examine some of the major hypotheses that have been tested in regard to the mitochondrial free radical theory of aging: (a) long-lived animals produce fewer ROS than short-lived ones, (b) aged animals show an increased accumulation of cellular oxidative end products, (c) damage to the mitochondrial genome results in an aged phenotype, and (d) alterations in antioxidant systems/enzymes and oxidative damage repair enzymes affect life span.

An obvious assertion that arises from the free radical theory is that long-lived animals will produce fewer ROS than short-lived animals. Several groups have undertaken the task of determining a correlation between length of life and ROS production. Various authors have examined this hypothesis in comparative physiology experiments looking at mitochondria isolated from relatively long-lived mammals and birds compared to shorter-lived species (Ku & Sohal, 1993; Ku et al., 1993; Barja & Herrero, 2000; Barja, 2002). They did indeed find that long-lived species had lower rates of ROS production. Some criticism of these data arose when it was pointed out that allometry, which takes into account body size, was not considered in these studies (Speakman, 2005). However, recent work has shown that mitochondria isolated from long-lived animals produce measurably smaller amounts of ROS during reverse electron transport than shorter-lived animals even after correction for the effects of body mass and phylogeny (Lambert et al., 2007). This effect appears to be caused primarily by differences in the concentration of complex I (Lambert et al., 2010).

Another means of establishing a relationship between ROS and aging is to examine the accumulation of oxidative end products. The accumulation of lipofuscin (red-brown pigment deposits or "age spots") in postmitotic tissues is correlated with age and is the product of oxidation (Jung et al., 2007). This evidence has been criticized as largely correlative and in fact may be due to factors other than ROS production, such as declining lysosomal function with age. However, lipofuscin content displays an oxidative stress age-associated trend. Generally speaking, all the biological macromolecules—lipids, proteins, and nucleic acids—are at risk of oxidative damage. The oxidative sensitivity of lipids is well known (consider rancid oils), and this certainly occurs in vivo (Gutteridge & Halliwell, 1990). It has been suggested that diseases of aging, such as atherosclerosis, occur as a result of lipid peroxidation in membranes (Spiteller, 2007). Oxidized proteins also increase with age and can form aberrant carbonyl side chains and cross-linked protein aggregates (Stadtman, 2006).

Of particular interest to the mitochondrial free radical theory of aging is oxidative damage to genetic material. MtDNA is uniquely at risk for damage because of its close proximity to the ROS-generating sites in mitochondria, its lack of protective histones, and its high density of coding regions, as well as its limited DNA repair enzyme activity. There is evidence that DNA is modified by oxidative stress. A correlation between mtDNA deletions and increased content of oxidatively modified bases (8-OHdG) was noted in a study of the postmortem human heart (Hayakawa et al., 1992). It was also demonstrated that treatment of human skin fibroblasts with a sublethal dose of *tert*-butylhydroperoxide to simulate oxidative stress resulted in the formation and accumulation of the common age-associated 4977-bp deletion in mtDNA (Dumont et al., 2000).

Correspondingly, a rather large body of evidence associates aging phenotypes with mtDNA mutations and deletions (Hiona et al., 2007; Kujoth et al., 2005). Postmitotic tissues with high respiratory demand, such as skeletal muscle, show decreased aerobic capacity in aged humans, and this has been associated with a decline in electron transport chain complexes (Short et al., 2005). A commonly used marker for mitochondrial electron transport chain abnormalities is the presence of ragged red fibers, which are muscle fibers containing structurally aberrant mitochondria that have a loss of cytochrome *c* oxidase (complex IV) and often a concomitant increase in succinate dehydrogenase activity (Bourgeois & Tarnopolsky, 2004). Muscle that exhibits a large degree of sarcopenia, the progressive loss of tissue mass with age, exhibits more electron transport chain abnormalities than tissues with less sarcopenia (Bua et al., 2002). In this regard, there are several common deletions and mutations that are observed in aged tissues, and it is from these observations that the vicious cycle hypothesis has gained support. The earliest suggestion that some mitochondrial mutations could stimulate ROS production was by Bandy & Davison in 1990. Their original conclusion that increased ROS could occur with some mtDNA mutations was tempered with the observation that some mutations in respiratory chain components would certainly result in the elimination of ROS. This, along with other work, eventually evolved into the vicious cycle hypothesis (Hiona et al., 2007). Both the idea that mtDNA mutations perpetuated aging and the belief that this was in essence a vicious cycle were uniquely tested with the generation of the mitochondrial mutator mouse, or the POLG mouse.

The POLG mouse is a knock-in genetic model expressing a version of the mitochondrial DNA polymerase-γ lacking the proofreading exonuclease domain. Specifically, these mice showed a marked increase in mtDNA point mutations and deletions (Trifunovic et al., 2004). These mutations were associated with a severely limited life span and early onset of aging phenotypes such as alopecia, osteoporosis, and sarcopenia. Importantly, the authors found that mtDNA mutations severely compromised mitochondrial respiratory capacity and ATP production. Although there was a strong aging-like phenotype, POLG mice did not display increased ROS production or increased signs of oxidative stress (Trifunovic et al., 2005). This is strong evidence against the vicious cycle hypothesis, but it is difficult to interpret as evidence that ROS damage does not play a role in aging. It merely indicates that ROS production is not enhanced in mutants with extremely damaged mtDNA. Also, a recent analysis of the POLG mouse by Bailey et al. suggests that the animal is under significant replication stress because of the high level of damaged DNA. Bailey et al. further suggest that it is not necessarily the downstream effects of expressing mutant DNA, but the continued strain of trying to prevent the accumulation of mutations that results in decreased ROS production and loss of homeostasis (Bailey et al., 2009).

Another challenge to the vicious cycle hypothesis was presented in data by Hutter et al. showing that normally aged human skeletal muscle produces fewer ROS than tissue from young donors (Hutter et al., 2007). Somewhat surprisingly and contrary to previous reports (Short et al., 2005; Bua et al., 2002), Hutter et al. did not find any impairment of mitochondrial function in the aged muscle. It should be noted that they did not examine the mtDNA nor did they indicate whether study participants showed typical aged muscular phenotypes, such as sarcopenia. Nonetheless, of particular interest is the authors' finding that ROS production was decreased in aged subjects; one could postulate that this is due to either some unrecognized change in electron transport chain function or a change in antioxidant enzyme behavior.

Manipulation of antioxidant enzymes has proved a valuable tool in the analysis of the roles of mitochondrial ROS in aging. As described in detail by Lustgarten et al. in Chapter 8 of this book, numerous genetic models have been developed to test the hypothesis that ROS neutralization is crucial in life span determination (Muller et al., 2007). In particular, Mn-SOD, catalase, and thioredoxin have been explored in mammalian systems. As mentioned earlier, Mn-SOD is an essential mitochondrial antioxidant enzyme. Mn-SOD knockout mice are short-lived and exhibit a severe phenotype including anemia, degeneration of neurons, and cardiomyopathy, as well as increased susceptibility to oxidative mitochondrial injury in neurons, cardiac myocytes, and other metabolically active tissues (Lebovitz et al., 1996). Conversely, overexpression of Mn-SOD has been shown to protect against oxidative damage and increase resistance to inducers of mitochondrial permeability (Silva et al., 2005). Hu et al. reported modest increases in mean and maximum life span (4 and

18%, respectively) in mice overexpressing Mn-SOD (Hu et al., 2007), but Jang et al., despite observing a decrease in oxidative damage, did not observe any significant increase in mean or maximum life span (Jang et al., 2009). This may be explained by the overall shorter-lived colony in the Hu study. This conflict does not give a clear picture of the role of ROS in lifespan determination.

A mouse model overexpressing catalase targeted to mitochondria showed a significant (20%) increase in both median and maximal life span (Schriner et al., 2005). The authors analyzed end-of-life pathology and found that catalase expression was associated with reduced tumor burden, reduced cardiac lesions, and lowered systemic inflammation. The mice also showed a lower overall disease burden, and they did not show any detrimental clinical effects (Treuting et al., 2008). Another study found that cardiac aging was attenuated in animals in which catalase was targeted to heart mitochondria (Dai et al., 2009). As catalase expression presumably lowers the levels of ROS released from mitochondria, these observations offer strong support for the free radical aging hypothesis.

Another mitochondrial enzyme that has been examined in genetic models is thioredoxin-2. As described earlier, thioredoxin is an important player in the maintenance of the reducing mitochondrial environment. The homozygous thioredoxin knockout is embryonic lethal and the timing of the lethality coincides with the maturation of the mitochondria and beginning of oxidative phosphorylation (Nonn et al., 2003). However, the heterozygous knockout is viable, but shows decreased mitochondrial function, decreased ATP production, and reduced activity of electron transport chain complexes. These mice also showed increased ROS production compared to wild-type mice and increased oxidative damage to nuclear DNA, lipids, and proteins in liver (Perez et al., 2008). The same group, however, has not found that it affected life span in studies conducted thus far (Perez et al., 2009). It has been shown that cells overexpressing thioredoxin-2 were less sensitive to oxidative stress (Chen et al., 2002). Whether thioredoxin-modulated ROS supports the free radical aging hypothesis will depend on the outcome of the longevity studies.

The above analysis lends some support to the mitochondrial free radical hypothesis of aging, in that long-lived animals tend to produce fewer ROS and there is evidence that aged animals have greater oxidative damage to cellular components. The POLG mouse does not support the hypothesis if one perceives it as an adequate model of aging. However, if one interprets it as a severely compromised mouse model that shows an aging-like phenotype, then it does not necessarily undermine the hypothesis. The genetic models involving antioxidant enzymes appear to challenge the hypothesis. Other than the catalase-overexpressing mouse, the data do not strongly support a free radical aging mechanism. However, as we describe under Mitochondrial Antioxidant Systems above, the reducing environment of the cell is a highly intricate system and is maintained by a number of enzymes and small molecules working in concert. It is perhaps not necessary to discard the hypothesis fully because knockouts of particular antioxidant enzymes do not significantly reduce life span, or overexpression of them does not lengthen life span. The variation in radical production within different systems, as well as the sheer complexity of the antioxidant system, indicates that it is a highly regulated system and increases the probability that compensatory action is taking place in the cell. However, interested readers are encouraged to read the accompanying chapter in this volume by Lustgarten et al. (Chapter 8) or examine the thorough review by Muller et al. for further analysis of the antioxidant genetic models (Muller et al., 2007).

CONCLUSIONS

Is mitochondrial ROS production responsible for cellular senescence? What we know so far about the mechanisms of ROS production and the biology of aging does not give a clear answer to this question. In this chapter, we have endeavored to describe the mitochondrial ROS-producing mechanisms in the context of aging hypotheses. We described the major mitochondrial ROS-producing sites as respiratory chain complexes I and III, with some consideration given to the less well described mitochondrial enzymes. ROS production was framed in terms of mitochondrial bioenergetics and physiology. With the purpose of instructing those that would set out to characterize ROS production in their system, particular consideration was given to the use of respiratory chain inhibitors to study ROS production in vitro. We examined the mitochondrial antioxidant system with a view toward placing ROS neutralization into context alongside ROS production. We provided some interpretation of the existing body of experimental evidence that both supports and refutes the mitochondrial aging hypothesis. What can be taken away from this interpretation is that ROS production is a physiological, and at times probably pathological, process. The hypothesis that mitochondrial ROS are the mechanism by which aging occurs is still as interesting now as it was at its inception in 1956, but to assess the role of oxidative damage in the aging process properly, we need to enhance our understanding of these mechanisms. Despite our current understanding of these processes, we remain woefully uninformed on the physiological ROS production mechanisms and what homeostatic imbalances occur that lead to oxidative damage in cells. The growing interest in this field has led to some excellent studies that have furthered our understanding of the oxidant/antioxidant system in biological systems. It is this work that will ultimately lead to a clear picture of the role that free radicals play in aging and disease.

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Chapter | 4 |

Aging and Apoptosis in Muscle

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CHAPTER CONTENTS

Overview of Apoptosis in Aging Muscles 64
  Myonuclear Apoptosis 64
  Myonuclear Apoptotic Signaling in Skeletal Muscle 65
Intrinsic Apoptotic Pathway 66
  Cellular Conditions for Activation of the Intrinsic Apoptotic Pathway 66
  Mitochondrial Dysfunction as a Molecular Initiator of Apoptotic Signaling 67
    Mitochondrial Dysfunction in Sarcopenia 67
    Mitochondrial Bioenergetics in Aging Muscle 67
    Mitochondrial Bioenergetics and Apoptotic Signaling 68
    Mitochondrial Permeability 68
    Oxidative Stress and Mitochondria 69
    Perturbations Increasing Oxidative Stress Leading to Apoptosis Signaling 70
  The BCL-2 Protein Family 70
  Caspase-Dependent Signaling 71
  Caspase-Independent Signaling 71
  Apoptotic Suppressors 72
  Mitochondria-Mediated Signaling for Apoptosis in Sarcopenia 72
Extrinsic Apoptotic Signaling in Skeletal Muscle 77
  Cellular Conditions for Activation of the Extrinsic Apoptotic Pathway 77
  TNF-α and Death Receptor Signaling 77
  Cross Talk between Extrinsic and Intrinsic Apoptotic Signaling 81
Sarcoplasmic/Endoplasmic Reticulum–Calcium Stress Apoptotic Signaling 82
  Cellular Conditions that Activate the SR/ER–Ca^{2+} Stress Apoptotic Pathway 82
  Signaling via the SR–Ca^{2+} Stress Apoptotic Pathway 83
  Cross Talk of the SR/ER–Ca^{2+} Stress and the Intrinsic Apoptotic Pathway 84
Apoptosis Occurs in Satellite Cells/Muscle Precursor Stem Cells 84
  The Role of MyoD in Apoptosis of MPCs 84
  Role of Inhibitory of Differentiation-2 in Apoptosis of MPCs 85
Modulation of Apoptosis in Aging Muscle 85
  Interventions by Muscle Loading 85
  Apoptotic Elimination of MPCs Reduces Muscle Hypertrophic Adaptation to Loading 94
  Unloading and Disuse in Skeletal Muscle 94
  Regulation of Apoptosis by Aerobic Exercise 95
  Reduction of Apoptosis by Caloric Restriction 96
Aging and Cardiomyocyte Apoptosis 96
  Cardiomyocyte Apoptosis and Heart Failure 97
  Apoptosis and the Aging Heart 97
  Modulation of Bcl-2 and Bax in the Aging Heart 98
  Human Src Homology and Collagen Protein of 66 kDa and Myocardial Apoptosis 99
  Longevity Intervention and Cardiomyocyte Apoptosis 99
Conclusions 100
Acknowledgments 101
References 101

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OVERVIEW OF APOPTOSIS IN AGING MUSCLES

Frailty in the healthy elderly has become a widespread problem central to the care of geriatric populations. The condition of sarcopenia, an age-associated loss of muscle mass and function (Evans, 1995), is often a component of the geriatric syndrome of frailty. All elderly people show evidence of sarcopenia and loss of function particularly after the seventh decade of life, with ~40% decline in muscle mass by the age of 80 (Evans, 1995). The loss of muscle mass and strength with age (particularly in men over the age of 60) is associated with increased mortality (Metter et al., 2002) and increased incidence of falling and hip fractures (Lloyd et al., 2009). Although the mechanisms leading to sarcopenia are multifactorial, apoptosis has been shown to be part of the events that lead to muscle loss with aging. Cardiovascular dysfunction and, particularly, heart failure further contribute to the geriatric syndrome of frailty, thereby severely limiting the function, the quality of life, and the life expectancy of the elderly who have these health problems (Afilalo et al., 2009; Cesari & Pahor, 2008).

Apoptosis is a fundamental biological process that is highly conserved among species ranging from worms to humans (Ellis et al., 1991; Sulston & Horvitz, 1977; Yuan, 1996). The steps in apoptosis that lead to the eventual elimination of cells from tissues require ATP. The sequential events in apoptosis were first described in the nematode *Caenorhabditis elegans* by Kerr and colleagues (Kerr et al., 1972). The distinctive morphological characteristics of apoptosis include cell shrinkage, cell membrane blebbing, chromatin condensation, internucleosomal degradation of chromosomal DNA, and formation of membrane-bound fragments called apoptotic bodies (Kerr et al., 1972). The morphological and biochemical characteristics of apoptosis are unique and clearly distinguish it from necrotic cell death.

Data obtained in the past 2 decades have provided a better understanding of the biological role of apoptosis. Apoptosis is necessary for the elimination of damaged, aberrant, or harmful cells. Apoptosis also participates in normal embryonic development, tissue turnover, and immunological function (Thompson, 1995). Apoptosis regulates part of the balance among cell proliferation, differentiation, and cell death in multicellular organisms. Therefore, it is reasonable to conclude that health would be threatened if apoptosis is not adequately maintained or if it is disrupted. In fact, aberrant regulation of apoptosis contributes to the pathogenesis of several diseases, including viral infections, cancers, autoimmune diseases (e.g., systemic lupus erythematosus and rheumatoid arthritis), loss of pancreatic β cells in diabetes mellitus, toxin-induced liver disease, acquired immune deficiency syndrome, myocardial and cerebral ischemic injuries, neurodegenerative diseases (e.g., Alzheimer & Parkinson diseases) (Cacciapaglia et al., 2009; Campisi & Sedivy, 2009; Duke et al., 1996; Lee & Pervaiz, 2007; McMullen et al., 2009; Thompson, 1995; Williams, 1991; Yuan & Yankner, 2000), and muscle loss (e.g., sarcopenia, disuse) associated with aging (Adhihetty et al., 2008, 2009; Adhihetty & Hood, 2003; Dirks & Leeuwenburgh, 2005; Marzetti et al., 2008c, 2010a; Pistilli et al., 2006b; Primeau et al., 2002; Siu et al., 2006; Siu & Alway, 2005a, 2006b).

Myonuclear Apoptosis

Apoptosis was initially described as a series of events that was ultimately responsible for the elimination of entire cells, and this was essential for maintaining the homeostasis of cell growth and death, especially in cells with a high proliferative rate. In the context of single cells, the term apoptosis is a clearly defined process leading to elimination of the nucleus and therefore the cell. However, the better term to describe this same process in multinucleated postmitotic cell populations including cardiomyocytes and skeletal myofibers is "myonuclear apoptosis." This is because elimination of a single nucleus can occur without the death of the entire (multinucleated) muscle cell, although this may result in smaller cells. Myonuclear apoptosis (or nuclear apoptosis) can occur without inflammation or disturbing adjacent proteins or organelles.

Accumulating evidence has shown that apoptosis is a significant contributor to muscle degeneration and sarcopenia (Adhihetty et al., 2008, 2009; Adhihetty & Hood, 2003; Dirks & Leeuwenburgh, 2005; Lees et al., 2009; Marzetti et al., 2008c, 2010a; Pistilli et al., 2006b; Primeau et al., 2002; Siu et al., 2006; Siu & Alway, 2005a, 2006b; Smith et al., 2009; Tews, 2005). However, apoptosis in skeletal muscle is unique for several reasons. First, skeletal muscle is multinucleated, so that the removal of one myonucleus by apoptosis will not produce "wholesale" muscle cell death. However, elimination of a myonucleus will lead to loss of gene expression within the cytoplasmic domain that had been controlled by that myonucleus. This loss of gene control cannot be fully assumed by an adjacent myonucleus, and unless the lost nucleus is replaced, the muscle fiber will atrophy. Second, muscle contains subsarcolemmal and intermyofibrillar mitochondria. These are two morphologically and biochemically distinct subfractions of mitochondria that exist in different regions of the fiber. They have regional differences in sensitivity to apoptotic stimuli within muscle fibers (Adhihetty et al., 2007a, 2008, 2009; Williamson et al., 2010). Third, skeletal muscle is a malleable tissue capable of changing its mitochondrial content and/or composition in response to chronic alterations in muscle use (Adhihetty et al., 2003; Irrcher et al., 2003; Schmutz et al., 2010) or disuse (Adhihetty et al., 2008; Chabi et al., 2009; Leivo

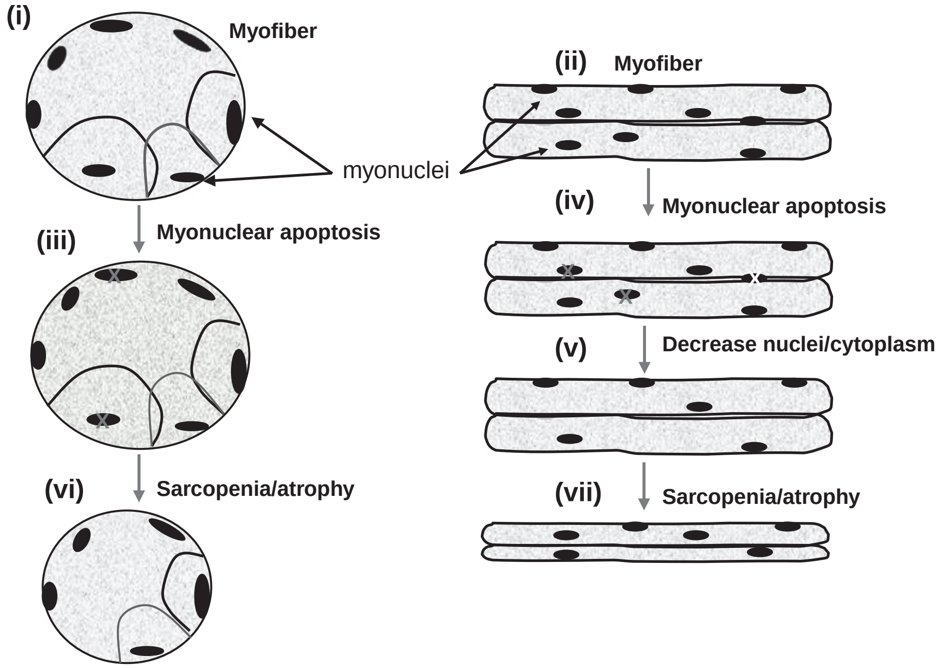

Figure 4.1 Muscle fibers are illustrated in cross section (i, iii, vi) or longitudinally (ii, iv, v, vii). Myonuclei in muscle fibers control a fixed cytoplasmic domain (iii). Nuclei are targeted for elimination by apoptosis (asterisks; iii, iv). Fewer nuclei are unable to maintain the cytoplasmic area (v) and this results in fiber atrophy and ultimately sarcopenia (vi and vii).

et al., 1998). Such variations in mitochondrial content and/or composition can undoubtedly influence the degree of organelle-directed apoptotic signaling in skeletal muscle.

The potential for a single nucleus in a multinucleated cell (e.g., myofiber) to be targeted for destruction, while largely preserving the other organelles and preventing total destruction of the cell, is particularly intriguing. This suggests that apoptosis in muscle cells represents a series of cell signaling events that are extremely precise and targeted and not a blanket response to a stimulus.

Evidence that not all myonuclei in a single myofiber become apoptotic during muscle loss has been observed in experimental denervation and denervation-associated disease (e.g., infantile spinal muscular atrophy; Fidzianska, 2002; Simic et al., 2000; Simic, 2008). This further supports the hypothesis of myonuclear apoptosis in modulating the myofiber volume by controlling the successive myofiber segments. The concept of myonuclear apoptosis is consistent with the proposed "nuclear domain hypothesis", which explains the phenomenon of cell size remodeling of myofiber by adding or subtracting nuclei because each nucleus controls a specifically defined cytoplasmic area (Figure 4.1). The skeletal myofiber is a differentiated but highly plastic cell type, which adapts to loading and unloading (Allen et al., 1996; Carson & Alway, 1996; Degens & Alway, 2003; Hikida et al., 1997; Phelan & Gonyea, 1997). The nuclear domain hypothesis predicts that a nucleus controls a defined volume of cellular territory in each myofiber (Allen et al., 1999). Therefore, addition of extra nuclei (from satellite cells) into the myofiber is essential to support the increment of cell size to achieve muscle hypertrophy, and removal of the myonuclei is required for muscle atrophy. If fewer nuclei are available, less transcriptional and translational support can be provided to a given cytoplasmic area and this area must therefore be reduced. Generally, there is a tight relationship between nuclear number and muscle fiber cross-sectional area and volume. Nevertheless, this relationship is not perfect, because the myonuclear domain increases slightly with age (i.e., fewer nuclei/cytoplasm area). With age there is also a loss of satellite cells or muscle precursor cells (MPCs), which reduces the muscle's ability to replace nuclei (Brack et al., 2005, 2007; Brack & Rando, 2007; Bruusgaard et al., 2006; Korolchuk et al., 2009; Yang et al., 2009). This results in a somewhat transient increase in the nuclear domain with aging, but the excessive domain size triggers fiber atrophy (Brack et al., 2005), which in turn restores the original nuclear domain size, but also contributes to sarcopenia (Figure 4.1).

Myonuclear Apoptotic Signaling in Skeletal Muscle

One of the distinctive characteristics of apoptosis is that it allows the execution of cells in the absence of inflammation and therefore it does not disturb neighboring cells. This characteristic of apoptosis permits highly selective dismissal of targeted individual cells among

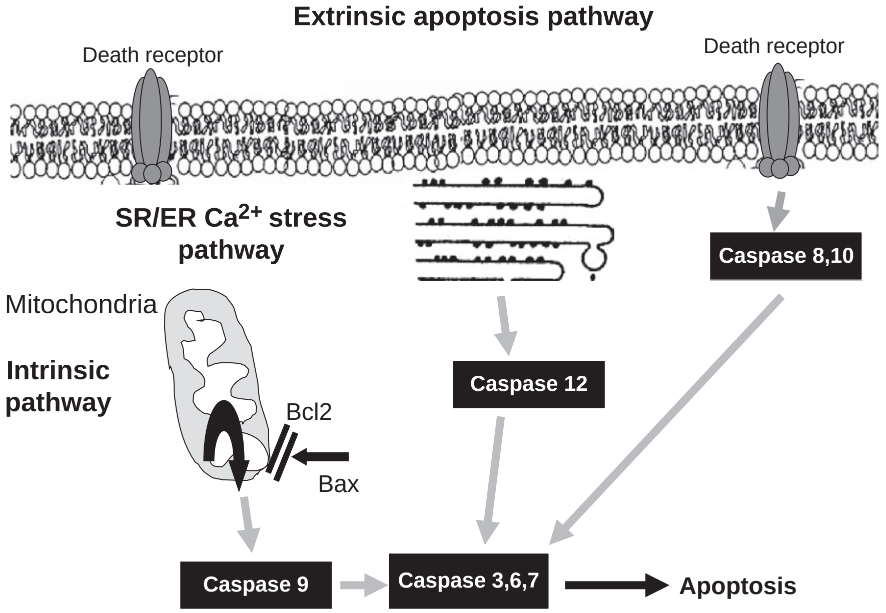

Figure 4.2 Three apoptotic pathways have been identified in sarcopenia. These include the intrinsic (mitochondria) pathway, which involves mitochondrial dysfunction and increased mitochondrial permeability. A series of downstream signaling events results in activation of initiator caspases (e.g., caspase-9) and effector caspases (e.g., caspase-3) and finally apoptosis. The endoplasmic reticulum (ER)–calcium stress pathway activates initiator caspases (e.g., caspase-12) and then effector caspases (e.g., caspase-3 or -7). The extrinsic pathway is activated by a ligand (e.g., TNF-α) and activates initiator caspases (e.g., caspase-8) and the effector caspases (e.g., caspase-3) and, through this, apoptosis.

the whole cell population. In the case of muscle, nuclei are eliminated without destroying adjacent structures. Apoptosis-induced myonuclear debris removal probably involves the ubiquitin–proteasome pathway, as well as autophagy in many cell types (Korolchuk et al., 2009; Yang et al., 2009), including skeletal muscle (Attaix et al., 2005; Combaret et al., 2009).

Three primary apoptotic pathways have been implicated for mediating cellular signaling transduction leading to the implementation of apoptosis in skeletal muscle cells (Figure 4.2). These apoptotic pathways include mitochondria-dependent (intrinsic), death receptor-mediated (extrinsic), and sarcoplasmic/endoplasmic reticulum–calcium stress-induced pathways (Gorman et al., 2000; Li et al., 1998; Nakagawa et al., 2000; Phaneuf & Leeuwenburgh, 2002; Puzianowska-Kuznicka & Kuznicki, 2009; Spierings et al., 2005). Various proteins regulate the induction of apoptosis. These include B cell leukemia/lymphoma-2 (BCL-2) family proteins; cysteine-dependent aspartic acid-specific proteases (caspases); inhibitors of apoptosis proteins (IAPs); caspase-independent apoptotic factors including apoptosis inducing factor (AIF), endonuclease G (EndoG), and heat-requirement A2 protein (HtrA2/Omi); and other apoptosis-related proteins such as cytochrome *c*, apoptosis protease activating factor-1 (Apaf-1), apoptosis repressor with caspase recruitment domain, Smac/Diablo, p53, and heat-shock proteins.

The participation of these apoptotic factors is selective in nature and largely dependent on which apoptotic pathway is activated. For example, when apoptosis is stimulated by TNF-α and Fas ligand (FasL), which subsequently activate the death receptor apoptotic pathway, the initiator caspase, caspase-8, is responsible for mediating the downstream signal transduction that leads to apoptosis (Li et al., 1998; Sun et al., 1999). Apoptotic signaling initiated by intracellular calcium disturbance and endoplasmic reticulum/sarcoplasmic reticulum stress is attributed to initial activation of caspase-12 (Nakagawa et al., 2000; Nakagawa & Yuan, 2000). Caspase-9 mediates the mitochondria-dependent apoptosis through the interaction of procaspase-9 with Apaf-1, dATP/ATP, and cytochrome *c*. Although different initiator caspases (caspase-8, -9, and -12) are responsible for initial apoptotic signaling, these signals eventually converge on the activation of common effector caspases-3, -6, and -7, which facilitate the progression to the terminal disassembly of the nucleus.

INTRINSIC APOPTOTIC PATHWAY

Cellular Conditions for Activation of the Intrinsic Apoptotic Pathway

Several cellular conditions can contribute to alterations in mitochondrial function and predispose muscle fibers for subsequent apoptotic events. These include elevations in oxidative and mechanical stresses that occur with muscle loading, muscle unloading, muscle disuse/denervation, and aging. Other conditions that are associated with aging include mutations in mitochondrial DNA, loss of mitochondrial enzyme activity, and a reduction in the number of mitochondria in a muscle fiber. Such perturbations "prime" the muscle cells for apoptosis (similar to cocking a gun) so that the apoptosis process is fully engaged once a sufficient stimulus threshold is reached.

Mitochondrial Dysfunction as a Molecular Initiator of Apoptotic Signaling

Mitochondrial Dysfunction in Sarcopenia

Aging is characterized by a progressive loss of mitochondrial function (Hepple, 2009; Wallace et al., 1995; Wallace, 2000, 2001). This leads to a loss of muscle oxidative capacity (Essen-Gustavsson & Borges, 1986; Rasmussen et al., 2003). Indirect assessments of mitochondrial function by measuring phosphocreatine recovery after knee extensor exercise has shown a greater reduction in mitochondrial function in muscles of aged subjects than could be accounted for by the reduction in mitochondrial volume density as determined by electron microscopy from muscle biopsy samples. These data suggest that aging in humans is associated with a reduced oxidative capacity per volume of mitochondria in skeletal muscle (Conley et al., 2000). The intimate connection between mitochondrial function and the viability of skeletal muscle suggests that this organelle probably plays a significant role in the progression of sarcopenia. A consequence of apoptosis is a loss in myonuclear number, resulting in a reduction in myofiber diameter to maintain a constant myonuclear domain size (Alway & Siu, 2008; Dirks & Leeuwenburgh, 2005; Pistilli et al., 2006b; Pistilli & Alway, 2008; Wang et al., 2008). This decrease in fiber area results in whole muscle atrophy. This is especially evident in fast muscles, which have a high percentage of type II myosin heavy chain. This suggests that there is a significant mitochondrial involvement in the progression of sarcopenia. Although there are fewer mitochondria in type II fibers than in type I fibers, type II fibers appear to have the greatest levels of oxidative stress (e.g., aging, muscle disuse), and this fiber type is susceptible to oxidative damage and apoptosis (Pistilli et al., 2006b; Siu et al., 2008). In addition, type II muscle fibers are preferentially lost in the elderly (Conley et al., 2007a). It is possible that a high percentage of the mitochondria in type II muscle fibers have some level of dysfunction, and this directly contributes to elevated levels of apoptosis and eventual loss of type II fibers in aged muscles.

The role of mitochondrial dysfunction in promoting sarcopenia was identified by studies showing that muscle fibers containing dysfunctional mitochondria were atrophied compared to fibers that were of normal size (Herbst et al., 2007; McKenzie et al., 2002). Mitochondrial dysfunction leads to reduced oxidative phosphorylation and increased permeability of the mitochondria. In skeletal muscle, this causes a release of proapoptotic factors from the mitochondria, which activates downstream signaling for apoptosis (Adhihetty et al., 2008; Alway & Siu, 2008; Marzetti et al., 2010b; Siu, 2009).

Mitochondrial Bioenergetics in Aging Muscle

Aged mammalian tissues show a decreased capacity to produce ATP by oxidative phosphorylation because of dysfunctional mitochondria. Production of reactive oxygen species (ROS) is probably one source of insult that contributes to mitochondrial dysfunction. The involvement of mitochondria both as producers and as targets of ROS has been the basis for the mitochondrial theory of aging (Harman, 1956; Lenaz et al., 1999). Oxidation of key mitochondrial structural elements and enzymes contributes to mitochondrial dysfunction. This can occur in response to elevations in ROS as a result of aging per se (Chen et al., 2010; Figueiredo et al., 2009; Thompson et al., 2006) and also after increased (Ryan et al., 2008) or decreased muscle activity (Jang et al., 2009; Powers et al., 2005; Siu et al., 2008).

Complexes I, III, and IV of the electron transport chain (ETC) move protons across the inner mitochondrial membrane into the intermembrane space, creating an electrochemical gradient, which is then utilized by complex V for ATP generation. Studies that have examined the impact of aging on skeletal muscle mitochondrial ETC complex function have not provided consistent results. While some investigations report no age-related changes in mitochondrial function (Rasmussen et al., 2003), most studies indicate that mitochondria isolated from skeletal muscle of aged animals have impaired respiratory function (Ames et al., 1995; Drew et al., 2003; Figueiredo et al., 2009; Shigenaga et al., 1994). For example, State 3 activity of the electron transport chain has been reported to be significantly lower in skeletal muscle mitochondria of aged rats compared to young rats (Figueiredo et al., 2009). In addition, complex IV of the electron transport chain exhibits a disproportionate decline in activity with aging relative to other mitochondrial enzymes in brain and liver tissue (Navarro & Boveris, 2007). This has also been seen in mitochondria that were isolated from aged skeletal muscles (Hagen et al., 2004; Hepple et al., 2005). Many mitochondria in aging muscles are dysfunctional and fragile (Terman et al., 2006;

Terman & Brunk, 2004; Tonkonogi et al., 2003). It is therefore possible that only the most robust mitochondria are isolated and selected for study in most in vitro experiments. If this is the case, isolated mitochondria preparations might underestimate mitochondrial dysfunction in whole muscles of old animals (Tonkonogi et al., 2003).

A reduction in oxidative phosphorylation has been confirmed by in vivo studies in muscles of aged humans (Amara et al., 2007; Petersen et al., 2003). In addition, aging is associated with a decrease in mitochondrial membrane potential, a decrease in peroxide production, and changes in mitochondria morphology and protein composition (Gomez et al., 2009; Hagen et al., 1997; Sastre et al., 2002). Many important mitochondrial-encoded proteins originate from myonuclei and therefore must be transported into the mitochondria for proper mitochondrial function and biogenesis. Aging results in decreased mitochondrial protein transport (Huang et al., 2010; Sastre et al., 2002) and therefore, this may have some role in reducing mitochondria number, size, or function in aging muscles.

The age-related reductions in maximal mitochondrial ATP production have also been associated with concomitant reductions in skeletal muscle mitochondrial enzyme activities (Coggan et al., 1993), protein synthesis and expression (Coggan et al., 1993; Rooyackers et al., 1996), and mtDNA abundance in humans (Amara et al., 2007; Coggan et al., 1993; Conley et al., 2007a; Short et al., 2005). These changes probably contribute to altered bioenergetics in mitochondria from aged muscles. In contrast, other data suggest that mitochondrial oxidative phosphorylation in State 4 respiration was not changed in muscle mitochondria with aging (Figueiredo et al., 2009).

Age-associated changes in mitochondrial bioenergetics appear to be specific, affecting some proteins but not others. For example, mtDNA-encoded proteins such as cytochrome *c* oxidase are lost with aging, but succinate dehydrogenase proteins are not reduced in muscle mitochondria from old individuals (Coggan et al., 1993; Muller-Hocker et al., 1992). In addition, mitochondria isolated from aged mice that were activated by ADP to simulate exercise had a greater loss of cytochrome *c* from the mitochondria to the cytosol than isolated control mitochondria exposed to the same conditions (Figueiredo et al., 2009). The loss of these proteins from the mitochondria to the cytosol with aging has an important role to play in the initiation of apoptosis (Kadenbach et al., 2004, 2009).

Mitochondrial Bioenergetics and Apoptotic Signaling

An accumulating body of evidence suggests that disruptions in mitochondrial function precede the activation of the intrinsic apoptotic pathway in sarcopenia of aging (Chabi et al., 2008; Pistilli et al., 2006b; Seo et al., 2008; Siu et al., 2005b), as well as disuse-associated muscle atrophy (Adhihetty et al., 2007b; Siu & Alway, 2005b). This is not surprising because mitochondria play such a critical role in maintaining cellular integrity and ATP levels (Figure 4.3).

Disruptions of the electron transport chain in mitochondria result in diminished ATP production and, consequently, in a marked perturbation of the bioenergetic state of the cell. This disturbance has the potential to impact signaling for cell death (Honda et al., 2003; Izyumov et al., 2004). The inhibition of ATP production has been observed in both apoptosis and necrosis. However, this phenomenon occurs relatively late in apoptosis, as the complete apoptotic program requires the energy-dependent formation of the apoptosome and cleavage and activation of key proapoptotic proteins. By contrast, necrosis is characterized by an early loss of ATP synthesis and seems to proceed under conditions of low cytosolic ATP levels (Kim et al., 2003, 2004). Thus, the mitochondria's ability to maintain adequate cellular levels of ATP appears to be important in determining whether the necrotic or the apoptotic cell death pathway will be followed in response to various environmental or metabolic stresses.

Mitochondrial Permeability

An important step in mitochondrial regulation of apoptosis begins with an increase in mitochondrial permeability. When mitochondrial-housed proteins are released into the cell's cytosol, they can initiate a cascade of proteolytic events that converge on the nucleus. This ultimately leads to fragmentation of DNA and elimination of the nucleus. While cell death is the automatic conclusion of uninucleated nonmuscle cells (Bernardi et al., 1999; Kim et al., 2003), this is not necessarily the case in a multinucleated muscle cell. However, removal of a sufficient number of nuclei will ultimately lead to cell death.

The release of proapoptotic proteins from the mitochondria occurs in response to cellular stressors such as ROS and includes cytochrome *c*, EndoG, Smac/Diablo, and AIF. Release of these proapoptotic factors occurs through either the mitochondrial permeability transition pore (mtPTP) (Forte & Bernardi, 2006; Kroemer et al., 2007; Precht et al., 2005; Rasola & Bernardi, 2007) or the homo-oligomeric Bax mitochondrial apoptotic channels (MAC) in the outer mitochondrial membrane (Dejean et al., 2006a,b; Martin et al., 2007). Putative components of the MAC channel are Bax and Bak, whereas Bcl-2 acts as a negative regulator of this channel (Dejean et al., 2005, 2006a,b). Opening of the mtPTP in the inner membrane causes depolarization of the mitochondrial membrane potential and swelling of the matrix

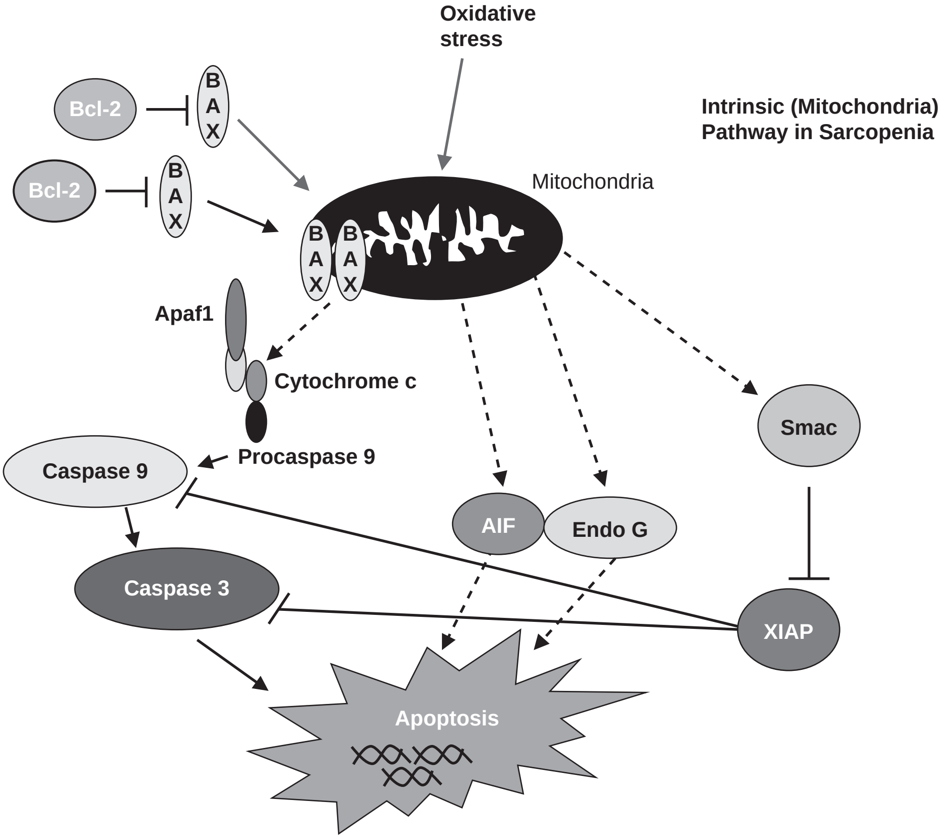

Figure 4.3 The intrinsic (mitochondria) pathway is activated in sarcopenia. Proapoptotic factors (e.g., Bax) heterodimerize to form a mitochondrial channel that releases caspase-dependent (e.g., cytochrome c) or caspase-independent (e.g., AIF, EndoG, Smac/Diablo) proapoptotic factors and results in DNA fragmentation and nuclear apoptosis in muscle.

space. This swelling ruptures the outer membrane, and cytochrome *c* and other proapoptotic proteins are released into the cytosol (Bernardi et al., 2001, 2006; Bernardi & Rasola, 2007; Forte & Bernardi, 2006; Giorgio et al., 2005; Krauskopf et al., 2006; Petronilli et al., 2001). However, cytochrome *c* release can occur in the absence of mitochondrial depolarization and without loss of outer membrane integrity (Rasola & Bernardi, 2007). Mitochondria that were isolated from muscles of older but not senescent mice have been shown to have greater release of cytochrome *c* even at basal resting levels (Figueiredo et al., 2009). This implies that mitochondrial permeability increases with age in mitochondria, and this predisposes the muscle cells to induction of apoptosis. Together these observations indicate that mitochondrial disruption is not required to initiate apoptosis, but mitochondrial permeabilization is essential in apoptotic signaling events (De Giorgi et al., 2002; Dejean et al., 2006a,b; Liu et al., 1996; Wei et al., 2001; Yang et al., 1997).

Oxidative Stress and Mitochondria

The free radical theory of aging was initially proposed by Harman more than 5 decades ago (Harman, 1956). This theory suggests that mitochondrial dysfunction from oxidative damage to mtDNA is caused by ROS, and ROS damage is a central factor contributing to aging (Harman, 1956, 2003, 2006; Kadenbach et al., 2009; Malinska et al., 2009).

The potential for ROS to induce oxidative damage has significant implications for postmitotic tissues such as neurons, myocardium, and skeletal muscle (Hagen et al., 2004; Meissner, 2007; Meissner et al., 2008; Murray et al., 2007; Ricci et al., 2007). Mitochondria are a primary source of ROS in both myonuclei and quiescent muscle stem cells (i.e., satellite cells), which have been shown to be prone to random superoxide “sparks” (Wang et al., 2008).

ROS-induced defects in mtDNA are thought to underlie at least part of the loss of mitochondrial function with aging (Kujoth et al., 2005, 2006;

Wallace & Fan, 2009). Mitochondrial DNA is particularly susceptible to oxidative damage (Hagen et al., 2004; Meissner, 2007; Meissner et al., 2008; Murray et al., 2007; Ricci et al., 2007) because of its proximity to the electron transport chain, the lack of protective histones, and an inefficient repair system compared to nuclear DNA (Lee & Wei, 2007; Ma et al., 2009; Wei & Lee, 2002). The accumulation of mutations of mtDNA induced by exposure to ROS is thought to lead to errors in the mtDNA-encoded polypeptides. Such alterations would affect the four mitochondrial complexes involved in energy production and would result in defective electron transfer, oxidative phosphorylation, and ATP production (Marcinek, 2004; Marcinek et al., 2005). Respiratory chain defects may further increase ROS production, thus establishing a vicious cycle of ROS production and mitochondrial dysfunction (Ozawa, 1995). Genetic or ROS-induced mutations in mtDNA can also contribute to mitochondrial dysfunction, and this will prime the apoptotic pathway so that apoptosis can be quickly initiated upon sensing an appropriate stimulus.

Several lines of evidence support the idea that mtDNA damage and mutations contribute to aging in muscle (reviewed in Dirks Naylor & Leeuwenburgh, 2008; Dirks & Leeuwenburgh, 2006; Marzetti et al., 2010a). For example, mice expressing an aberrant mtDNA polymerase accumulate mtDNA mutations and display a premature aging phenotype, including extensive sarcopenia, compared to wild-type littermates (Kujoth et al., 2005, 2006). Thus, ROS-induced accumulations in damaged and oxidized proteins, lipids, and mtDNA would result in a cycle of cellular dysfunction that leads to the onset of phenotypes associated with aging. These observations are among the most prominent features of the oxidative stress theory of aging (Harman, 1956, 2006).

Perturbations Increasing Oxidative Stress Leading to Apoptosis Signaling

Although muscle loading is a reasonable countermeasure for sarcopenia, exercise also causes an increase in the generation of free radicals in a wide range of cells, including muscles (Davies et al., 1982; McArdle & Jackson, 2000; Ryan et al., 2008). Further, exhaustive or fatiguing exercise causes oxidation of glutathione and the release of cytosolic enzymes (Sastre et al., 1992) and leads to cell damage (Jackson et al., 2007; McArdle et al., 2001; McArdle & Jackson, 2000). Oxidative stress is greater in muscles of old versus young adult animals (Ryan et al., 2008) and exercise increases ROS-induced oxidative damage in skeletal muscle of old rats (Ryan et al., 2008; Thomas et al., 2010). Excessive exercise-induced ROS burdens would be expected to induce mitochondrial dysfunction and increase apoptotic signaling in muscles and mitochondria of aged animals.

ROS elevation and damage are not limited to excessive muscle use. Disuse atrophy has also been shown to result in oxidative injury in skeletal muscle under experimental atrophic conditions such as limb immobilization or hind-limb suspension in young and aged animals (Kondo et al., 1991, 1992, 1993a,b, 1994; Siu et al., 2008). In addition, age-dependent increases in lipid peroxidation (e.g., MDA/4-HAE) and oxidative stress (e.g., H_2O_2) and decreases in antioxidant enzymes [e.g., superoxide dismutase (Mn-SOD) protein abundance] occur in skeletal muscles of aged animals in response to disuse (Siu et al., 2008). Together these data suggest that aging elevates muscle levels of oxidative stress and exacerbates the disturbance of redox balance during muscle unloading.

Mitochondrial disruption can occur by ROS-induced oxidation of key mitochondrial components. For example, cardiolipin, a mitochondrial-specific phospholipid that regulates numerous enzyme activities, including those related to oxidative phosphorylation and coupled respiration, is oxidized by ROS (Huang et al., 2008; Jiang et al., 2008). Cardiolipin peroxidation by ROS appears to be an important step that initiates the release of cytochrome *c* from the mitochondrial intermembrane space (Choi et al., 2007; Gonzalvez & Gottlieb, 2007; Kagan et al., 2009). Cardiolipin decreases with aging, and it is also a ROS target in aged myocardium (Lesnefsky & Hoppel, 2008). Although cardiolipin abundance may not change in the mitochondria of aged skeletal muscles (Figueiredo et al., 2009), it is not yet known if cardiolipin is a key ROS target under conditions of loading or unloading in aged skeletal muscles.

The BCL-2 Protein Family

The BCL-2 family serves as an important upstream intracellular checkpoint that plays a crucial role in the coordination of the apoptotic signaling (Danial & Korsmeyer, 2004). BCL-2 family members share homology within four conserved sequence motifs, which comprise the BH1, BH2, BH3, and BH4 family proteins. In general, the BCL-2 family consists of three subclasses: (i) antiapoptotic proteins (e.g., Bcl-2, Bcl-X_L, Bcl-W, A1, and Mcl-1), (ii) multidomain proapoptotic proteins ((Bax, Bak, and Bcl-2-related ovarian killer (Bok)), and (iii) BH3-only proapoptotic proteins [Bid, Bad, Bim, Bik, Dp5/Hrk, phorbol-12 myristate-13 acetate-induced protein 1 (Noxa), and Puma] (Chao & Korsmeyer, 1998; Danial & Korsmeyer, 2004; Mikhailov et al., 2001, 2003). All proapoptotic members and most antiapoptotic members contain the BH3 domain and this domain is believed to be essential for the interactions among the family members (Chao & Korsmeyer, 1998; Korsmeyer, 1995, 1999). The BH3 sequence motif has a hydrophobic α-helix, which is favorable for protein interaction. This is the putative region that is thought to be responsible for the association among the BCL-2 family members through homo- or hetero-oligomerization (Chao & Korsmeyer, 1998; Er et al., 2007; Mikhailov et al., 2001, 2003). The strict control that balances cell survival and apoptotic cell death is purported to be regulated by the relative ratio of pro- and antiapoptotic BCL-2 members (Chao et al., 1995; Chao & Korsmeyer, 1998; Danial & Korsmeyer, 2004; Korsmeyer et al., 1995) with a given cell or cell region.

Proapoptotic Bax and antiapoptotic Bcl-2 proteins are members of the BCL-2 family. These proteins comprise the main components that regulate mitochondrial apoptotic channels or pores. Bcl-2 forms a heterodimer with Bax and prevents its translocation to the mitochondria under nonapoptotic conditions. However, an apoptotic stimulus translocates Bax to the mitochondria and phosphorylates it. Bax undergoes conformational change to expose its N-terminus (Basanez et al., 1999; Cartron et al., 2002; Desagher & Martinou, 2000; Hsu et al., 1997; Wolter et al., 1997), thereby allowing Bax–Bax oligomerization and insertion of Bax into the outer mitochondrial membrane (Zha et al., 1996). This mediates the subsequent release of the apoptogenic factors (e.g., cytochrome *c*, EndoG, AIF) from the mitochondrial intermembrane space (Kroemer et al., 2007; Narita et al., 1998; Reed et al., 1998; Shimizu et al., 1999; Tsujimoto et al., 2006; Tsujimoto & Shimizu, 2000). Bcl-2 functions to bind to Bax, thereby preventing the Bax–Bax oligomerization and insertion into the mitochondrial membrane. Therefore, Bcl-2 opposes the proapoptotic activity of Bax (Antonsson et al., 2000; Korsmeyer et al., 1995; Korsmeyer, 1995, 1999; Kroemer et al., 2007; Lalier et al., 2007; Reed, 1997, 2006; Reed et al., 1998; Yin et al., 1994).

Caspase-Dependent Signaling

Several members of the proapoptotic caspase family appear to have critical roles in apoptotic signaling transduction (Chang & Yang, 2000; Degterev et al., 2003; Earnshaw et al., 1999; Grutter, 2000). Caspases are synthesized as inactive procaspases. When procaspases undergo cleavage or oligomerization-mediated self-/autoactivation by an apoptotic signal, they are converted from their inactive procaspase form to the active protease configuration (Chang & Yang, 2000; Deveraux et al., 1999; Deveraux & Reed, 1999; Earnshaw et al., 1999; Grutter, 2000; Stennicke et al., 1999; Stennicke & Salvesen, 1999).

Caspase-9 is an initiator caspase that mediates the mitochondria-mediated apoptosis signaling. Caspase-9 participates in formation of a protein complex called the apoptosome (Figure 4.3). The interaction of procaspase-9 with Apaf-1, cytochrome *c* (which is released from the mitochondria), and ATP/dATP in the cytosol activates caspase-9, which cleaves procaspase-3 and activates it (Acehan et al., 2002; Chang & Yang, 2000; Shi, 2002a,b, 2004). Caspase-3 is a common downstream effector (executer) caspase for initiating DNA destruction. Cellular substrates for caspase-3 cleavage include proteins responsible for cell cycle regulation (e.g., $p21^{Cip1/Waf1}$), apoptotic cell death (e.g., Bcl-2 and IAP), DNA repair [e.g., poly(ADP-ribose) polymerase and inhibitor of caspase-activated DNase], cell signal transduction (e.g., Akt/PKB), the cytoskeletal structural scaffold (e.g., gelsolin), and others (Chang & Yang, 2000).

Caspase-Independent Signaling

Several proapoptotic proteins are normally housed in the mitochondrial intermembrane space, but an apoptotic stimulus causes them to be released from the mitochondria into the cytosol where they initiate an apoptotic program of cell death (Blink et al., 2004; Joza et al., 2001; Li et al., 2001). AIF, EndoG, and HtrA2/Omi have been shown to be able to induce apoptosis without the involvement of caspases (Blink et al., 2004; Joza et al., 2001; Li et al., 2001). AIF is a mitochondrial flavoprotein that has both oxidoreductase and apoptosis-inducing properties (Cande et al., 2002a,b; Joza et al., 2001, 2005). The apoptotic function of AIF may be the result of a putative DNA binding site that results in chromatin condensation and DNA fragmentation (Lipton & Bossy-Wetzel, 2002; Ye et al., 2002). Another mitochondria-housed proapoptotic protein is EndoG, a well-conserved nuclear-encoded endonuclease, which can induce chromosomal DNA cleavage in a caspase-independent manner (Li et al., 2001). In contrast, the apoptotic properties of the serine protease HtrA2/Omi are less well defined. It has been proposed that HtrA2/Omi may induce apoptosis via a mechanism that is similar to that of Smac/Diablo, in which the apoptosis-suppressing activities of IAPs are removed through a caspase-regulated process (Hegde et al., 2002; Shi, 2004; Shiozaki & Shi, 2004). However, it has also been shown that the apoptosis-inducing ability of HtrA2/Omi can function via its proteolytic activity in the absence of caspase activation (Blink et al., 2004; Suzuki et al., 2004).

It is known that cytosolic and nuclear levels of AIF and EndoG are elevated in skeletal muscles of old animals (Leeuwenburgh et al., 2005; Marzetti et al., 2008c; Siu & Alway, 2006a). This suggests a central role for apoptosis in sarcopenia, but the extent to which caspase-dependent versus caspase-independent signaling dominates apoptotic elimination of nuclei has not yet been established. Although a role for HtrA2/Omi has been suggested in response to myocardial injury or heart failure (Bhuiyan & Fukunaga, 2007; Siu et al., 2007), it has not been established that HtrA2/Omi is elevated in sarcopenia.

Apoptotic Suppressors

A group of endogenous proteins has been shown to function in suppressing proapoptotic signaling. Members of the IAP family of apoptotic suppressors include X-linked inhibitor of apoptosis (XIAP), apoptosis repressor with caspases recruitment domain protein (ARC), and Fas-associated death domain protein-like interleukin-1α-converting enzyme-like inhibitory protein (FLIP). XIAP is a gene product that is well conserved among many species (Deveraux et al., 1998; Shi, 2002b). The antiapoptotic ability of XIAP is attributed to the conserved baculovirus inhibitor of an apoptosis repeat (BIR) motif, which is essential for the inhibition of initiator as well as effector caspases. XIAP is increased in muscles of aged birds compared to young birds, and this appears to provide a level of protection against aging-associated loss in these muscles (Siu et al., 2005c). All protein members of the IAP family studied to date carry a minimum of one BIR motif (Chowdhury et al., 2008; Deveraux et al., 1998, 1999; Sanna et al., 2002).

ARC and FLIP are two endogenous apoptosis-suppressing proteins with high expression levels in muscle tissue (Irmler et al., 1997; Koseki et al., 1998). It is possible that the high resistance of mature muscle tissues to apoptosis is related, at least in part, to the abundant levels of these two apoptotic suppressors, although this has not been definitively shown. The apoptotic-suppressive effects of ARC and FLIP are thought to be due to their inhibiting interactions on selective caspases. In particular, caspase-8, which is the initiator caspase in the death receptor-mediated apoptosis, is targeted by ARC and FLIP (Heikaus et al., 2008; Irmler et al., 1997; Koseki et al., 1998; Yu et al., 2009). Additional observations indicate that ARC interacts with the proapoptotic Bax protein and in doing so suppresses mitochondria-mediated apoptotic signaling (Gustafsson et al., 2004).

Mitochondria-Mediated Signaling for Apoptosis in Sarcopenia

Several factors contribute to the complex problem of sarcopenia. These include inflammation, oxidative stress, loss of systemically or locally generated growth signals and neural factors, and reduced muscle progenitor stem cell function. Not only do postmitotic myocytes exhibit apoptosis during atrophy that is initiated by denervation and unloading (Allen et al., 1997; Alway et al., 2003a,b; Jin et al., 2001; Siu et al., 2005b; Siu & Alway, 2005a), but apoptosis is thought to have an important role in the aging-associated loss of muscle mass or sarcopenia (Alway et al., 2002a, 2003b; Dirks & Leeuwenburgh, 2006; li-Youcef et al., 2007; Pistilli et al., 2006b; Senoo-Matsuda et al., 2003; Siu et al., 2004, 2005b; Siu & Alway, 2005a, 2006a,b; Zheng et al., 2005).

Aging has been shown to elevate levels of apoptosis through the intrinsic apoptotic pathway in both slow-contracting muscles, which have a majority of fibers that contain type I myosin heavy chain, and predominately fast-contracting muscles that have a preponderance of type II myosin heavy chain. In general, there are a greater number of apoptotic gene changes in fast-contracting fibers, and this is consistent with the observation that slow-fibered muscles are more resistant to aging-associated muscle loss than fast-contracting fibers. Estimations of percentage changes in mitochondria-associated apoptotic signaling are given in Table 4.1. Evidence for myonuclei undergoing apoptosis via the intrinsic pathway in aging has been shown by severalfold increases in TUNEL-positive nuclei and increases in the frequency of nuclei with DNA strand breaks and in the expression of proapoptotic genes and proteins including Bax, caspase-3, AIF, and Apaf-1 in aged and atrophied muscles in mammals and non-mammals, including birds, worms, and flies (Alway et al., 2002a, 2003b; Dirks & Leeuwenburgh, 2006; li-Youcef et al., 2007; Pistilli et al., 2006b; Senoo-Matsuda et al., 2003; Siu et al., 2004, 2005b; Siu & Alway, 2005a, 2006a,b; Zheng et al., 2005). Cytosolic levels of cytochrome *c* do not appear to increase in either slow- or fast-contracting skeletal muscles with age (Table 4.1). The published data show a wide range in caspase-9 levels, which range from large to modest increases or no changes in fast or slow skeletal muscles (Table 4.1). The responses of caspase-independent signaling including AIF and EndoG have been reported to increase from ~50 to 5000% in fast-contracting muscles with aging (Table 4.1). Bax has been reported to increase in fast- and slow-contracting muscles of most studies, but a few studies have found decreases in this protein with increased age (Table 4.1). The changes in Bcl-2 are less clear in fast muscles, because reports vary from decreases to increases in this protein with increased age. Inhibitor of differentiation-2 (Id2) increases markedly in both fast- and slow-contracting muscles with aging, and recent data indicate that Id2 can activate mitochondrial-associated apoptosis in muscle cells (Butler et al., 2009).

Table 4.1 Effect of aging on mitochondria-associated apoptotic signaling in skeletal muscle

APOPTOTIC MARKER	SPECIES	MUSCLE	ASSAY	AGES (MONTHS)	CHANGE WITH AGE (VS YOUNGEST) (%)	REFERENCE
Slow (oxidative) skeletal muscles						
DNA fragmentation	Male FBN	Soleus	TUNEL	6, 32	+200	Ogata et al., 2009
DNA fragmentation	Male FBN	Soleus	ELISA	5–7, 33	+300	Pistilli et al., 2006a
DNA fragmentation	Male FBN	Soleus	TUNEL	6, 30, 36	+3100 (30 months) +6600 (36 months)	Rice & Blough, 2006
DNA fragmentation	Male FBN	Soleus	Western blot	8, 28	No change	Marzetti et al., 2008a
Caspase-3	Male FBN	Soleus	ICC	6, 32	+900	Ogata et al., 2009
Caspase-3	Male FBN	Soleus	Western blot	8, 28	+30 (cleaved)	Marzetti et al., 2008a
Caspase-3 (full length)	Male FBN	Soleus	Western blot	6, 30, 36	No change (30 months) −30 (36 months)	Rice & Blough, 2006
Caspase-3	Male FBN	Soleus	Western blot	9, 33	No change	Alway et al., 2003a
Caspase-3	Male FBN	Soleus	Fluorimetry	5–7, 33	+20	Pistilli et al., 2006a
Caspase-9 (full length)	Male FBN	Soleus	Western blot	6, 30, 36	+44 (30 months) −48 (36 months)	Rice & Blough, 2006
Bax	Male FBN	Soleus	Western blot	6, 32	No change (cytosolic) +170 (mitochondrial)	Ogata et al., 2009
Bax	Male FBN	Soleus	Western blot	6, 30, 36	No change	Rice & Blough, 2006
Bcl-2	Male FBN	Soleus	Western blot	6, 32	+475 (cytosolic) +50 (mitochondrial)	Ogata et al., 2009
Bcl-2	Male FBN	Soleus	Western blot	6, 30, 36	+15 (30 mo) +88 (36 mo)	Rice & Blough, 2006
Bcl-2	Male F344	Soleus	Western blot	3, 24	−36	Song et al., 2006
Bax/Bcl-2	Male F344	Soleus	Western blot	3, 24	+310	Song et al., 2006
Cytochrome c	Male FBN	Soleus	Western blot	6, 32	No change (cytosolic) −58 (mitochondrial)	Ogata et al., 2009
Id2	Male FBN	Soleus	Western blot	9, 33	+1300	Alway et al., 2003a
Fast (glycolytic) skeletal muscles						
DNA fragmentation	Male FBN	EDL	Western blot	8, 28	+200	Marzetti et al., 2008a
DNA fragmentation	Male FBN	Extraocular	TUNEL	6, 18,30	No change	McMullen et al., 2009
DNA fragmentation	Male FBN	Extraocular	ELISA	6, 18, 30	No change	McMullen et al., 2009
DNA fragmentation	Male FBN	Gastrocnemius	DNA laddering	16, 29	+42	Chung & Ng, 2006
DNA fragmentation	Male FBN	Plantaris	ELISA	5–7, 33	+600	Pistilli et al., 2006b
DNA fragmentation	Male C57Bl/6	Gastrocnemius	Fluorimetry	5, 25	+23	Braga et al., 2008

(*Continued*)

Table 4.1 (Continued)

APOPTOTIC MARKER	SPECIES	MUSCLE	ASSAY	AGES (MONTHS)	CHANGE WITH AGE (VS YOUNGEST) (%)	REFERENCE
DNA fragmentation	Male FBN	Gastrocnemius	ELISA	8, 18, 29, 37	+1100	Marzetti et al., 2008c
DNA fragmentation	Male FBN	EDL	TUNEL	6, 30, 36	+1600 (30 months) +3100 (36 months)	Rice & Blough, 2006
DNA fragmentation	Male FBN	Plantaris	TUNEL	9, 33	+600	Pistilli et al., 2006a
DNA fragmentation	Japanese quail	Patagialis	ELISA	2, 48	+18	Siu & Alway, 2006a
DNA fragmentation	Male F344	Gastrocnemius	ELISA	6, 24	+50	Dirks & Leeuwenburgh, 2002
DNA fragmentation	Male F344	Gastrocnemius	ELISA	3, 24	+67	Song et al., 2006
DNA fragmentation	Male F344	Gastrocnemius	Western blot	12, 26	+40	Dirks & Leeuwenburgh, 2004
Caspase-3	Male FBN	Gastrocnemius	Western blot	8, 18, 29, 37	+260	Marzetti et al., 2009b
Caspase-3	Male FBN	Gastrocnemius	Western blot	8, 18, 29, 37	+260	Marzetti et al., 2009b
Caspase-3	Male FBN	EDL	Western blot	8, 28	+100	Marzetti et al., 2008a
Caspase-3	Male FBN	EDL	Western blot	8, 28	+30 (cleaved)	Marzetti et al., 2008a
Caspase-3	Male FBN	Extraocular	Fluorimetry	6, 18, 30	No change	McMullen et al., 2009
Caspase-3	Male FBN	Plantaris	RT-PCR	8, 30, 35	+400 (30 months) +2800 (35 months)	Baker & Hepple, 2006
Caspase-3 (full length)	Male FBN	EDL	Western blot	6, 30, 36	+87 (30 months) +284 (36 months)	Rice & Blough, 2006
Caspase-3	Male F344	Gastrocnemius	Western blot	3, 24	+100,000	Song et al., 2006
Caspase-3	Male F344	Gastrocnemius	Western blot	12, 26	+69	Dirks & Leeuwenburgh, 2004
Caspase-3 (cleaved)	Male F344	Gastrocnemius	Western blot	12, 26	+129	Dirks & Leeuwenburgh, 2004
Caspase-3 activity	Male F344	Gastrocnemius	Fluorimetry	12, 26	No change	Dirks & Leeuwenburgh, 2004
Caspase-3	Male FBN	Gastrocnemius	Western blot	9, 33	+100	Alway et al., 2003a
Caspase-3	Male F344	Gastrocnemius	ELISA	6, 24	No change	Dirks & Leeuwenburgh, 2002
Caspase-3	D257A	Gastrocnemius	Western blot	3, 13	+80	Kujoth et al., 2005
Caspase-9	Male FBN	Extraocular	Fluorimetry	6, 18, 30	No change	McMullen et al., 2009
Caspase-9	Male FBN	Gastrocnemius	Western blot	8, 18, 29, 37	No change	Marzetti et al., 2008c
Caspase-9	Male C57Bl/6	Gastrocnemius	Fluorimetry	5, 25	+250	Braga et al., 2008
Caspase-9	Male FBN	Plantaris	RT-PCR	8, 30, 35	+360 (30 months) +1600 (35 months)	Baker & Hepple, 2006
Caspase-9	Male FBN	Gastrocnemius	Western blot	16, 29	+210 (cleaved)	Chung & Ng, 2006
Caspase-9 (full length)	Male FBN	EDL	Western blot	6, 30, 36	No change (30 months) +30 (36 months)	Rice & Blough, 2006
Caspase-9	Male FBN	Plantaris	RT-PCR	9, 33	No change	Pistilli et al., 2006b
Caspase-9	Male F344	Gastrocnemius	Western blot	12, 26	No change	Dirks & Leeuwenburgh, 2004

Caspase-9 (activity)	Male F344	Gastrocnemius	Western blot	12, 26	No change	Dirks & Leeuwenburgh, 2004
Caspase-9	Male FBN	Plantaris	Western blot	9, 33	+300	Alway et al., 2002a
Caspase-2	Male C57Bl/6	Gastrocnemius	Western blot	5, 25	+650	Braga et al., 2008
Cytochrome *c*	Male FBN	Gastrocnemius	Western blot	8, 18, 29, 37	No change	Marzetti et al., 2008c
Cytochrome *c*	*Coturnix* quail	Patagialis	ELISA	2, 48	No change (cytosolic)	Siu & Alway, 2006a
Cytochrome *c*	Male F344	Gastrocnemius	ELISA	12, 26	−22	Dirks & Leeuwenburgh, 2004
Cytochrome *c*	Male F344	Gastrocnemius	ELISA	6, 24	No change (cytosolic)	Dirks & Leeuwenburgh, 2002
Apaf-1	Male FBN	Plantaris	RT-PCR	8, 30, 35	−62 (30 months) −90 (35 months)	Baker & Hepple, 2006
Apaf-1	Male FBN	Plantaris	RT-PCR	9, 33	No change	Pistilli et al., 2006b
Apaf-1	Male FBN	Gastrocnemius	Western blot	16, 29	+803	Chung & Ng, 2006
Apaf-1	Male F344	Gastrocnemius	Western blot	12, 26	+20	Dirks & Leeuwenburgh, 2004
Apaf-1	Male F344	Gastrocnemius	Western blot	6, 24	No change	Dirks & Leeuwenburgh, 2002
AIF	Male FBN	Gastrocnemius	Western blot	8, 18, 29, 37	+150 (cytosolic) +25 (nuclear)	Marzetti et al., 2008c
AIF	Male FBN	Plantaris	RT-PCR	8, 30, 35	No change (30 months) +5000 (35 months)	Baker & Hepple, 2006
AIF	Male FBN	Plantaris	RT-PCR	9, 33	No change	Pistilli et al., 2006b
AIF	Male F344	Gastrocnemius	Western blot	12, 26	+55 (total) No change (nuclear)	Dirks & Leeuwenburgh, 2004
EndoG	Male FBN	Gastrocnemius	Western blot	8, 18, 29, 37	+70 (cytosolic) +46 (nuclear)	Marzetti et al., 2008c
ARC	*Coturnix* quail	Patagialis	Western blot	2, 48	No change	Siu & Alway, 2006a
ARC	*Coturnix* quail	Patagialis	RT-PCR	2, 16–24	No change	Siu et al., 2005c
ARC	*Coturnix* quail	Patagialis	Western blot	2, 10, 20, 30, 40 & 50 days	No change	Siu et al., 2005c
ARC	Male F344	Gastrocnemius	Western blot	12, 26	+32 (cytosolic) +47 (mitochondrial)	Dirks & Leeuwenburgh, 2004
XIAP	*Coturnix* quail	Patagialis	Western blot	2, 16–24	+98	Siu et al., 2005c
XIAP	Male F344	Gastrocnemius	Western blot	12, 26	+30	Dirks & Leeuwenburgh, 2004
XIAP	*Coturnix* quail	Patagialis	ELISA	2, 48	No change (cytosolic)	Siu & Alway, 2006a
XIAP	*Coturnix* quail	Patagialis	RT-PCR	2, 16–24	No change	Siu et al., 2005c
Bax	Male FBN	Gastrocnemius	Western blot	8, 18, 29, 37	+140 (mitochondrial)	Marzetti et al., 2008c
Bax	Male C57Bl/6	Gastrocnemius	Western Blot	5, 25	No change	Braga et al., 2008
Bax	Male FBN	Plantaris	RT-PCR	8, 30, 35	−74 (30 months) −96 (35 months)	Baker & Hepple, 2006

(*Continued*)

Table 4.1 (Continued)

APOPTOTIC MARKER	SPECIES	MUSCLE	ASSAY	AGES (MONTHS)	CHANGE WITH AGE (VS YOUNGEST) (%)	REFERENCE
Bax	Male FBN	EDL	Western blot	6, 30, 36	+53 (30 months) +136 (36 months)	Rice & Blough, 2006
Bax	Male FBN	Gastrocnemius	Western blot	16, 29	+226	Chung & Ng, 2006
Bax	Male FBN	Plantaris	RT-PCR	9, 33	+150	Pistilli et al., 2006b
Bax	Male FBN	Plantaris	Western blot	9, 33	+900	Pistilli et al., 2006b
Bax	Male F344	Gastrocnemius	Western blot	3, 24	+60	Song et al., 2006
Bax	Male FBN	Plantaris	Western blot	9, 33	+9500	Alway et al., 2002a
Bax	Male F344	Gastrocnemius	ELISA	6, 24	No change	Dirks & Leeuwenburgh, 2002
Bcl-2	Male FBN	Gastrocnemius	Western blot	8, 18, 29, 37	+350 (mitochondrial)	Marzetti et al., 2008c
Bcl-2	Male C57Bl/6	Gastrocnemius	RT-PCR	5, 25	+50	Braga et al., 2008
Phospho-Bcl-2	Male C57Bl/6	Gastrocnemius	Western blot	5, 25	+1950	Braga et al., 2008
Bcl-2	Male FBN	Plantaris	RT-PCR	8, 30, 35	−66 (30 months) −90 (35 months)	Baker & Hepple, 2006
Bcl-2	Male FBN	Gastrocnemius	Western blot	16, 29	+68	Chung & Ng, 2006
Bcl-2	Male FBN	EDL	Western blot	6, 30, 36	+34 (30 months) +58 (36 months)	Rice & Blough, 2006
Bcl-2	Male FBN	Plantaris	RT-PCR	9, 33	No change	Pistilli et al., 2006b
Bcl-2	Male FBN	Plantaris	Western blot	9, 33	+600	Pistilli et al., 2006b
Bcl-2	Male F344	Gastrocnemius	Western blot	3, 24	−20	Song et al., 2006
Bcl-2	Male F344	Gastrocnemius	ELISA	6, 24	No change	Dirks & Leeuwenburgh, 2002
Bax/Blc-2	Male FBN	Gastrocnemius	Western blot	16, 29	+92	Chung & Ng, 2006
Bax/Bcl-2	Male F344	Gastrocnemius	Western blot	3, 24	+98	Song et al., 2006
Id2	Male FBN	Plantaris	RT-PCR	9, 33	+100	Pistilli et al., 2006b
Id2	Male FBN	Gastrocnemius	Western blot	6, 30	+65 (nuclear) +60 (cytosolic)	Siu et al., 2006
Id2	Male FBN	Gastrocnemius	Western blot	9, 33	+340	Alway et al., 2003a
Id2	Male FBN	Plantaris	RT PCR	9, 33	+150	Alway et al., 2002a
Id2	Male FBN	Plantaris	Western blot	9, 33	+200	Alway et al., 2002a
Phospho-JNK	Male C57Bl/6	Gastrocnemius	Western blot	5, 25	+80	Braga et al., 2008
p53	Male FBN	Gastrocnemius	Western blot	6, 30	+35 (nuclear) +65 (cytosolic)	Siu et al., 2006
p53	Male FBN	Gastrocnemius	DNA laddering	16, 29	+90	Chung & Ng, 2006

Examples of studies that have described apoptotic signaling in slow oxidative and fast glycolytic skeletal muscles in aged animals are given. In some cases, the authors have estimated the percentage changes in signaling genes or proteins from the data presented in the respective article. FBN, Fischer 344 × Brown Norway rats; F344, Fischer 344 rats; D257A, mice containing a mitochondrial DNA mutation; EDL, extensor digitorum longus, ICC, immunocytochemistry.

EXTRINSIC APOPTOTIC SIGNALING IN SKELETAL MUSCLE

Cellular Conditions for Activation of the Extrinsic Apoptotic Pathway

One potential mechanism contributing to the onset of sarcopenia is the loss of growth factors in the serum that feed aging cells (Bruunsgaard, 2002; Pedersen et al., 2003). Another possibility is that the age-induced increase in circulating serum cytokines contributes to sarcopenia by activating the extrinsic apoptotic pathway. In humans, serum levels of catabolic cytokines, such as TNF-α (Sandmand et al., 2003; Schaap et al., 2009) and IL-6 (Bruunsgaard, 2002; Forsey et al., 2003; Pedersen et al., 2003; Schaap et al., 2009), are increased in healthy elderly compared to young adults. Serum concentrations of TNF-α have been proposed as a prognostic marker of all-cause mortality in centenarians (Bruunsgaard et al., 2003a) and of age-associated pathology and mortality in 80-year-old adults (Bruunsgaard et al., 2003b). TNF-α is also known to activate the extrinsic apoptotic pathway and induce DNA fragmentation in skeletal muscles (Carbo et al., 2002).

TNF-α and Death Receptor Signaling

Several studies have found associations between increases in circulating cytokines and sarcopenia (Pedersen et al., 2003; Schaap et al., 2006, 2009; Visser et al., 2002). Specifically, elevated circulating levels of TNF-α are associated with lower appendicular skeletal muscle mass (Pedersen et al., 2003) and reduced knee extensor and grip strength (Visser et al., 2002).

TNF-α is a pleiotropic cytokine that has an important role in many different physiological and pathological processes, including immune and inflammatory responses (Wajant et al., 2003; Wajant, 2009) and activation of the extrinsic apoptotic pathway (Ricci et al., 2007). The contribution of the extrinsic apoptotic pathway to skeletal muscle mass losses, especially during aging, has been less well studied than the intrinsic pathway (Marzetti et al., 2009b; Phillips & Leeuwenburgh, 2005; Pistilli et al., 2006a). Nevertheless, activation of the extrinsic pathway appears to play a role in aging-associated muscle loss (Figure 4.4).

We have found extensive activation of the extrinsic apoptotic signaling pathway in muscles of old rats (Pistilli et al., 2006a, 2007; Siu et al., 2008), and therefore we speculate that circulating TNF-α may be the initiator of this pathway in skeletal muscle. TNF-α has been shown to promote protein degradation (Garcia-Martinez et al., 1993a,b; Llovera et al., 1997, 1998) and apoptosis directly within skeletal muscle (Carbo et al., 2002; Figueras et al., 2005). Furthermore, intravenous injection of recombinant TNF-α increases protein degradation in rat skeletal muscles and this is associated with increased activity of the ubiquitin-dependent proteolytic pathway (Garcia-Martinez et al., 1993a, 1995; Llovera et al., 1997, 1998). In addition, elevated TNF-α concentrations in cell culture for 24–48 h increase apoptosis in

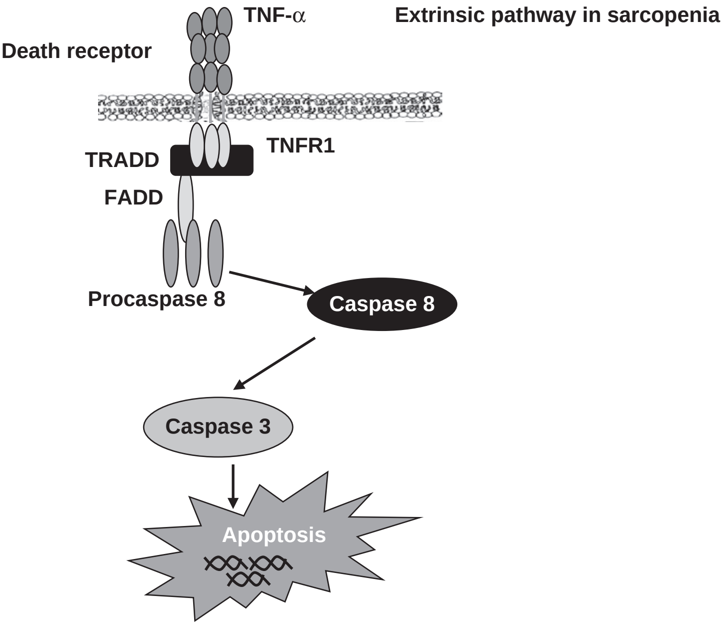

Figure 4.4 The extrinsic (death receptor) pathway is activated in aging and contributes to sarcopenia. A ligand (e.g., TNF-α) binds to the death receptor and TNFR1 and activates procaspase-8 and caspase-8 in turn activates caspase-3 and DNA fragmentation.

skeletal myoblasts as determined by DNA fragmentation (Foulstone et al., 2001; Meadows et al., 2000). A reduction of procaspase-8 occurs within 6 h of incubating myoblasts in vitro with recombinant TNF-α, suggesting a TNF-α-mediated cleavage and activation of this initiator caspase in myoblast cultures (Meadows et al., 2000).

The effects of TNF-α on apoptosis are not limited to in vitro conditions, because a systemic elevation of TNF-α in vivo increases DNA fragmentation within rodent skeletal muscle (Carbo et al., 2002). Based on the observation that TNF-α mRNA was not different between muscles from young adult and those from aged rats, it is reasonable to assume that muscle-derived TNF-α does not act in an autocrine manner to stimulate the proapoptotic signaling observed in this study.

The increase in circulating concentrations of TNF-α in aged animals may initiate proapoptotic signaling upon binding to the type 1 TNF receptor (TNFR1) in aged skeletal muscles. Upon binding, a death-inducing signaling complex (DISC) is formed at the cytoplasmic portion of the TNFR. This is composed of adaptor proteins such as Fas-associated death domain protein (FADD), TNFR-associated death domain protein (TRADD), and procaspase-8 (reviewed in Sprick & Walczak, 2004). Formation of the DISC stimulates cleavage of procaspase-8 into the functional initiator caspase-8. Once cleaved, caspase-8 stimulates cleavage and activation of the executioner caspase-3 (Ricci et al., 2007). Thus, this pathway represents an extrinsic pathway of apoptosis activated by binding of a ligand (i.e., TNF-α) to a cell surface death receptor (type 1 TNFR).

Nuclear factor-κB (NF-κB) is the best-known mediator of TNF-α-associated cellular responses. NF-κB is a group of dimeric transcription factors that are members of the NF-κB/Rel family, including p50, p52, p65 (Rel-A), Rel-B, and c-Rel (Kearns & Hoffmann, 2009; Shih et al., 2009). The activity of NF-κB is normally regulated by the IκB family of inhibitors, which bind to and sequester NF-κB in the cytoplasm (Shih et al., 2009). Activation of NF-κB is triggered by IκB phosphorylation by IKK kinases and subsequent proteasomal degradation, which allows NF-κB to translocate to the nucleus, where it binds to the κB consensus sequences and modulates specific target genes (Kearns & Hoffmann, 2009; Vallabhapurapu & Karin, 2009). NF-κB provides a protective role during TNF-α-induced apoptosis. This is because NF-κB is a transcriptional activator of antiapoptotic proteins including cFLIP, Bcl-2, and Bcl-X_L (Vallabhapurapu & Karin, 2009). However, NF-κB can also promote apoptosis when activated by proapoptotic proteins including p53, Fas, and FasL (Burstein & Duckett, 2003; Dutta et al., 2006; Fan et al., 2008).

p53 upregulated modulator of apoptosis (PUMA) is a downstream target of p53 and a BH3-only Bcl-2 family member (Chipuk & Green, 2009; A. P. Ghosh et al., 2009; Lee et al., 2009). It is induced by p53 after exposure to DNA-damaging agents, such as γ-irradiation and commonly used chemotherapeutic drugs or oxidative stress (Chipuk & Green, 2009; S. P. Ghosh et al., 2009). It is also activated by a variety of nongenotoxic stimuli independent of p53, such as serum starvation, kinase inhibitors, glucocorticoids, endoplasmic reticulum stress, and ischemia/reperfusion (Nickson et al., 2007; Yu & Zhang, 2008). The proapoptotic function of PUMA is mediated by its interactions with antiapoptotic BCL-2 family members such as Bcl-2 and Bcl-X_L. This leads to Bax/Bak-dependent mitochondrial dysfunction, mitochondria permeability, and caspase activation (Chipuk & Green, 2009) in the intrinsic apoptotic pathway as discussed above. In addition, PUMA is directly activated by NF-κB and contributes to TNF-α-induced apoptosis (Wang et al., 2009).

Based on the well-documented increase in circulating TNF-α levels with aging (Bruunsgaard et al., 2001, 2003a,b; Bruunsgaard, 2002; Pedersen et al., 2003; Sandmand et al., 2003; Schaap et al., 2006, 2009; Visser et al., 2002) and increases in apoptosis of myonuclei in aged skeletal muscles (Allen et al., 1997; Pistilli et al., 2006b; Siu et al., 2005b), we examined whether apoptotic signaling via the extrinsic pathway contributed to sarcopenia. Our data show that pro- and antiapoptotic proteins in the extrinsic apoptotic pathway are affected by aging in fast (plantaris) and slow (soleus) skeletal muscles of rats (Pistilli et al., 2006a,b). Similarly, Marzetti and co-workers (Marzetti et al., 2009b, 2010a) report elevated TNF-α and TNFR1 in muscles of old rodents. Together, these data suggest that TNF-α-mediated signaling may be an important element triggering the extrinsic apoptotic pathway in and leading to sarcopenia in aging muscles.

Table 4.2 summarizes age-associated apoptotic changes in the extrinsic pathway in slow- and fast-contracting muscles. Muscles from aged rats are significantly smaller and exhibit a larger incidence in fragmented DNA than muscles in young adult rats. This suggests there is a higher level of nuclear apoptosis in muscles from aged animals. In addition, muscles from aged rodents have higher TNFR and FADD mRNA content (measured by semiquantitative reverse transcriptase-polymerase chain reaction) and protein contents for FADD, Bid, and FLIP and enzymatic activities of caspase-8 and caspase-3, compared to muscles from young adult rodents. Although there is an increase in mRNA expression for the TNFR as measured by the semiquantitative approach, the protein content for the TNFR appears to be unchanged (Pistilli et al., 2006a,b). This may be explained by the fact that the TNFR antibody utilized in Western immunoblots recognizes the soluble form of the receptor. Thus, the changes in the membrane-bound form of the receptor, measured by PCR, and the amount of the soluble TNFR may not be equivalent. While fast-contracting muscles are generally more susceptible to apoptosis and sarcopenic muscle

Table 4.2 Effect of aging on death receptor (extrinsic) apoptotic signaling in slow oxidative skeletal muscles and fast glycolytic muscles

APOPTOTIC MARKER	SPECIES	MUSCLE	ASSAY	AGES (MONTHS)	CHANGE WITH AGE (VS YOUNGEST) (%)	REFERENCE
Slow (oxidative) muscles						
DNA fragmentation	Male FBN	Soleus	ELISA	5–7, 33	+300	Pistilli et al., 2006a
Caspase-8	Male FBN	Soleus	Fluorimetry	5–7, 33	+33	Pistilli et al., 2006a
Caspase-8	Male F344	Soleus	Western blot	6, 26	No difference	Phillips & Leeuwenburgh, 2005
Caspase-8	Male FBN	Soleus	Western blot	9, 33	No difference	Alway et al., 2003a
Caspase-8	Male FBN	Soleus	Western blot	8, 28	No difference	Marzetti et al., 2008a
Bid	Male FBN	Soleus	Western blot	5–7, 33	+1090	Pistilli et al., 2006a
TRADD	Male FBN	Soleus	RT-PCR	5–7, 33	No difference	Pistilli et al., 2006a
FADD	Male FBN	Soleus	RT-PCR	5–7, 33	+94	Pistilli et al., 2006a
FADD	Male FBN	Soleus	Western blot	5–7, 33	+300	Pistilli et al., 2006a
FADD	Male F344	Soleus	Western blot	6, 26	No difference	Phillips & Leeuwenburgh, 2005
TNFR1	Male FBN	Soleus	RT-PCR	5–7, 33	+60	Pistilli et al., 2006a
TNFR1	Male FBN	Soleus	Western blot	5–7, 33	No difference	Pistilli et al., 2006a
TNFR1	Male F344	Soleus	Western blot	6, 26	No difference	Phillips & Leeuwenburgh, 2005
TNFR1	Male FBN	Soleus	Western blot	8, 28	No difference	Marzetti et al., 2008a
TNF-α	Male FBN	Soleus	RT-PCR	5–7, 33	No difference	Pistilli et al., 2006a
TNF-α	Male FBN	Soleus	Western blot	8, 28	No difference	Marzetti et al., 2008a
TNF-α	Male F344	Plasma	ELISA	6, 26	+215	Phillips & Leeuwenburgh, 2005
IL-15	Male FBN	Soleus	RT-PCR	5–7, 33	+20	Pistilli et al., 2007
IKKγ	Male F344	Soleus	Western blot	6, 26	+111	Phillips & Leeuwenburgh, 2005
IκB-α	Male F344	Soleus	Western blot	6, 26	+80	Phillips & Leeuwenburgh, 2005
NF-κB activity	Male F344	Soleus	Western blot	6, 26	No difference	Phillips & Leeuwenburgh, 2005
Fast (glycolytic) skeletal muscles						
DNA fragmentation	Male FBN	Plantaris	ELISA	5–7, 33	+600	Pistilli et al., 2006a
Caspase-8	Male FBN	Gastrocnemius	Western blot	8, 18, 29, 37	+140	Marzetti et al., 2009b
Caspase-8	Male FBN	Extraocular	Fluorimetry	6, 18, 30	No difference	McMullen et al., 2009
Caspase-8	Male FBN	Plantaris	RT-PCR	8, 30, 35	+300 (30 months) +700 (35 months)	Baker & Hepple, 2006

(Continued)

Table 4.2 (Continued)

APOPTOTIC MARKER	SPECIES	MUSCLE	ASSAY	AGES (MONTHS)	CHANGE WITH AGE (VS YOUNGEST) (%)	REFERENCE
Caspase-8	Male FBN	Plantaris	Fluorimetry	5–7, 33	+72	Pistilli et al., 2006a
Caspase-3	Male FBN	Plantaris	Fluorimetry	5–7, 33	+27	Pistilli et al., 2006a
Caspase-8	Male FBN	Gastrocnemius	Western blot	8, 18, 29, 37	+140	Marzetti et al., 2009b
Caspase-8	Male F344	SVL	Western blot	6, 26	+200	Phillips & Leeuwenburgh, 2005
Caspase-8	Male FBN	Gastrocnemius	Western blot	9, 33	+50	Alway et al., 2003a
Caspase-8	Male FBN	EDL	Western blot	8, 28	+100	Marzetti et al., 2008a
Bid	Male FBN	Plantaris	Western blot	5–7, 33	+1150	Pistilli et al., 2006a
TRADD	Male FBN	Plantaris	RT-PCR	5–7, 33	No difference	Pistilli et al., 2006a
FADD	Male FBN	Plantaris	RT-PCR	5–7, 33	+50	Pistilli et al., 2006a
FADD	Male FBN	Plantaris	Western blot	5–7, 33	+650	Pistilli et al., 2006a
FADD	Male F344	SVL	Western blot	6, 26	+50	Phillips & Leeuwenburgh, 2005
TNFR1	Male FBN	Plantaris	RT-PCR	5–7, 33	+43	Pistilli et al., 2006a
TNFR1	Male FBN	Plantaris	Western blot	5–7, 33	No difference	Pistilli et al., 2006a
TNFR1	Male FBN	Gastrocnemius	Western blot	8, 18, 29, 37	+400	Marzetti et al., 2009b
TNFR1	Male F344	SVL	Western blot	6, 26	No difference	Phillips & Leeuwenburgh, 2005
TNFR1	Male FBN	EDL	Western blot	8, 28	+220	Marzetti et al., 2008a
TNF-α	Male FBN	Gastrocnemius	Immunoassay	8, 18, 29, 37	+200	Marzetti et al., 2009b
TNF-α	Male FBN	Plantaris	RT-PCR	5–7, 33	No difference	Pistilli et al., 2006a
TNF-α	Male FBN	EDL	Western blot	8, 28	No difference	Marzetti et al., 2008a
TNF-α	Male FBN	Gastrocnemius	Immunoassay	8, 18, 29, 37	+200	Marzetti et al., 2009b
TNF-α	Male F344	Plasma	ELISA	6, 26	+215	Phillips & Leeuwenburgh, 2005
IL-15	Male FBN	Gastrocnemius	Immunoassay	8, 18, 29, 37	−50	Marzetti et al., 2009b
IL-15	Male FBN	Plantaris	RT-PCR	5–7, 33	No difference	Pistilli et al., 2007
IKKγ	Male F344	SVL	Western blot	6, 26	No difference	Phillips & Leeuwenburgh, 2005
IκB-α	Male F344	SVL	Western blot	6, 26	No difference	Phillips & Leeuwenburgh, 2005
NF-κB activity	Male F344	SVL	Western blot	6, 26	−14	Phillips & Leeuwenburgh, 2005

In some cases, the percentage changes were estimated from data presented in the referenced article. FBN, Fischer 344 × Brown Norway rats; F344, Fischer 344 rats; EDL, extensor digitorum longus; ICC, immunocytochemistry; SVL, superficial vastus lateralis muscle.

loss, proapoptotic changes have been reported to be expressed in a similar fashion in both plantaris and soleus muscles. Nevertheless, strong relationships between markers of apoptosis and muscle loss have been observed in the fast plantaris muscle that were not observed in the soleus (Pistilli et al., 2006a,b). These data show that type II fibers are preferentially affected by aging and suggest that type II fiber-containing skeletal muscles may be more susceptible to muscle mass losses via the extrinsic apoptotic pathway (Pistilli et al., 2006b).

Regulation of the extrinsic pathway is very complex, with some proteins appearing to have dual roles. For example, cFLIP(L) is widely regarded as an inhibitor of initiator caspase-8 activation and cell death in the extrinsic pathway; however, it is also capable of enhancing procaspase-8 activation through heterodimerization of their respective protease domains. Cleavage of the intersubunit linker of cFLIP(L) by procaspase-8 potentiates the activation process by enhancing heterodimerization between the two proteins and elevates the proteolytic activity of unprocessed caspase-8 (Yu et al., 2009). FLIP's role in the regulation of apoptosis may be in part related to individual splice variants [i.e., protein isoforms, for example, FLIP(S) versus FLIP(L) or FLIPc]. For example, disruption of NF-κB regulation of FLIPc has been implicated in muscle wasting diseases such as limb-girdle muscular dystrophy type 2A (Benayoun et al., 2008), although it is not known if similar deregulations occur in aging muscles.

Cross Talk between Extrinsic and Intrinsic Apoptotic Signaling

Cross talk between extrinsic and intrinsic apoptotic pathways was recently reviewed (Sprick & Walczak, 2004). Cross talk between these pathways is the result of the cleavage of the proapoptotic BCL-2 family member Bid (Crompton, 2000; Gillick & Crompton, 2008). Cleaved and activated caspase-8 can not only serve to activate caspase-3, which is the executioner caspase, but also cleave full-length Bid into a truncated version (tBid) (Gillick & Crompton, 2008). tBid then interacts with proapoptotic Bax to stimulate downstream apoptotic signaling from the mitochondria (Grinberg et al., 2005). As has been previously shown, apoptotic signaling from the mitochondria stimulates cleavage of procaspase-9, which then serves to activate caspase-3 (Johnson & Jarvis, 2004). Thus, both the extrinsic and the intrinsic apoptotic pathways converge on caspase-3, which then fully engages proapoptotic signaling. Skeletal muscles from aged rodents contain a greater protein abundance of full-length Bid, which raises the possibility that cross talk between the extrinsic pathway and the intrinsic pathway may occur in aged skeletal muscles (Figure 4.5).

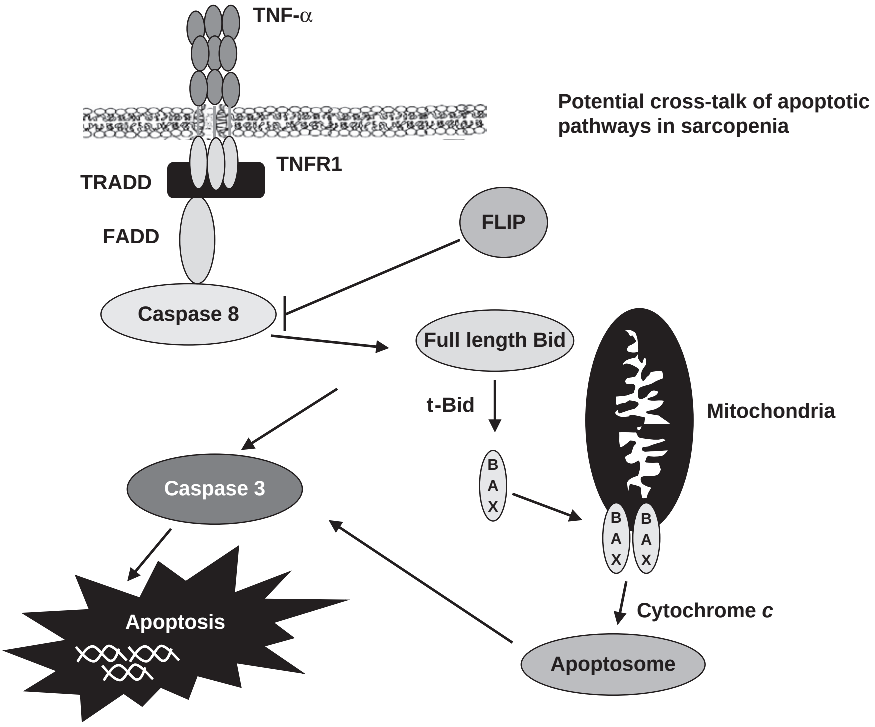

Figure 4.5 The potential cross talk between the extrinsic and the intrinsic apoptotic signaling pathways is shown.

Certain cell types seem to be more susceptible to proapoptotic signaling via the extrinsic pathway without involving the mitochondria. These cell types, termed "type I" cells, differ from "type II" cells based on the accumulation of DISC after ligand binding (Scaffidi et al., 1998). Upon ligand binding, type I cells accumulate a larger amount of DISC on the cytoplasmic side of the death receptor and do not require additional proapoptotic signaling arising from the mitochondria to activate apoptosis fully. In contrast, type II cells accumulate considerably less DISC and require mitochondrial signaling to activate fully an apoptotic program. Skeletal muscle cells may act as type II cells (Henriques-Pons & Nagaraju, 2009; Nagaraju et al., 2000), in that apoptotic signaling arising from the death receptor can include subsequent mitochondrial apoptotic signaling through Bid activation. Future studies should directly address the ability of truncated Bid to mediate messages from the death receptor to the mitochondria from aging muscles.

SARCOPLASMIC/ENDOPLASMIC RETICULUM–CALCIUM STRESS APOPTOTIC SIGNALING

Cellular Conditions that Activate the SR/ER–Ca^{2+} Stress Apoptotic Pathway

Data suggest that the ER–Ca^{2+} stress pathway (in skeletal muscle this is the SR–Ca^{2+} stress pathway) may be an important regulator of the induction of apoptosis (Marciniak et al., 2004; Oyadomari & Mori, 2004; Szegezdi et al., 2006b, 2009). In skeletal muscle, SR stress occurs by alterations in Ca^{2+} homeostasis, in a fashion similar to that seen for ER stress in nonmuscle cells (reviewed in Puzianowska-Kuznicka & Kuznicki, 2009), and accumulation of unfolded proteins and oxidatively damaged proteins in the SR (Figure 4.6).

Intracellular fluxes in Ca^{2+} ion concentration regulate processes such as proliferation, transcription, contraction, exocytosis, immune response, and apoptosis (reviewed in Berridge et al., 2003; Carafoli, 2002; Saris & Carafoli, 2005). Muscle has a highly developed endoplasmic reticulum called the sarcoplasmic reticulum, which is responsible for tight control, storage, and release of Ca^{2+}. Various calcium pump channels in the plasma membrane, endoplasmic and sarcoplasmic reticula, and mitochondria are responsible for the transport of Ca^{2+}. In skeletal muscle, isoforms of the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump regulate calcium uptake by the SR. With aging, there is a decreased Ca^{2+} uptake by the SR (Zhao et al., 2008) and an increase in cytosol calcium in skeletal muscle, which is probably due at least in part to SERCA pump dysfunction (Brini & Carafoli, 2009; Periasamy & Kalyanasundaram, 2007). SERCA proteins are particularly sensitive to ROS-induced oxidative damage (Yin et al., 2000), and therefore dysfunctional Ca^{2+} handling leading to high intracellular calcium levels might be related to ROS-induced damage of this key calcium regulator protein. Thus, mechanical conditions (e.g., fatiguing muscle contractions, which elevate intracellular calcium) and oxidative stresses (which is elevated with aging and muscle disuse) disturb SR function and can also lead to the accumulation of unfolded proteins in

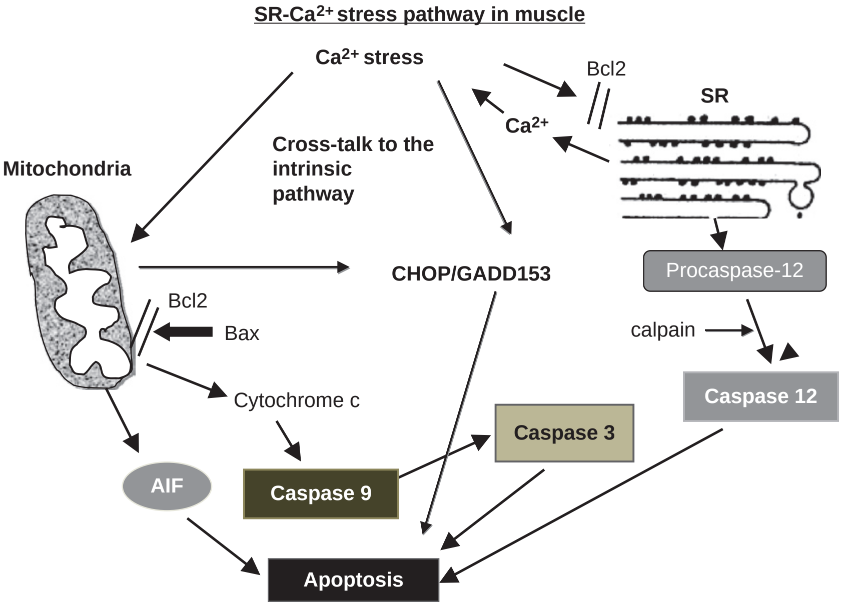

Figure 4.6 Signaling via the SR–Ca^{2+} stress pathway and the potential cross talk between this pathway and the intrinsic apoptotic signaling pathway in muscle.

the lumen of the endoplasmic reticulum. If the stress is prolonged, apoptotic signaling can be initiated through the SR/ER–Ca^{2+} stress pathway (Ogata et al., 2009; Oyadomari & Mori, 2004).

Signaling via the SR–Ca^{2+} Stress Apoptotic Pathway

The SR–Ca^{2+} stress signaling in skeletal muscle activates the cell death caspase cascade (Oyadomari & Mori, 2004). Skeletal muscle has a large amount of Ca^{2+}, which is stored in the SR, but sustained increases in muscle cytosolic levels of Ca^{2+} in response to repeated action potentials (i.e., for muscle contractions) in the face of dysfunctional SERCA pumps provide a potential source for SR–Ca^{2+} stress in aging muscle. In addition, accumulation of oxidatively damaged proteins is increased in skeletal muscle with aging (Marzani et al., 2008; Pietrangelo et al., 2009; Semba et al., 2007). Therefore, the SR–Ca^{2+} stress pathway may also play a more important role than previously appreciated in apoptosis induction in skeletal muscle with aging.

Although a large body of research has primarily focused on the actions of the BCL-2 protein members at the mitochondria, it has been known for more than a decade that Bcl-2 has an antiapoptotic role at the ER of nonskeletal muscle cells and presumably also the SR of skeletal muscle cells (Annis et al., 2001; Puzianowska-Kuznicka & Kuznicki, 2009; Zhu et al., 1996). Interestingly, the antiapoptotic function of Bcl-2 in protecting cells against Ca^{2+}-dependent death stimuli (Szegezdi et al., 2009) is dependent on its phosphorylation state. Bcl-2 and Bcl-X_L promote cell survival by maintaining a reduced SR–Ca^{2+} concentration and consequently limiting the amount of Ca^{2+} that can be released by the SR/ER upon cellular stress. Phosphorylated Bcl-2 is predominantly localized to the light membrane fraction rich in SR, and it is unable to dimerize with the proapoptotic BCL-2 family proteins and is therefore unable to reduce Ca^{2+}-dependent cell death (Bassik et al., 2004; Lin et al., 2006; Oakes et al., 2005, 2006). Bcl-2 and Bcl-X_L may also participate in Ca^{2+} release from the SR in a non-voltage-dependent fashion in response to stress (Oakes et al., 2005).

Although the SR–Ca^{2+} stress pathway has not been well studied in aging muscles, the data published to date suggest that proapoptotic proteins in this pathway are elevated in muscles of aged animals (Table 4.3). For example, GRP78, a chaperone protein that is an initiator of SR stress in muscle cells (and ER stress in nonmuscle cells), is upregulated in soleus muscles of 32-month-old rats compared to young adult rats (Ogata et al., 2009). In addition, SR/ER-Ca^{2+} stress proteins, such as caspase-12 and CHOP/GADD153, are upregulated in skeletal muscle from aged animals (Chung & Ng, 2006; Dirks & Leeuwenburgh, 2004; Schroder, 2008; Szegezdi et al., 2006a,b). The increase in SR/ER–Ca^{2+} stress proteins appears to be aging specific and not the result of atrophy per se, because hind-limb suspension induces significant atrophy in the soleus of young animals without a change in CHOP/GADD153 protein abundance (Hunter et al., 2001; Ogata et al., 2009).

Table 4.3 Effect of aging on sarcoplasmic/endoplasmic reticulum–calcium stress-associated apoptotic signaling in slow- and fast-contracting skeletal muscle

APOPTOTIC MARKER	SPECIES	MUSCLE	ASSAY	AGES (MONTHS)	CHANGE WITH AGING (VS YOUNGEST) (%)	REFERENCE
GRP78	Male FBN	Soleus	Western blot	6, 32	+162	Ogata et al., 2009
CHOP/ GADD153	Male FBN	Soleus	Western blot	6, 32	+241	Ogata et al., 2009
Caspase-12	Male FBN	Extraocular	Fluorimetry	6, 18, 30	No difference	McMullen et al., 2009
Caspase-12	Male FBN	Soleus	Western blot	6, 32	+200	Ogata et al., 2009
Caspase-12	Male F344	Gastrocnemius	Western blot	12, 26	+350 (total) No difference (cleaved)	Dirks & Leeuwenburgh, 2004
Caspase-12	Male F344	Gastrocnemius	Western blot	16, 32	+135 (procaspase) No difference (cleaved)	Chung & Ng, 2006

When the percentage changes were not directly reported they were estimated from data presented in the referenced article.
FBN, Fischer 344 × Brown Norway rats; F344, Fischer 344 rats.

Procaspase-12 is localized on the outer membrane of the SR and it has been implicated in apoptosis mediated by SR–Ca^{2+} stress. Aging increases procaspase-12 abundance by threefold in the rodent gastrocnemius (Ogata et al., 2009). Unlike the intrinsic and extrinsic pathways, apoptosis in the SR–Ca^{2+} stress pathway is thought to be mediated by calpains (Sanges & Marigo, 2006). Consequently, apoptosis is mediated by cleavage of procaspase-12 by calpains and translocation of cleaved caspase-12 to the nucleus to initiate DNA fragmentation (Ogata et al., 2009).

Cross Talk of the SR/ER–Ca^{2+} Stress and the Intrinsic Apoptotic Pathway

Cross talk between the SR/ER–Ca^{2+} stress pathway and the intrinsic apoptotic pathway is extensive (Figure 4.6). Apoptosis initiated by SR stress is also highly dependent on the release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol. This event is associated with the opening of the mitochondria permeability transition pore and a collapse in the mitochondrial transmembrane potential, which occurs as a result of the movement of Ca^{2+} after its release into the cytosol from the SR. Once released, cytochrome *c* and procaspase-9 are recruited to Apaf-1 to form the apoptosome complex, which subsequently cleaves and activates procaspase-9 to active caspase-9 in the intrinsic pathway.

Cross talk between the SR stress pathway and the mitochondria signaling pathway appears to be important for strengthening the response to an appropriate apoptotic stress. For example, upregulation of CHOP/GADD153 in the SR/ER–Ca^{2+} apoptotic pathway appears to reflect accumulations of misfolded proteins in mitochondria (Aldridge et al., 2007; Horibe & Hoogenraad, 2007). In addition, the mitochondria-associated AIF primarily controls apoptosis caused by changes in Ca^{2+} homeostasis, whereas caspase-12 has a major role in apoptotic signaling in response to protein misfolding. Nevertheless, caspase-12 and AIF colocalize to the nucleus to induce apoptosis in response to SR–Ca^{2+} stresses (Lees et al., 2009). Thus, both AIF and caspase-12 appear to cooperate and, in doing so, each reinforces the actions of the other during apoptotic signaling.

APOPTOSIS OCCURS IN SATELLITE CELLS/MUSCLE PRECURSOR STEM CELLS

Lees and co-workers (2009) have shown that satellite cells/MPCs isolated from hind-limb muscles of old rats have increased TNF-α-induced NF-κB activation and expression of mRNA levels for TRAF2 and the cell death-inducing receptor, Fas (CD95), in response to prolonged (24 h) TNF-α treatment compared to MPCs isolated from muscles of young animals. These findings suggest that age-related differences may exist in the regulatory mechanisms responsible for NF-κB inactivation, which may in turn have an effect on TNF-α-induced apoptotic signaling. Systemic and muscle levels of TNF-α increase with aging, and this should result in an even more profound increase in activation of apoptotic gene targets through the extrinsic pathway, compared to MPCs in muscles of young adult rats (Renault et al., 2002; Siu et al., 2005b).

We have shown that MPCs that had more recently proliferated (e.g., in response to a loading stimulus) were also more susceptible to apoptosis than myonuclei that had been in the muscle fiber for a much longer period of time (Krajnak et al., 2006; Siu et al., 2005b). Based on these data and the age-related increase in the susceptibility of myoblasts to apoptosis (Jejurikar et al., 2006), we hypothesize that a diminished hypertrophic response at old age compared to young animals or humans is due to a lower MPC recruitment because of an increased extent of apoptosis (Alway et al., 2002a; Dirks & Leeuwenburgh, 2004; Leeuwenburgh, 2003; Siu et al., 2005b) and consequently a lower number of MPCs. Since exercise may reduce apoptosis (Peterson et al., 2008; Siu et al., 2004) we expect that chronic lifelong resistance exercise or overload may attenuate the age-related apoptosis and maintain a higher muscle mass than can be achieved by short-term resistance training or overload initiated at old age.

The Role of MyoD in Apoptosis of MPCs

MyoD is a helix–loop–helix muscle-specific transcription factor that has long been recognized for its crucial role in myoblast proliferation and differentiation. MPC proliferation is markedly reduced by aging (Carson & Alway, 1996; Lees et al., 2006), and this has been tied, at least in part, to reduced MyoD in skeletal muscles of aged animals (Alway et al., 2001, 2002a; Brack et al., 2005; Tamaki et al., 2000). Lower MyoD protein abundance or limited increases in MyoD in response to a growth stimulus (Alway et al., 2001, 2002a; Tamaki et al., 2000) would presumably limit the potential for muscle accumulation and growth. However, MyoD has recently been shown to have a role in regulating apoptosis in a subset of myoblasts that were induced to differentiate. p53-independent regulation of the proapoptotic Bcl2 family member PUMA by MyoD provides one mechanism that targets MPCs during differentiation (Asakura et al., 2007). However, differentiation of activated MPCs appears to be very complicated because MyoD levels in MPCs are also linked with increased apoptosis and lower survival (Asakura et al., 2007) and this may be a very important contributor to lower increases in muscle mass with aging in response to overload. While it seems counterintuitive to think that apoptosis signaling would increase during periods of high MPC proliferation/differentiation under loading conditions, there is evidence to suggest that the muscle regulatory factor MyoD may in fact trigger MPC apoptosis during differentiation (Asakura et al., 2007; Shaltouki et al., 2007). Thus, MyoD may be a signaling molecule that is common to both differentiation and apoptosis.

Role of Inhibitory of Differentiation-2 in Apoptosis of MPCs

Id proteins lack the DNA-binding basic domain found in basic helix–loop–helix family members. It is this basic domain that is responsible for binding to the consensus E-box sequence and subsequent gene transcription in muscles (Benezra et al., 1990; Christy et al., 1991; Norton et al., 1998; Norton, 2000; Norton & Atherton, 1998). Although it is only speculative, it is possible that elevated levels of Id2 proteins in atrophied muscles of aged animals (Alway et al., 2002a; Krajnak et al., 2006) activate proapoptotic pathways via Bax, as occurs at least in part in muscle damage and cardiovascular and muscle disease (Dalla et al., 2001; Sandri et al., 1997; Sandri & Carraro, 1999). In support of this idea, we were unable to detect Bax in many control or hypertrophied muscles of young adult rats, while Bax and caspase-9 were elevated in both control and overloaded muscles of aged rats (Alway et al., 2002a). Furthermore, Id2 protein levels significantly increase with overload in muscles of old rats compared to young adult rats (Alway et al., 2003b; Siu et al., 2006; Siu & Alway, 2005a). While overexpression of Id3 has been shown to induce apoptosis in fibroblasts and other cell types (Norton & Atherton, 1998), we do not think that Id3 plays an important role in apoptosis of aged skeletal muscles. Evidence for this conclusion comes from observations of no change in Id3 protein abundance with overload in muscles from young rats and a decrease in Id3 protein abundance in overloaded muscles of old rats compared to control muscles (Alway et al., 2002a). As Bax and Id3 change in opposite directions during muscle hypertrophy at old age, it is unlikely that Id3 has any direct role in regulating apoptosis of aged muscles at least during loading conditions.

We have observed greater Id2 protein levels and apoptosis in muscles from emphysematous animals (Degens et al., 2007) and in old compared with young adult rats (Alway et al., 2003a,b; Siu & Alway, 2006b). Finally, we have found that the phospho-ablated form of Id2 has a proapoptotic role in myoblasts (Butler et al., 2009). Although this does not establish a causal link, together these data support the idea that Id2 has a dual role in skeletal muscle, but that the apoptotic role of Id2 is limited to old age. Therefore, in future studies it is important to determine if Id2 plays a mechanistic role in pathways regulating apoptosis of skeletal muscle in aged animals and in vivo during muscle loading.

MODULATION OF APOPTOSIS IN AGING MUSCLE

Various perturbations have been used to determine if aging increases the sensitivity of skeletal muscle to apoptosis and apoptosis signaling cascades. These include increases in muscle loading; muscle unloading; loading followed by a period of unloading, disuse, or denervation; and caloric restriction. Extensive data show that these perturbations regulate apoptotic signaling in slow- and fast-contracting aging skeletal muscle via both intrinsic (Table 4.4) and extrinsic (Table 4.5) pathways. In general, long-term adaptations to caloric restriction, increased loading, and aerobic exercise reduce apoptotic signaling in both intrinsic and extrinsic pathways, whereas disuse, denervation, and muscle unloading in aged animals increase apoptotic signaling in these pathways. There are very limited data that address how perturbations to aging skeletal muscle affect the SR/ER–Ca^{2+} apoptotic pathway (Table 4.6).

Interventions by Muscle Loading

The evidence presented above indicates that mitochondrial dysfunction is a major contributing factor to the pathophysiology of aging including sarcopenia. While muscle disuse decreases mitochondrial function leading to apoptosis (Adhihetty et al., 2007b; Siu & Alway, 2005a), chronic exercise improves mitochondrial function (Lanza et al., 2008; Lanza & Nair, 2010) and reduces apoptotic signaling (Siu & Alway, 2006a; Song et al., 2006). The elevated basal levels of myonuclear apoptosis and apoptotic signaling in muscles from aged animals may contribute to the poorer hypertrophic response to muscle loading in aging (Alway et al., 2002a; Alway & Siu, 2008; Degens & Alway, 2003, 2006).

Adaptation to chronic loading has been shown to improve antiapoptotic proteins in skeletal muscle, including XIAP (Siu et al., 2005c) and Bcl-2 (Alway et al., 2002a), and to reduce DNA fragmentation (Siu & Alway, 2006a) and lower proapoptotic proteins including Bax (Siu & Alway, 2006a), ARC (Pistilli et al., 2006b; Siu & Alway, 2006a), and AIF (Siu et al., 2006; Siu & Alway, 2006a).

Table 4.4 Effect of aging and muscle perturbations on mitochondria-associated apoptotic signaling in slow oxidative and fast glycolytic skeletal muscle

APOPTOTIC MARKER	SPECIES	PERTURBATION	MUSCLE	ASSAY	AGES (MONTHS)	EFFECT OF AGING (%)	MUSCLE RESPONSE (%)	REFERENCE
Slow oxidative skeletal muscles								
DNA fragmentation	Male FBN	4 weeks running	Soleus	Western blot	8, 28	No difference	No change	Marzetti et al., 2008a
Caspase-3	Male FBN	4 weeks running	Soleus	Western blot	8, 28	No difference	No change	Marzetti et al., 2008a
Caspase-3	FBN	Denervation	Soleus	Western blot	9, 33	No difference	+60	Alway et al., 2003a
AIF	Male FBN	4 weeks running	Soleus	Western blot	8, 28	No difference (cytosolic)	No change (cytosolic)	Marzetti et al., 2008a
EndoG	Male FBN	4 weeks running	Soleus	Western blot	8, 28	No difference (cytosolic)	No change (cytosolic)	Marzetti et al., 2008a
Bax	Male F344	12 weeks running	Soleus	Western blot	3, 24	No difference	−60	Song et al., 2006
Bax	FBN	Denervation	Soleus	Western blot	9, 33	+900	+1087	Alway et al., 2003a
Bcl-2	Male F344	12 weeks running	Soleus	Western blot	3, 24	+9	+86	Song et al., 2006
Bax/Bcl-2	Male F344	12 weeks running	Soleus	Western blot	3, 24	No difference	−80	Song et al., 2006
Id2	FBN	Denervation	Soleus	Western blot	9, 33	+1200	No change	Alway et al., 2003a
Fast glycolytic skeletal muscle								
DNA fragmentation	Male F344	CR	Plantaris	ELISA	6, 24	−55	−35	Wohlgemuth et al., 2010
DNA fragmentation	Male F344	CR + running	Plantaris	ELISA	6, 24	−55	−34	Wohlgemuth et al., 2010
DNA fragmentation	Male FBN	CR	Gastrocnemius	ELISA	8, 18, 29, 37	+750	−50	Marzetti et al., 2009b
DNA fragmentation	Male FBN	4 weeks running	EDL	Western blot	8, 28	No difference	−60	Marzetti et al., 2008a
DNA fragmentation	Male FBN	HLS	Plantaris	TUNEL	9, 33	+135	No change	Pistilli et al., 2006b
DNA fragmentation	Male FBN	HLS	Plantaris	ELISA	9, 33	+600	No change	Pistilli et al., 2006b
DNA fragmentation	*Coturnix* quails	7 days stretch loading	Patagialis	ELISA	2, 48	+85	No change	Siu & Alway, 2006a
DNA fragmentation	*Coturnix* quails	21 days stretch loading	Patagialis	ELISA	2, 48	No difference	−61	Siu & Alway, 2006a

DNA fragmentation	Male F344	12 weeks running	Gastrocnemius	ELISA	3, 24	No difference	−45	Song et al., 2006
DNA fragmentation	F344	CR	Gastrocnemius	ELISA	12, 26	No difference	−23	Dirks & Leeuwenburgh, 2004
Caspase-3 (cleaved)	Male F344	CR	Plantaris	Fluorimetry	6, 24	−50	−20	Wohlgemuth et al., 2010
Caspase-3 (cleaved)	Male F344	CR + running	Plantaris	Fluorimetry	6, 24	−50	No change	Wohlgemuth et al., 2010
Caspase-3	Male FBN	CR	Gastrocnemius	Western blot	8, 18, 29, 37	−60 (cleaved)	−64 (cleaved)	Marzetti et al., 2009a
Caspase-3	Male FBN	4 weeks running	EDL	Western blot	8, 28	−20 (cleaved)	−100 (cleaved)	Marzetti et al., 2008a
Caspase-3 (cleaved)	Male F344	12 weeks running	Gastrocnemius	Western blot	3, 24	+150,000	−95	Song et al., 2006
Caspase-3	F344	CR	Gastrocnemius	Western blot	12, 26	No difference	−92 (total) −126 (cleaved)	Dirks & Leeuwenburgh, 2004
Caspase-3 activity	F344	CR	Gastrocnemius	Fluorimetry	12, 26	No difference	No change	Dirks & Leeuwenburgh, 2004
Caspase-3	FBN	Denervation	Gastrocnemius	Western blot	9, 33	No difference	+60	Alway et al., 2003a
Caspase-9 (cleaved)	Male F344	CR	Plantaris	Fluorimetry	6, 24	−60	−20	Wohlgemuth et al., 2010
Caspase-9 (cleaved)	Male F344	CR + running	Plantaris	Fluorimetry	6, 24	−60	+10	Wohlgemuth et al., 2010
Caspase-9	Male FBN	HLS	Plantaris	RT-PCR	9, 33	No difference	No change	Pistilli et al., 2006b
Caspase-9	F344	CR	Gastrocnemius	Western blot	12, 26	No difference	No change	Dirks & Leeuwenburgh, 2004
Caspase-9 (cleaved)	F344	CR	Gastrocnemius	Western blot	12, 26	No difference	No change	Dirks & Leeuwenburgh, 2004
Cytochroze *c* (cytosolic)	*Coturnix* quails	7 days stretch loading	Patagialis	ELISA	2, 48	+40	No change	Siu & Alway, 2006a
Cytochrome *c* (cytosolic)	*Coturnix* quails	21 days stretch loading	Patagialis	ELISA	2, 48	+100	−50	Siu & Alway, 2006a
Cytochrome *c*	F344	CR	Gastrocnemius	ELISA	12, 26	−29	No change	Dirks & Leeuwenburgh, 2004
Apaf-1	F344	CR	Gastrocnemius	Western blot	12, 26	−25	−45	Dirks & Leeuwenburgh, 2004
Apaf-1	Male FBN	HLS	Plantaris	RT-PCR	9, 33	+120	+130	Pistilli et al., 2006b
AIF	Male FBN	4 weeks running	EDL	Western blot	8, 28	No difference (cytosolic)	No change (cytosolic)	Marzetti et al., 2008a

(*Continued*)

Table 4.4 (Continued)

APOPTOTIC MARKER	SPECIES	PERTURBATION	MUSCLE	ASSAY	AGES (MONTHS)	EFFECT OF AGING (%)	MUSCLE RESPONSE (%)	REFERENCE
AIF (cytosolic)	*Coturnix* quails	7 days stretch loading	Patagialis	ELISA	2, 48	No difference	−40	Siu & Alway, 2006a
AIF (cytosolic)	*Coturnix* quails	21 days stretch loading	Patagialis	ELISA	2, 48	No difference	No change	Siu & Alway, 2006a
AIF	Male FBN	HLS	Plantaris	RT-PCR	9, 33	No difference	+95	Pistilli et al., 2006b
AIF	F344	CR	Gastrocnemius	Western blot	12, 26	No difference (total) −35 (nuclear)	−46 (total) −16 (nuclear)	Dirks & Leeuwenburgh, 2004
ARC	*Coturnix* quails	7 days stretch loading	Patagialis	ELISA	2, 48	−40	−40	Siu & Alway, 2006a
ARC	*Coturnix* quails	21 days stretch loading	Patagialis	ELISA	2, 48	No difference	No change	Siu & Alway, 2006a
ARC	*Coturnix* quails	14 days loading	Patagialis	Western blot	2, 16–24	No difference	No change	Siu et al., 2005c
ARC	*Coturnix* quails	14 days loading + 7 days unloading	Patagialis	Western blot	2, 16–24	No difference	No change	Siu et al., 2005c
ARC	*Coturnix* quails	14 days loading + 14 days unloading	Patagialis	Western blot	2, 16–24	No difference	No change	Siu et al., 2005c
ARC	F344	CR	Gastrocnemius	Fluorimetry	12, 26	+45 (cytosolic) No difference (mitochondria)	+77	Dirks & Leeuwenburgh, 2004
XIAP	*Coturnix* quails	7 days stretch loading	Patagialis	ELISA	2, 48	+40	+110	Siu & Alway, 2006a
XIAP	*Coturnix* quails	21 days stretch loading	Patagialis	ELISA	2, 48	No difference	+60	Siu & Alway, 2006a
XIAP	*Coturnix* quails	14 days loading	Patagialis	RT-PCR	2, 16–24	No difference	No change	Siu et al., 2005c
XIAP	*Coturnix* quails	14 days loading + 7 days unloading	Patagialis	RT-PCR	2, 16–24	+45	+47	Siu et al., 2005c
XIAP	*Coturnix* quails	14 days loading + 14 days unloading	Patagialis	RT-PCR	2, 16–24	No difference	No change	Siu et al., 2005c
XIAP	*Coturnix* quails	14 days loading	Patagialis	Western blot	2, 16–24	+86	+116	Siu et al., 2005c
XIAP	*Coturnix* quails	14 days loading + 7 days unloading	Patagialis	Western blot	2, 16–24	+300	+67	Siu et al., 2005c

XIAP	*Coturnix* quails	14 days loading + 14 days unloading	Patagialis	Western blot	2, 16–24	+200	+57	Siu et al., 2005c
XIAP	F344	CR	Gastrocnemius	Fluorimetry	12, 26	No difference	−30	Dirks & Leeuwenburgh, 2004
FLIP_L	Male FBN	CR	Gastrocnemius	Western blot	8, 18, 29,37	No difference	No change	Marzetti et al., 2009a
FLIP	*Coturnix* quails	14 days loading	Patagialis	RT-PCR	2, 16–24	No difference	No change	Siu et al., 2005c
FLIP	*Coturnix* quails	14 days loading + 7 days unloading	Patagialis	RT-PCR	2, 16–24	No difference	No change	Siu et al., 2005c
FLIP	*Coturnix* quails	14 days loading + 14 days unloading	Patagialis	RT-PCR	2, 16–24	No difference	No change	Siu et al., 2005c
cIAP-1/2	Male FBN	CR	Gastrocnemius	Western blot	8, 18, 29, 37	No difference	+25	Marzetti et al., 2009a
EndoG	Male FBN	4 weeks running	EDL	Western blot	8, 28	No difference (cytosolic)	No change (cytosolic)	Marzetti et al., 2008a
Bax	Male FBN	HLS	Plantaris	RT-PCR	9, 33	+40	+25	Pistilli et al., 2006b
Bax	Male FBN	HLS	Plantaris	Western blot	9, 33	+600	+30	Pistilli et al., 2006b
Bax	*Coturnix* quails	7 days stretch loading	Patagialis	Western blot	2, 48	No difference	No change	Siu & Alway, 2006a
Bax	*Coturnix* quails	21 days stretch loading	Patagialis	Western blot	2, 48	No difference	No change	Siu & Alway, 2006a
Bax	Male F344	12 weeks running	Gastrocnemius	Western blot	3, 24	−80	−92	Song et al., 2006
Bax	FBN	Denervation	Gastrocnemius	Western blot	9, 33	+400	+49	Alway et al., 2003a
Bcl-2	Male FBN	HLS	Plantaris	RT-PCR	9, 33	No difference	+61	Pistilli et al., 2006b
Bcl-2	Male FBN	HLS	Plantaris	Western blot	9, 33	+500	+110	Pistilli et al., 2006b
Bcl-2	Male F344	12 weeks running	Gastrocnemius	Western blot	3, 24	+94	+166	Song et al., 2006
Bax/Bcl-2	Male F344	12 weeks running	Gastrocnemius	Western blot	3, 24	−97	−97	Song et al., 2006
Id2	Male FBN	HLS	Plantaris	RT-PCR	9, 33	+110	+100	Pistilli et al., 2006b
Id2 (mRNA)	Male FBN	14 days HLS	Gastrocnemius	RT-PCR	6, 30	No difference	No change	Siu et al., 2006
Id2 (nuclear)	Male FBN	14 days HLS	Gastrocnemius	Western blot	6, 30	+63	No change	Siu et al., 2006
Id2 (cytosolic)	Male FBN	14 days HLS	Gastrocnemius	Western blot	6, 30	+44	+135	Siu et al., 2006
Id2	FBN	Denervation	Gastrocnemius	Western blot	9, 33	+350	No change	Alway et al., 2003a
p53 (mRNA)	Male FBN	14 days HLS	Gastrocnemius	RT-PCR	6, 30	No difference	No change	Siu et al., 2006
p53 (nuclear)	Male FBN	14 days HLS	Gastrocnemius	Western blot	6, 30	+57	No change	Siu et al., 2006
p53 (cytosolic)	Male FBN	14 days HLS	Gastrocnemius	Western blot	6, 30	+81	+52	Siu et al., 2006

Effect of aging refers to the effect of aging on muscle response to perturbation (young vs old perturbations). Muscle response refers to muscle response to perturbation in old age (perturbed vs control muscles in aged animals). When the percentage changes were not directly reported they were estimated from data presented in the referenced article. FBN, Fischer 344 × Brown Norway rats; F344, Fischer 344 rats; HLS, hind-limb tail suspension; CR, caloric restriction; EDL, extensor digitorum longus.

Table 4.5 Effect of aging and perturbations on death receptor (extrinsic) apoptotic signaling in slow oxidative and fast glycolytic skeletal muscle

APOPTOTIC MARKER	SPECIES	PERTURBATION	MUSCLE	ASSAY	AGES (MONTHS)	EFFECT OF AGING (%)	MUSCLE RESPONSES (%)	REFERENCE
Slow oxidative muscles								
DNA fragmentation	Male FBN	4 weeks running	Soleus	Western blot	8, 28	No difference	No change	Marzetti et al., 2008a
Caspase-8	Male F344	CR	Soleus	Western blot	6, 26	N/A	No change	Phillips & Leeuwenburgh, 2005
Caspase-8	FBN	Denervation	Soleus	Western blot	9, 33	+20	+167	Alway et al., 2003a
Caspase-8	Male FBN	4 weeks running	Soleus	Western blot	8, 28	No difference	No change	Marzetti et al., 2008a
FADD	Male F344	CR	Soleus	Western blot	6, 26	N/A	No change	Phillips & Leeuwenburgh, 2005
TNFR1	Male FBN	4 weeks running	Soleus	Western blot	8, 28	No difference	No change	Marzetti et al., 2008a
TNF-α	Male FBN	4 weeks running	Soleus	Western blot	8, 28	No difference	No change	Marzetti et al., 2008a
TNF-α	Male F344	CR	Plasma	ELISA	6, 26	N/A	−65	Phillips & Leeuwenburgh, 2005
TNF-α	Male F344	CR	Soleus	ICC	6, 26	N/A	No change	Phillips & Leeuwenburgh, 2005
IL-15	Male FBN	***	Soleus	RT-PCR	5–7, 33	−24	No change	Pistilli et al., 2007
IKKγ	Male F344	CR	Soleus	Western blot	6, 26	N/A	−60	Phillips & Leeuwenburgh, 2005
IκB-α	Male F344	CR	Soleus	Western blot	6, 26	N/A	−50	Phillips & Leeuwenburgh, 2005
NF-κB activity	Male F344	CR	Soleus	ELISA	6, 26	N/A	No change	Phillips & Leeuwenburgh, 2005
Fast glycolytic skeletal muscles								
DNA fragmentation	Male FBN	CR	Gastrocnemius	ELISA	8, 18, 29, 37	−55	No change	Marzetti et al., 2009b
DNA fragmentation	Male FBN	4 weeks running	EDL	Western blot	8, 28	No difference	−60	Marzetti et al., 2008a
Caspase-8	FBN	Denervation	Soleus	Western blot	9, 33	+20	+167	Alway et al., 2003a
Caspase-8	FBN	Denervation	Gastrocnemius	Western blot	9, 33	+116	+190	Alway et al., 2003a
Caspase-8	Male FBN	CR	Gastrocnemius	Western blot	8, 18, 29, 37	+50 (cleaved)	−40 (cleaved)	Marzetti et al., 2009b
Caspase-8	Male FBN	4 weeks running	EDL	Western blot	8, 28	No difference	−100	Marzetti et al., 2008a

FADD	Male F344	CR	SVL	Western blot	6, 26	N/A	−40	Phillips & Leeuwenburgh, 2005
TNF-α	Male FBN	CR	Gastrocnemius	Immunoassay	8, 18, 29, 37	+150	−30	Marzetti et al., 2009b
TNFR1	Male FBN	CR	Gastrocnemius	Western blot	8, 18, 29, 37	+95	−60	Marzetti et al., 2009b
TNFR1	Male F344	CR	SVL	Western blot	6, 26	N/A	No change	Phillips & Leeuwenburgh, 2005
TNFR1	Male FBN	4 weeks running	EDL	Western blot	8, 28	No difference	−65	Marzetti et al., 2008a
TNF-α	Male FBN	4 weeks running	EDL	Western blot	8, 28	No difference	No change	Marzetti et al., 2008a
TNF-α	Male F344	CR	SVL	ICC	6, 26	N/A	+7	Phillips & Leeuwenburgh, 2005
IL-15	Male FBN	CR	Gastrocnemius	RT-PCR	8, 18, 29, 37	No difference	No change	Marzetti et al., 2009b
IL-15	Male FBN	14 days HLS	Plantaris	RT-PCR	5–7, 33	No difference	+71	Pistilli et al., 2007
IL-15	*Coturnix* quail	14 days loading +7 days unloading	Patagialis	RT-PCR	2, 24	+35	+19	Pistilli et al., 2007
IKK-γ	Male F344	CR	SVL	Western blot	6, 26	N/A	No change	Phillips & Leeuwenburgh, 2005
IκB-α	Male F344	CR	SVL	Western blot	6, 26	N/A	No change	Phillips & Leeuwenburgh, 2005
NF-κB activity	Male F344	CR	SVL	ELISA	6, 26	N/A	No change	Phillips & Leeuwenburgh, 2005

Effect of aging refers to the effect of aging on muscle responses to perturbations (young vs old perturbations). Muscle responses refers muscle responses to perturbations in old age (control vs perturbed muscles in aged animals). When the percentage changes were not directly reported they were estimated from data presented in the referenced article. FBN, Fischer 344 × Brown Norway rats; F344, Fischer 344 rats; HLS, hind-limb tail suspension; CR, caloric restriction; EDL, extensor digitorum longus; SVL, superficial vastus lateralis.

Table 4.6 Effect of aging and muscle perturbations on the extrinsic apoptotic pathway in fast-contracting skeletal muscle

APOPTOTIC MARKER	SPECIES	PERTURBATION	MUSCLE	ASSAY	AGES (MONTHS)	EFFECT OF AGING (%)	MUSCLE RESPONSES (%)	REFERENCE
Caspase-12	F344	CR	Gastrocnemius	Western blot	12, 26	+350 (total) No difference (cleaved)	+86 (total) −25 (cleaved)	Dirks & Leeuwenburgh, 2004

Effect of aging refers to the effect of aging on muscle responses to perturbations (young vs old perturbations). Muscle responses refers to the muscle responses to perturbations in old age (control vs perturbed muscles in aged animals). CR, caloric restriction.

Table 4.7 Effect of aging on mitochondria-associated apoptotic signaling in the myocardium

APOPTOTIC MARKER	SPECIES	ASSAY	AGES (MONTHS)	CHANGE WITH AGE (VS YOUNGEST) (%)	REFERENCE
DNA fragmentation	Male monkey	TUNEL	3–9 months, 17–26 years	+400	Zhang et al., 2007
DNA fragmentation	Female monkey	TUNEL	2–12 months, 18–24 years	No difference	Zhang et al., 2007
DNA fragmentation	F344	ELISA	3, 24	+ 62	Kwak et al., 2006
DNA fragmentation	F344	TUNEL	3, 24	3.2	Kwak et al., 2006
DNA fragmentation	SD	DNA ladder	6–8, 22–24	+115	Liu et al., 1998
DNA fragmentation	F344	TUNEL	6–8, 22–24	+100	Azhar et al., 1999a
DNA fragmentation	FBN	ELISA	6, 36	+160	Ljubicic et al., 2010
Caspase-3	F344	Western blot	3, 24	+114	Kwak et al., 2006
Casapse-3	Male F344	Fluorescence	6, 16, 24	No difference	Phaneuf & Leeuwenburgh, 2002
Caspase-3 (cleaved)	D257A	ELISA	3, 13	+80	Kujoth et al., 2005
Caspase-3 (cleaved)	F344	Western blot	6, 30, 36	+139 (17 kDa), +192 (19 kDa)	Kakarla et al., 2010
Caspase-9	F344	Western blot	3, 24	+89	Kwak et al., 2006
Caspase-9 (cleaved)	FBN	Western blot	6, 30, 36	+35	Kakarla et al., 2010
Cytochrome c	Male F344	Immunoassay	6, 16, 24	+23	Phaneuf & Leeuwenburgh, 2002
Cytochrome c	FBN	Western blot	6, 36	+130 (cytosolic)	Ljubicic et al., 2010
Apaf-1	Male F344	Western blot	6, 16, 24	No difference	Phaneuf & Leeuwenburgh, 2002
AIF	FBN	Western blot	6, 36	+130 (cytosolic)	Ljubicic et al., 2010
Bax	F344	Western blot	3, 24	+176	Kwak et al., 2006
Bax	Male F344	Western blot	6, 16, 24	No difference	Phaneuf & Leeuwenburgh, 2002
Bax	FBN	mRNA	4, 19	+27	Liu et al., 2002
Bax	SD	Western blot	6–8, 22–24	+95	Liu et al., 1998
Bax	SD	Western blot	6–8, 22–24	+95	Liu et al., 1998
Bax	F344	Western blot	6–8, 22–24	+94	Azhar et al., 1999a
Bax	FBN	Western blot	6, 36	+73 (mitochondrial)	Ljubicic et al., 2010
Bcl-2	F344	Western blot	3, 24	−26	Kwak et al., 2006
Bcl-2	Male F344	Western blot	6, 16, 24	−15	Phaneuf & Leeuwenburgh, 2002
Bcl-2	FBN	RT-PCR	4, 19	No difference	Liu et al., 2002
Bcl-2	SD	Western blot	6–8, 22–24	+50	Liu et al., 1998
Bcl-2	F344	Western blot	6–8, 22–24	No difference	Azhar et al., 1999a
Bax/Bcl-2	F344	Western blot	3, 24	+272	Kwak et al., 2006
Bax/Bcl-2	FBN	RT-PCR	4, 19	No difference	Liu et al., 2002
Bax/Bcl-2	SD	Western blot	6–8, 22–24	+20	Liu et al., 1998
Bax/Bcl-2	F344	Western blot	6–8, 22–24	No difference	Azhar et al., 1999a
Bax/Bcl-xl	F344	Western blot	6–8, 22–24	No difference	Quindry et al., 2005
p66Shc	FBN	Western blot	6, 36	+57 (mitochondrial)	Ljubicic et al., 2010
p46Shc	FBN	Western blot	6, 36	+310 (mitochondrial)	Ljubicic et al., 2010

When the percentage changes were not directly reported they were estimated from the data presented in the referenced article. FBN, Fischer 344 × Brown Norway rats; F344, Fischer 344 rats; SD, Sprague Dawley; CR, caloric restriction.

Table 4.8 Effect of aging and perturbations on mitochondria-associated apoptotic signaling in cardiac muscle

APOPTOTIC MARKER	SPECIES	PERTURBATION	ASSAY	AGES (MONTHS)	EFFECT OF AGING (%)	MUSCLE RESPONSES (%)	REFERENCE
DNA fragmentation	F344	12 weeks running	ELISA	3, 24	No difference	−44	Kwak et al., 2006
DNA fragmentation	F344	12 weeks running	TUNEL	3, 24	+1.6	−1.62	Kwak et al., 2006
DNA fragmentation	FBN	MI/R	ELISA	4, 19	+260	+720	Liu et al., 2002
DNA fragmentation	SD	MI/R	DNA ladder	6–8, 22–24	−44	+90	Liu et al., 1998
DNA fragmentation	F344	Hypoxia/R	TUNEL	6–8, 22–24	+40	+440	Azhar et al., 1999a
DNA fragmentation factor	Male B6C3F1 mice	CR	Microarray	5, 30	—	−430	Lee et al., 2002
Caspase-3	F344	12 weeks running	Western blot	3, 24	+115	−31	Kwak et al., 2006
Caspase-9	F344	12 weeks running	Western blot	3, 24	No difference	−74	Kwak et al., 2006
Caspase-9	Male B6C3F1 mice	CR	Microarray	5, 30	—	−190	Lee et al., 2002
Bcl-2	FBN	MI/R	RT-PCR	4, 19	−74	−81	Liu et al., 2002
Bcl-2	F344	12 weeks running	Western blot	3, 24	−37	+118	Kwak et al., 2006
Bcl-2	F344	Hypoxia/R	TUNEL	6–8, 22–24	No difference	+220	Azhar et al., 1999a
Bax	F344	12 weeks running	Western blot	3, 24	+120	−53	Kwak et al., 2006
Bax	Male B6C3F1 mice	CR	Microarray	5, 30	—	−180	Lee et al., 2002
Bax	FBN	MI/R	RT-PCR	4, 19	+23	+61	Liu et al., 2002
Bax	SD	MI/R	Western blot	6–8, 22–24	No difference	−50	Liu et al., 1998
Bax	F344	Hypoxia/R	TUNEL	6–8, 22–24	No difference	No change	Azhar et al., 1999a
Bax/Bcl-2	F344	12 weeks running	Western blot	3, 24	No difference	−79	Kwak et al., 2006
Bax/Bcl-2	FBN	MI/R	RT-PCR	4, 19	+370	+730	Liu et al., 2002
Bax/Bcl-2	SD	MI/R	Western blot	6–8, 22–24	−40	−60	Liu et al., 1998
Bax/Bcl-2	F344	Hypoxia/R	TUNEL	6–8, 22–24	−57	−200	Azhar et al., 1999a
Bad	Male B6C3F1 mice	CR	Microarray	5, 30	—	−340	Lee et al., 2002
Bcl-X$_L$	F344	Hypoxia/R	TUNEL	6–8, 22–24	No difference	+150	Azhar et al., 1999a
Bax/Bcl-X$_L$	F344	Hypoxia/R	TUNEL	6–8, 22–24	−120	−150	Azhar et al., 1999a

Effect of aging refers to the effect of aging on muscle responses to perturbations (young vs old perturbations). Muscle responses refers to the muscle responses to perturbations in old age (control vs perturbed muscles in aged animals). When the percentage changes were not directly reported they were estimated from data presented in the referenced article. MI/R, myocardial ischemia/reperfusion injury; Hypoxia/R, hypoxia/reperfusion; FBN, Fischer 344 × Brown Norway rats; F344, Fischer 344 rats; CR, caloric restriction.

Our lab (Ferketich et al., 1998; Roman et al., 1993) and others (Charette et al., 1991; Deschenes & Kraemer, 2002; Mayhew et al., 2009; Parise & Yarasheski, 2000; Welle et al., 1995) have shown that resistance exercise is an effective tool to reduce but not eliminate sarcopenia in aging humans. Although aging has generally been shown to attenuate the absolute extent of muscle adaptations that are possible with increased loading (Cutlip et al., 2006; Degens & Alway, 2003; Ryan et al., 2008) it is not known how much of this might be the result of increased nuclear apoptosis in skeletal muscle. Interestingly, several studies have reported unexpected improvements in mitochondrial function in both young adult and aged subjects in response to resistance exercise training. For example, the mitochondrial capacity for ATP synthesis increases after resistance training in older subjects (Conley et al., 2007b; Jubrias et al., 2001; Tarnopolsky, 2009). Resistance exercise also increases antioxidant enzymes and decreases oxidative stress in muscles of older subjects (Parise et al., 2005). Furthermore, 26 weeks of whole-body resistance exercise was shown to reverse the gene expression of mitochondrial proteins that were associated with normal aging, to levels that were similar to those observed in young subjects (Tarnopolsky, 2009). Although we have found that resistance training did not increase the relative volume of mitochondria in muscle fibers of young adults, resistance exercise stimulated mitochondria biogenesis to maintain the myofibrillar-to-mitochondria volume (Alway et al., 1988; Alway, 1991). In addition, aging attenuates the adaptive response to improve the muscle's ability to buffer pro-oxidants in response to chronic muscle loading (Ryan et al., 2008). Nevertheless, there is some improvement in antioxidant enzymes and the ability to buffer oxidative stress in response to loading conditions (Parise et al., 2005; Ryan et al., 2008). Therefore, it is possible that resistance training could also improve mitochondrial function and stimulate mitochondrial biogenesis in aged individuals. Furthermore, chronic loading reduces proapoptotic signaling in muscles of aged animals (Siu & Alway, 2006a). Although it is speculative, if muscle loading not only improves antioxidant enzymes levels but also reduces apoptotic signaling such as Bax accumulation in mitochondria, we would expect that signaling via the intrinsic apoptotic signaling pathway will decrease in chronically exercised muscles of older hosts. We hypothesize that this would lead to improved muscle recovery after disuse and contribute to reduced sarcopenia.

Apoptotic Elimination of MPCs Reduces Muscle Hypertrophic Adaptation to Loading

It is thought that myonuclei maintain a constant cytoplasm-to-nuclei ratio (i.e., "nuclear domain", see Figure 4.1) and that hypertrophy requires that fibers add new nuclei (Rosenblatt et al., 1994; Schultz, 1996; Schultz & McCormick, 1994). Because myonuclei are postmitotic (Schultz, 1989, 1996; Schultz et al., 1994; Schultz & McCormick, 1994), satellite cells/MPCs provide the only important source for adding new nuclei to initiate muscle regeneration, muscle hypertrophy, and postnatal muscle growth in muscles of both young and aged animals (Adams, 2006; Allen et al., 1999; Carson & Alway, 1996; Hawke & Garry, 2001; Rosenblatt et al., 1994). MPCs are critical for muscle growth because muscle hypertrophy is markedly reduced or eliminated completely after irradiation to prevent MPC activation in mammals (Phelan & Gonyea, 1997; Rosenblatt et al., 1994). Growth of adult skeletal muscle requires activation and differentiation of satellite cells/MPCs and increased protein synthesis and accumulation of proteins, and this necessitates increased transcription of muscle genes (Degens & Alway, 2003). Thus, there is little doubt that MPC activation and differentiation are critical components in determining muscle adaptation and growth.

If MPCs are activated normally, but they either do not differentiate or do not survive to participate in increased protein synthesis, then muscle adaptation would be compromised (Carson & Alway, 1996). Elevation of apoptosis (lower MPC survival) in muscles from aged animals (Alway et al., 2002b; Dirks & Leeuwenburgh, 2002, 2004; Leeuwenburgh, 2003; Pollack et al., 2002; Siu et al., 2005b; Tamaki et al., 2000) could explain the poorer adaptation to repetitive loading in aging. We have shown that the most recently activated satellite cells/MPCs during loading are also the most susceptible to apoptosis during subsequent unloading (Siu et al., 2005b). Based on these data, we hypothesize that MPC contribution to chronic loading-induced adaptation (hypertrophy) is lower in muscles of old animals because apoptosis is higher (Alway et al., 2002a,b; Dirks & Leeuwenburgh, 2004; Leeuwenburgh, 2003; Pollack et al., 2002; Tamaki et al., 2000) and fewer MPCs survive to contribute to muscle adaptation.

Unloading and Disuse in Skeletal Muscle

In contrast to models involving increased loading, models of muscle unloading, muscle denervation, or hind-limb suspension show changes in proapoptotic signaling, including elevations in Bax, Apaf-1, and AIF (Alway et al., 2003a; Pistilli et al., 2006b; Siu et al., 2006; Siu & Alway, 2006b); the Bax/Bcl2 ratio (Alway et al., 2003a; Siu et al., 2005a,b); and cytosolic levels of Id2 and p53 (Alway et al., 2002a; Degens, 2007; Degens & Alway, 2003; Siu & Alway, 2005a). These modulations of apoptotic signaling have been summarized in Tables 4.4 and 4.5.

Models of muscle wasting and disuse have provided insight into potential mechanisms that link apoptotic signaling and sarcopenia (Siu, 2009; Siu & Alway, 2009). A 7-day wing unloading model that reversed the hypertrophy that was achieved by 14 days of wing loading (i.e., unloading-induced atrophy) in Japanese quails showed significantly reduced Bcl-2 protein abundance in both young and old birds (Siu et al., 2005b). In contrast, hind-limb unloading in rats appears to have a compensatory increase in Bcl-2 in muscles of both young and old animals, presumably to reduce the consequences of the suspension-induced increase in Bax protein (Pistilli et al., 2006b). Bax and cytochrome *c* release increases in unloaded muscles of both young and old animals but there is a greater increase in muscles from aged rodents. It is of interest to note that muscle disuse by hind-limb unloading in rats increased Apaf-1 in aged but not in young animals (Siu et al., 2005a). Hind-limb unweighting increased the proapoptotic proteins AIF, EndoG, and mitochondrial Smac/Diablo in muscles of old but not young animals (Dupont-Versteegden et al., 2006; Leeuwenburgh et al., 2005; Siu et al., 2005b). Caspase-9 and caspase-3 increase in muscles of both young and old animals in some, but not all, models of muscle wasting. Aging increases the release of the proapoptotic protein EndoG in muscles under conditions of unloading (Dupont-Versteegden et al., 2006; Leeuwenburgh et al., 2005).

Models of denervation-induced atrophy are relevant to understanding the muscle loss after neural or spinal cord injuries. In addition, some of the loss of skeletal muscle in aging is thought to be due to the loss of motor units and therefore loss of innervation (Siu, 2009). Denervation decreases muscle mitochondrial content and increases mitochondrial permeability, leading to elevated apoptosis signaling in skeletal muscle (Adhihetty et al., 2007b; Csukly et al., 2006). Interestingly, denervated rodent muscle has an increase in Bax, a decrease in Bcl-2, an increased Bax/Bcl-2 ratio, and an increase in the mitochondrial release of cytochrome *c*, Smac/Diablo, and AIF, as well as an upregulation of caspase-3 and caspase-9 mRNA, active protein fragment, and protease enzymatic activity, and a reduction in the antiapoptotic protein XIAP. DNA fragmentation is associated with the elevation in Bax in denervated muscle from animal models or humans (Adhihetty et al., 2007b; Csukly et al., 2006; Jejurikar et al., 2002; Migheli et al., 1997; Tews, 2002). Eliminating Bax or caspase-3 offsets much of the apoptotic-associated muscle loss in denervation (Plant et al., 2009; Siu & Alway, 2006b). This suggests that apoptosis in denervated muscle induces apoptosis, and apoptosis is not simply a consequence of the loss of muscle use. There is clear evidence to support a role for mitochondria-associated apoptotic signaling in rodent skeletal muscle in response to denervation (Siu, 2009; Siu & Alway, 2005b). The greater opening of the mitochondria permeability transition pore in denervated muscle explains the mechanism responsible for the release of several apoptotic factors such as cytochrome *c* and AIF (Csukly et al., 2006). Clearly, there is interplay between intrinsic apoptotic signaling and muscle wasting associated with disuse or denervation.

Regulation of Apoptosis by Aerobic Exercise

Although acute endurance exercise has been shown to increase apoptotic signaling under some conditions including dystrophies and other pathologies (Podhorska-Okolow et al., 1998, 1999; Sandri et al., 1997), long-term adaptation to endurance exercise has been shown to lower mitochondria-associated apoptosis in heart and skeletal muscle of rodents (Kwak et al., 2006; Peterson et al., 2008; Siu et al., 2004; Song et al., 2006). Nevertheless, aerobic exercise does not appear to improve muscle mass or act as a countermeasure to sarcopenia (Alway et al., 1996; Kwak et al., 2006; Peterson et al., 2008; Siu et al., 2004; Song et al., 2006). This might be in part due to aerobically induced pathways that are generally inhibitory to muscle growth (e.g., 5′ adenosine monophosphate activated protein kinase).

Apoptosis has been shown to occur in cardiac (Dalla et al., 2001; Hu et al., 2008; Molina et al., 2009) and skeletal muscles (Dalla et al., 2001; Libera et al., 2009; Vescovo & Dalla, 2006) of experimental models of chronic heart failure, and aerobic exercise is frequently prescribed as a therapeutic intervention for persons with heart disease. Limited data suggest that apoptosis signaling in the intrinsic pathway is reduced by aerobic exercise in cardiac and fast skeletal muscle of young, diseased, and aged animals (Kwak et al., 2006; Marzetti et al., 2008a,b; Peterson et al., 2008; Siu et al., 2004; Song et al., 2006). In contrast, most studies published to date do not find that aerobic exercise provides a substantial improvement in extrinsic apoptotic signaling of either slow- or fast-contracting skeletal muscles (Table 4.5).

Although nuclear apoptosis has been detected in muscles of humans with severe chronic heart failure (Adams et al., 1999, 2008; Vescovo et al., 2000), it does not appear to be a large component of skeletal muscle loss associated when the disease is less severe (Conraads et al., 2009). Complicating the treatment of heart failure and related cardiovascular diseases is the likelihood that drugs including statins, which are routinely prescribed to reduce hypercholesterolemia, may themselves have a proapoptotic role in skeletal muscle (Dirks & Jones, 2006). Such increases in apoptosis are likely to have devastating effects when statins are combined with sarcopenia, in which muscle loss is already high. Although aerobic exercise appears to reduce several skeletal muscle problems of persons suffering from severe chronic heart failure (Linke et al., 2005) and exercise increases antioxidant enzymes, and this is correlated with reduced apoptosis in skeletal muscle of persons with heart failure (Adams et al., 2008; Libera et al., 2009), currently there are no data showing that aerobic exercise reduces apoptosis and skeletal muscle cell loss in heart failure patients (Conraads et al., 2009).

Reduction of Apoptosis by Caloric Restriction

Caloric restriction, in which rodents are fed 30–40% fewer calories than their ad libitum fed littermates, extends the maximum life span 30–50%. Furthermore, caloric restriction slows both the rate of biological aging and the development of age-associated diseases. Lifelong caloric restriction reduces age-associated loss of muscle (Marzetti et al., 2008b, 2009a; Phillips & Leeuwenburgh, 2005). Even moderate lifelong caloric restriction of 8% has been shown to reduce DNA fragmentation and the proapoptotic markers caspase-9 and caspase-3 in fast-contracting rat muscles (Wohlgemuth et al., 2010). The mechanisms that account for this reduction in sarcopenia and lower proapoptotic signaling have not been fully elucidated. Nevertheless, it is likely that at least some of the protection occurs because caloric restriction attenuates oxidative stress. Reduced oxidative insult should better preserve mitochondrial function and integrity and therefore lead to lower mitochondria-associated apoptotic signaling than in ad libitum fed animals (Dirks & Leeuwenburgh, 2004; Marzetti et al., 2008b, 2009a,b; Phillips & Leeuwenburgh, 2005).

Another potential means whereby caloric restriction attenuates apoptosis is via a reduction in expression of inflammatory genes including NF-κB (Chung et al., 2009; Jung et al., 2009a,b). This may be a consequence of reducing oxidative stress in muscles of aging animals (Colom et al., 2007; Ferguson et al., 2008; Kim et al., 2008; Marzetti et al., 2009a; Rohrbach et al., 2006). Caloric restriction has also been shown to reduce TNF-α in fast muscles of old animals (Colom et al., 2007; Ferguson et al., 2008; Kim et al., 2008; Marzetti et al., 2009a; Rohrbach et al., 2006).

Six months of caloric restriction has been shown to improve mitochondria content, mitochondria biogenesis, and oxidative enzymes in skeletal muscles of human subjects ages 35–50 years (Civitarese et al., 2007). Presumably, an elevation in mitochondria content and function provides at least part of the mechanism whereby aerobic exercise reduces apoptotic signaling in muscles of aging rodents (Adhihetty et al., 2009; Hood, 2009; Kwak et al., 2006; Song et al., 2006).

Fast-contracting muscle fibers have a low mitochondria content relative to slow fibers (Alway et al., 1988; Alway, 1991), and low mitochondria content increases the muscle's sensitivity to oxidative stress-induced mitochondrial dysfunction and apoptosis (Bakarev & Nepomnyashchikh, 2004). As a result, it is possible that caloric-restriction-induced increases in mitochondria content (i.e., with "healthy mitochondria") would be most beneficial to muscles with a high type II fiber composition (i.e., fast fibers). Further work is, however, needed to determine if increasing the mitochondria content of type II fibers is a viable strategy for reducing targeted myonuclear apoptosis in fast fibers and thereby attenuating sarcopenia.

Activation of intrinsic, SR/ER–Ca^{2+} stress, and extrinsic apoptotic pathways is reduced in skeletal muscle by caloric restriction (Dirks & Leeuwenburgh, 2004). For example, caloric restriction reverses the age-related increase in gene expression of Bok, Noxa, Apaf-1, AIF, and PUMA in the aged gastrocnemius muscle compared with age-matched controls (Dirks & Leeuwenburgh, 2004). These modulations prevent the age-related elevation in the apoptotic potential and result in lower levels of Apaf-1, AIF, procaspase-3, cleaved caspase-3, and DNA fragmentation in aging muscles of rodents that are caloric restricted compared to ad libitum fed animals (Dirks & Leeuwenburgh, 2004; Marzetti et al., 2008b, 2009a). Caloric restriction also acts as a countermeasure for muscle apoptosis that is induced by TNF-α in aged rats (Phillips & Leeuwenburgh, 2005). Nevertheless, a direct causal link between dietary metabolic control and myonuclear apoptosis in muscle has remained elusive, although several studies have implicated Sirt1 as having a role in both events (Barger et al., 2008; Canto & Auwerx, 2009; Csiszar et al., 2009).

AGING AND CARDIOMYOCYTE APOPTOSIS

With aging the function of the heart declines and the incidence of heart failure increases (Lakatta & Levy, 2003). The loss of cardiac function is thought to involve aging-induced increases in fibrosis, coronary vascular dysfunction, cardiomyocyte dysfunction, and complete cardiomyocyte loss (Anversa et al., 1990). In fact it has been estimated that a healthy 70-year-old person will have lost 30% of his or her cardiomyocytes because of aging alone, in the absence of any cardiac disease (Olivetti et al., 1991, 1995). Since the heart is primarily composed of postmitotic cardiomyocytes, this cellular loss can significantly contribute to overall dysfunction of the heart, as opposed to other organs that can readily replace the damaged or lost cells through an increase in cell division.

Aging-related loss of cardiomyocytes has been shown to increase with age and occur via both necrosis and apoptosis (Kajstura et al., 1996; Nitahara et al., 1998; Torella et al., 2004). By injection of a monoclonal myosin antibody to detect necrosis and TUNEL staining and DNA laddering to confirm apoptosis in aging rat hearts, Kajstura and colleagues (1996) demonstrated that the proportion of apoptosis versus necrosis increases with age. Although necrosis was more prevalent at all ages in rodents, the number of apoptotic myocytes in the left ventricular free wall doubled from 16 to 24 months of age. This suggests that apoptosis accelerates with age. However, Torella and colleagues, examining nuclei of cardiomyocytes, showed that necrosis increased eightfold in mice from age 12 to 20–22 months, whereas apoptosis increased only threefold (Torella et al., 2004).

This increase in apoptotic myocytes was confirmed in aging Fischer 344 rats by another research group. Increases in apoptosis in 3-, 12-, and 24-month-old rat hearts correlated with increased diastolic calcium and DNase I and DNase II in cardiomyocytes with age without a change in p53, Bax, or Bcl-2 (Nitahara et al., 1998). DNase I activity in particular correlated well with the increase in apoptosis observed in rodents from 3 to 24 months of age. Although DNase I and II are endonucleases that can cleave internucleosomal DNA, and their activity has been shown to increase with apoptosis (Barry & Eastman, 1993; Giannakis et al., 1991), it is unclear whether the increase in DNase I activity plays a causal role in the increase in apoptosis with age. Taken together these studies suggest that necrosis is more prevalent than apoptosis in aging cardiomyocytes (especially in mouse hearts), but the incidence of both increases with aging. It is not yet clear if acceleration of apoptosis occurs as a universal process in the aging myocardium.

Cardiomyocyte Apoptosis and Heart Failure

Markers of increased cardiomyocyte apoptosis have been detected in various experimental models of heart failure, including myocardial infarction, pacing, and pressure overload (Condorelli et al., 1999; Holly et al., 1999; Lee et al., 1999; Li et al., 1997; Lim et al., 1999; Yue et al., 1998). There is considerable variation in the reported extent of myocyte apoptosis in these heart failure models, ranging from 0.02 to 14.7% (Holly et al., 1999; Yue et al., 1998). Studies of human end-stage heart failure patients also show an increase in apoptosis, with a wide range of incidence (0.2–35%) (Narula et al., 1996; Olivetti et al., 1997). Although these studies demonstrate that an increase in apoptosis correlates with heart failure, it is unclear whether apoptosis is causally related and contributes to the pathogenesis and/or progression of heart failure.

Support for a mechanistic role for apoptosis in heart failure has been provided by evidence from genetically engineered animal models. For example, loss of survival signaling through genetic deletion of the glycoprotein 130 receptor in a ventricle-specific manner resulted in massive apoptosis and rapid progression to heart failure after aortic constriction (Hirota et al., 1999). Additionally, Wencker and coworkers (2003) demonstrated that cardiac-specific expression of ligand-activated procaspase-8 was sufficient to induce a lethal dilated cardiomyopathy, which could be prevented by administration of a caspase inhibitor. Importantly, these mice had a low level of transgene expression and a low rate of cardiomyocyte apoptosis, which were comparable to those seen in human heart failure-developed cardiomyopathy. In addition, caspase inhibition in outbred rats mediates significant reductions in both apoptosis and infarct size after transient ischemia (Holly et al., 1999; Yaoita et al., 1998). Finally, enhanced survival signaling through adenoviral gene transfer of the antiapoptotic Bcl-2 or transgenic overexpression of insulin-like growth factor I lessens ventricular remodeling after ischemia (Chatterjee et al., 2002; Li et al., 1997). Thus, activation of apoptotic signaling and even low-level apoptosis can cause cardiac dysfunction, whereas inhibition of apoptosis reduces cardiac injury and preserves function.

Importantly, myocyte loss is not the only outcome of increased apoptosis in the heart. Low levels of apoptotic activity or interruption of apoptosis can lead to damaged, dysfunctional cardiomyocytes, as opposed to complete cell loss. Communal and coworkers demonstrated that a subset of myofibrillar proteins were targets for caspase-3 and that initiation of apoptosis in myocytes resulted in decreased contractile function (Communal et al., 2002). Additionally, inhibition of epidermal growth factor 2 receptor, which has been shown to cause heart failure in humans when used as a chemotherapeutic agent (Keefe, 2002; Seidman et al., 2002), causes only a low level of apoptosis, but results in a very dramatic activation of apoptotic signaling and mitochondrial dysfunction (Grazette et al., 2004). Thus, the mitochondrial dysfunction that is observed may significantly contribute to cardiac dysfunction and to the loss of cardiomyocytes. Both of these scenarios highlight the important concept that the contribution of apoptosis to heart failure may be underestimated by simply counting overtly apoptotic myocytes. In human studies, this model of apoptotic signaling contributing to overall dysfunction without complete cell loss is supported by the observation that extensive mitochondrial apoptotic signaling is accompanied by very little apoptosis (Narula et al., 1999).

Apoptosis and the Aging Heart

Aging causes a decline in cardiac function, which is characterized by decreases in stroke volume, ejection fraction, and cardiac output (Lakatta, 1986; Nussbacher et al., 1999). Remodeling of the aging heart involves a significant loss of cardiac myocytes (~30%), reactive hypertrophy of the remaining cells, and increased connective tissue and fibrosis (Anversa et al., 1990; Olivetti et al., 1991). The reduction in the number of cardiomyocytes contributes to the decline in cardiac functional capacity with aging. Although it is not clear that the increase in apoptosis observed in the heart is sufficient alone to cause heart failure, apoptosis represents an important process mediating the loss of cardiac myocytes with advanced age (Kajstura et al., 1996; Lee et al., 2002; Phaneuf & Leeuwenburgh, 2002). Furthermore, increases in the susceptibility to apoptosis in the aged heart combined with injury (myocardial ischemia, infarction) or chronic myocardial stress (hypertension) may precipitate cardiac dysfunction or overt failure. Finally, apoptotic changes with the aging myocardium may also have a gender component. This is illustrated from investigations of the long-tailed macaque (*Macaca fascicularis*) in which a fourfold greater level of apoptotic nuclei (by TUNEL) and a 51% increase in cardiomyocyte size have been observed in males, but no change in DNA fragmentation and only an 8% increase in myocyte size was found in females (Zhang et al., 2007). These modulations of apoptotic signaling have been summarized in Table 4.7. Further work is needed to determine if a gender-specific apoptotic response occurs in humans.

Modulation of Bcl-2 and Bax in the Aging Heart

Activation of the mitochondrial-mediated intrinsic apoptotic pathway, which has been documented in the aging and failing heart, may play a significant role in the development of cardiac dysfunction and pathogenesis. The levels of mitochondria-associated apoptotic markers, which increase during aging in normal mice, increase further in mouse strains expressing a proofreading-deficient version of mitochondrial DNA polymerase and correlate with the accumulation of mtDNA mutations in the heart (Kujoth et al., 2005). Changes in the pro- and antiapoptotic factors Bax and Bcl-2 in the intrinsic apoptotic pathway, in particular, may play a critical role in predisposing the aging heart to apoptosis. Phaneuf & Leeuwenburgh reported an increase in cytosolic cytochrome *c* (presumably secondary to release from the mitochondria), a marker of oxidative stress and a key cofactor in effector caspase activation, in hearts of 16- and 24-month-old Fischer 344 rats compared to 6-month-old animals (Phaneuf & Leeuwenburgh, 2002). Additionally they found a decrease in Bcl-2, but not Bax, protein with age. Since Bcl-2 can inhibit the release of cytochrome *c* from the mitochondria by preventing functional pore formation via proapoptotic proteins, including Bax (Oltvai et al., 1993), this decrease in Bcl-2 would be consistent and possibly mechanistically related to the increase in cytochrome *c* release. It is interesting to note that there was no detectable increase in caspase activity, despite the increase in cytochrome *c* release from the mitochondria (Oltvai et al., 1993). The authors (Oltvai et al., 1993) suggest that there could be a critical threshold of cytochrome *c* accumulation that is needed before caspase activation occurs. Another possibility is that the elevation in cytosolic levels of cytochrome *c* might represent a heightened basal apoptotic priming, so that when the aged heart receives an additional stress or insult, it will be more susceptible to apoptosis. A study comparing 3- versus 24-month old Fischer 344 (F344) rat hearts confirmed a decrease in Bcl-2 protein with aging; however, in contrast, they also reported an increase in Bax protein and apoptosis, as evidenced by a DNA fragmentation enzyme-linked immunoassay (ELISA), TUNEL staining, and caspase-3 cleavage (Kwak et al., 2006). The effect of aging on apoptotic signaling in the myocardium has been summarized in Table 4.7. These apoptotic events appear to be at least partially reversible, because chronic exercise training in the aged rats attenuated all of the apoptotic signaling increases due to aging (Table 4.8). Increased caspase-3 cleavage in the aged heart has also been reported by Kujoth and colleagues, who compared 5- and 30-month-old mice (Kujoth et al., 2005). This suggests that the aging-induced elevation in apoptotic signaling in the myocardium is not limited to rats.

Other groups have confirmed and expanded on findings in 36-month-old Fischer–Brown Norway (FBN) rat hearts, demonstrating increases cytochrome *c* and AIF release, Bax localization to the mitochondria, caspase activation, and apoptosis (Ljubicic et al., 2010). Discrepancies between the results of these various studies may be due to the rat strain that was studied (e.g., F344 vs FBN) or the techniques used to assay caspase activity or apoptosis (Table 4.7). Nonetheless, these studies indicate that the aging myocardium environment becomes increasingly proapoptotic, and the intrinsic/mitochondrial pathway is likely to be a key regulator of this progression in apoptotic signaling.

In a study of myocardial ischemia/reperfusion (MI/R) injury, 19-month-old rat hearts displayed higher levels of apoptosis by ELISA and TUNEL staining compared to 4-month-old rat hearts (Liu et al., 2002). Interestingly Bax protein levels were higher at baseline in the aged hearts, and after MI/R the ratio of Bax/Bcl-2 mRNA was increased, which further suggests a role for Bcl-2 and Bax in mediating the predisposition of the aged heart to apoptosis. In a different model of myocardial injury, permanent coronary artery occlusion, which is used to produce an infarction in the left ventricular wall, aged (22- to 24-month-old) compared to young (6- to 8-month-old) F344 rats were more susceptible to apoptosis as evidenced by increased DNA fragmentation that began earlier and peaked earlier during infarction (Liu et al., 1998). It is of interest that both Bax and Bcl-2 proteins were increased in the aged hearts, and the ratio of Bax to Bcl-2 declined after 3 h of infarction. In another study by the same group examining the cardiac effects of hypoxia/reoxygenation in old versus young rats, baseline Bcl-2 and Bcl-X_L levels were similar in young and old hearts; however, Bax expression was increased (Azhar et al., 1999a,b). The aged hearts showed more DNA fragmentation and TUNEL-positive cells, and similar to their previous findings with MI/R, the Bax/Bcl-2 and Bax/Bcl-X_L ratios were lower, favoring antiapoptotic conditions, after hypoxia/reoxygenation (Table 4.8). Although the antiapoptotic ratios observed in two of these studies do not explain the heightened sensitivity to apoptosis, reported increases in Bax protein levels in four of these studies suggest the importance of changes in Bax levels in mediating aging-related susceptibility to apoptosis.

Human Src Homology and Collagen Protein of 66 kDa and Myocardial Apoptosis

The Src homology and collagen protein of 66 kDa (p66Shc) regulates life span in mammals and is an important factor in the apoptotic response to oxidative stress via protein kinase Cβ (Pinton et al., 2007). Under basal conditions, a small amount of p66Shc localizes to the mitochondria (Nemoto et al., 2006; Orsini et al., 2004, 2006). However, oxidative stress triggers a mitochondrial accumulation of the protein, and once imported into the mitochondria, p66Shc interrupts normal mitochondrial structure and Ca^{2+} handling and induces apoptosis (Bianchi et al., 2006; Malhotra et al., 2009; Obreztchikova et al., 2006; Rota et al., 2006).

Several studies have demonstrated an association between p66Shc and cardiomyocyte apoptosis under conditions of cardiac stress including oxidative stress (Pinton et al., 2007). Results from Ljubicic and coworkers (2010) have linked mitochondrial dysfunction and increased DNA fragmentation in the aged myocardium with the specific mitochondrial localization of p66Shc and p46Shc. Together the data suggest that oxidative stress in the aging myocardium might trigger translocation of p66Shc and perhaps other Shc proteins to the mitochondria, to initiate an apoptotic cascade.

Longevity Intervention and Cardiomyocyte Apoptosis

No studies have definitively addressed whether life span can be increased by direct inhibition of aging-related cardiomyocyte apoptosis. Moreover, despite multiple reports demonstrating increased apoptosis in the aged myocardium in rodents, it is still unclear whether prevention of apoptosis can reduce morbidity and mortality by ameliorating cardiac dysfunction. Evidence from Hacker and colleagues (2006), who demonstrated that the decline in cardiac function is not causally related to mortality in aged rats, argues that studies in aging rodents will not provide a useful model for evaluating the effects of preventing cardiac dysfunction on longevity. However, we can gain some valuable insight into the importance of apoptosis in aged hearts by examining the effects of molecules or interventions that have been demonstrated to affect longevity (Alcendor et al., 2004, 2007; Cohen et al., 2004; Lee et al., 2002; Park & Prolla, 2005; Yan et al., 2007).

Calorie restriction has been well documented as an intervention that extends life span and slows the development of age-associated diseases. Improvement in cardiac function of aged mice has been demonstrated by calorie restriction (Barger et al., 2008). To examine changes in the expression of apoptotic genes in the aging heart, the effects of calorie restriction, a well-established antiaging intervention, on aged B6C3F_1 mice were evaluated by transcription profiling (Lee et al., 2002). Calorie restriction resulted in the transcriptional downregulation of proapoptotic genes, including Bad, Bax, DNA fragmentation factor, caspase-9, and caspase-11, while upregulation of the antiapoptotic genes Bcl-X and mouse inhibitor of apoptosis was observed.

Calorie restriction has been shown to induce mammalian Sir2 (SIRT1) and reduce stress-induced apoptosis by sequestration, and therefore inhibition, of Bax away from the mitochondria by Ku70 after deacetylation by SIRT1 (Cohen et al., 2004). To examine the effects of SIRT1 on apoptosis in the heart Alcendor and colleagues examined transgenic mice with cardiac-specific overexpression of SIRT1 (Alcendor et al., 2007). Low (2.5-fold) to moderate (7.5-fold) overexpression of SIRT1 reduced cardiac TUNEL staining to below nontransgenic levels in 2- to 3-, 6- to 7-, and 18-month-old mice; however, an increased TUNEL staining in nontransgenic mouse hearts did not manifest until 18 months. This suggests that SIRT1 inhibits not only aging-related apoptosis but also baseline levels of apoptosis, presumably because of inhibition of oxidative stress. This decrease in apoptosis was paralleled by a decrease in fibrosis and cardiac dysfunction in 18-month-old mice. It is tempting to speculate that the inhibition of apoptosis may play a causal role in preservation of heart function. Additionally, Bcl-2 and Bcl-X_L protein levels were reported to be higher in SIRT1 hearts at 2–3 months of age; however, it is unclear whether the increased levels persist at 18 months, and in their highest expressing line (12.5-fold), which demonstrates *increased* apoptosis, Bcl-2 was also elevated compared to nontransgenic mice, making it difficult to conclude that Bcl-2 is important for SIRT1-mediated decreases in cardiac apoptosis. Interestingly the authors stated that low levels of SIRT1 overexpression did not increase life span, which they attributed to the cardiac-specific nature of SIRT1 expression. However, it is worth mentioning that the mortality study was only ~20 months in duration, and at this point, the median life span was never reached for the mouse strain (FVB) studied (Alcendor et al., 2007). Global genetic deletion of adenylyl cyclase 5 (AC5) from mice results in an increase in mean and maximum life span of 8 and 4 months, respectively, which is associated with preserved cardiac function and enhanced resistance to oxidative stress (Yan et al., 2007). Comparing young (3- to 6-month-old) versus old (20- to 30-month-old) adenylyl cyclase 5 knockout (KO) mice showed an attenuation of aging-related increases in apoptosis, hypertrophy, and fibrosis. The decrease in apoptosis was attributed to an increase in activity of the prosurvival Raf/MEK/ERK pathway and an increase in antiapoptotic factors including RSK, p-Bad, Bcl-X_L, XIAP, and HSP70 in the heart. Overall isolated cells from the AC5 KO mice demonstrated increased resistance to oxidative stress, which also included an increase in Mn-SOD. Thus, a decrease in aging-related cardiac apoptosis is associated with preservation of function secondary to an upregulation of antiapoptotic factors. Speculation as to whether the preservation of heart function contributed to the life-span extension is complicated by the global (non-cardiac-specific) deletion of AC5 and dependent on cardiac dysfunction as a significant cause of mortality in mice (Yan et al., 2007).

In vivo measurements of cardiac function in mice that are homozygous null for the gene encoding fatty acid amide hydrolase (FAAH), an enzyme responsible for the metabolism of fatty acids including the endocannabinoid anandamide, showed attenuated aging-related decreases in both systolic and diastolic function in 28- to 31-month-old mice (Batkai et al., 2007). Although apoptosis was not measured in this study, it is reasonable to assume, based on studies in mice of a similar age, that apoptosis was present. Suppression of poly(ADP-ribose) polymerase cleavage and caspase-3 and caspase-9 activation in the FAAH KO versus wild-type hearts provided further evidence for the involvement and attenuation of apoptosis. Furthermore, gene expression of caspase-3 and caspase-9, along with TNF-α, was reduced in FAAH KO hearts, suggesting protection against the extrinsic death receptor pathway.

CONCLUSIONS

Apoptosis has recently emerged as an important mechanism in the pathophysiology of the loss of skeletal muscle with aging. Apoptosis also appears to have a role in aging myocardium, although it is not clear to what extent apoptosis might contribute to heart dysfunction with aging. Several lines of evidence support the hypothesis that mitochondrial (intrinsic), extrinsic (death receptor), and SR/ER–Ca^{2+} stress-activated apoptotic signaling occurs in skeletal muscles of old mammals. Furthermore, activation of mitochondrial apoptotic signaling during the early phases of disuse muscle atrophy, in heart failure, and in aging heart and skeletal muscles suggests that this may exist to balance muscle size and the metabolic or functional needs of the animal. If this is true, apoptosis may be a fundamentally important mechanism that regulates nuclei number and, therefore, controls the extent of muscle growth (or atrophy) in aging.

Mitochondrial dysfunction appears to be prevalent in cardiac muscle and skeletal muscles of senescent animals, and this is associated with a proapoptotic cellular environment and activation of the intrinsic apoptotic pathway. Nevertheless, it has not been determined to what extent skeletal and cardiac muscle loss would be reduced if apoptotic signaling could be fully blocked. As a result, we cannot rule out the possibility that the apoptotic signaling events in skeletal and cardiac muscles may primarily occur to eliminate dysfunctional nuclei and/or damaged muscle fibers, whose perseverance would be detrimental for organ function.

Evidence showing that muscle loss is reduced in Bax null mice that have been exposed to muscle-wasting conditions (Siu & Alway, 2006b) strongly suggests that a causal relationship exists between nuclear apoptosis and muscle loss. Nevertheless, definitively establishing a mechanistic link between the activation of apoptosis and sarcopenia or heart dysfunction in aging is particularly challenging because each of the proteins involved in the various apoptotic signaling pathways also possesses other functions when an apoptotic signal is absent. For example, Id2 is growth promoting by inducing proliferation of myoblasts, but is also able to induce myoblast death via apoptosis (Butler et al., 2009). Similarly, p66Shc is involved in normal mitochondrial function (Nemoto et al., 2006; Orsini et al., 2004), yet it is also activated and concentrated during elevated oxidative stress and aging and is associated with elevated apoptosis and DNA fragmentation in the aging heart (Ljubicic et al., 2010).

Although the loss of skeletal myocytes and cardiomyocytes takes place over the life span of an organism, the rate of this loss occurs more rapidly at the oldest ages. Therefore, to establish a definitive cause-and-effect relationship between apoptosis and loss of skeletal muscle or heart cells, it will be necessary to manipulate the expression and/or function of various apoptotic genes or proteins and study their effects on muscle retention over the life span of the animal.

Nevertheless, complicating the interpretation of data from such studies is the potential problem that mediators of other cellular events will also change over the life span of the animal. Therefore, the final experimental results could still not unequivocally attribute any loss in skeletal or cardiac muscle mass to solely apoptotic signaling. As a result, additional research is needed to clarify the role of nuclear apoptosis in the pathogenesis of sarcopenia and heart failure in aging. Studies are also needed to identify the relative importance of each of the apoptotic signaling pathways in loss of skeletal and cardiac muscle in humans.

Additional research is further warranted to examine the effects of combining long-term exercise and nutritional manipulations (e.g., increasing dietary antioxidants, restricting caloric intake, etc.) on the regulation of apoptotic signaling in skeletal and heart myocytes. It is possible that the combined effects of several therapies may reduce the cellular and systemic stresses in aging and thereby lower apoptotic loss of myonuclei and preserve or prevent aging-induced loss of skeletal and cardiac muscle mass and function.

Clearly, further research is required to understand more completely the complex cellular mechanisms underlying atrophy and loss that occur in aging skeletal and cardiac muscles and the importance of apoptosis in this process. Unraveling the regulatory factors in the apoptotic pathways will be a necessary step prior to having the ability to design effective interventions and countermeasures for reducing skeletal and cardiac muscle loss in aging.

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Chapter 5

Aging and Adipose Tissue

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CHAPTER CONTENTS

Introduction 119
Fat Tissue 120
Fat Tissue Composition 120
Fat Tissue Function 120
Aging, Lipotoxicity, and Fat Tissue 122
Aging and Fat Redistribution 122
Ectopic Fat Accumulation 122
Brown Adipose Tissue 123
Obesity and Aging 123
Inflammation 123
Cellular Senescence 125
Caloric Restriction 125
Fat Cell Function and Turnover 125
Preadipocytes 128
Conclusions and Future Areas of Research 130
Acknowledgments 130
References 131

INTRODUCTION

By the end of this century one-third of the population will be over 60 years of age (Lutz et al., 2008). As the population ages, age-related diseases and economic burden will increase significantly. Loss of functional capacity and increased morbidity in the elderly are frequently associated with metabolic disease, which, in turn, is associated with fat redistribution and dysfunction (Guo et al., 1999; Lutz et al., 2008). These strong relationships among fat tissue development and function, metabolic disease, and aging have become increasingly apparent and have stimulated increasing scientific interest (Figure 5.1).

Body composition changes dramatically throughout life. Total body weight increases until middle age (30–50 years of age) and slowly declines thereafter (Baumgartner et al., 1995; Carmelli et al., 1991; Guo et al., 1999; Kuk et al., 2009; Lei et al., 2006; Lutz et al., 2008). Frequently the elderly lose considerable fat-free mass, especially skeletal muscle and bone. This is associated with decreased mobility and physical dependency. Total fat mass peaks in early or middle old age (40–70 years of age), resulting in an increased percentage of body fat (Cartwright et al., 2007; Gallagher et al., 2000; Kuk et al., 2009; Raguso et al., 2006; Visser et al., 2005). By advanced old age

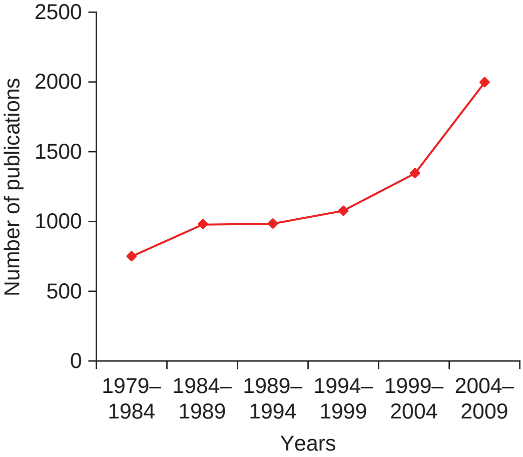

Figure 5.1 Increase in the number of publications about aging and fat tissue from 1979 to 2009 (from entries at http://www.ncbi.nlm.nih.gov/sites/entrez, accessed on December 4, 2001).

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(>85 years of age), adipose tissue is redistributed from subcutaneous to visceral depots and ectopic sites, such as muscle, liver, and bone marrow, with important metabolic implications (Kuk et al., 2009; Zamboni et al., 1997). Visceral fat content and intramuscular lipid are strong markers of impaired glucose metabolism, independently of total body obesity (Goodpaster et al., 2003, 2005).

This age-associated redistribution of fat leads to what is effectively a lipodystrophic state that is associated with metabolic complications, as occurs in genetic or acquired lipodystrophic syndromes (Garg & Agarwal, 2009; Zamboni et al., 1997). Epidemiological data indicate a strong relationship between age and prevalence of metabolic syndrome, independently of the population analyzed (Eckel et al., 2005). For example, in the United States the prevalence of metabolic syndrome is 7% in subjects from 20 to 29 years of age, compared to 44% in those from 60 to 69, and 42% in those 70 years and over (Eckel et al., 2005; Ford et al., 2002; Lechleitner, 2008).

Manipulating fat tissue abundance, function, and distribution has profound effects on life span and age-related disease onset. In animals, removal of visceral fat restores insulin responsiveness and improves glucose metabolism, while removal of subcutaneous fat has the opposite effect (Barzilai & Gupta, 1999; Weber et al., 2000). Also, in numerous studies over the past 70 years, caloric restriction has been noted to prolong life span (McCay et al., 1989; Mehta & Roth, 2009). This intervention, which leads to disproportionate loss of fat tissue (Bertrand et al., 1980), especially visceral fat (Barzilai & Gupta, 1999), delays multiple age-related changes in diverse species. Genetic interventions that reduce fat tissue mass are associated with increased life span in mice (Chiu et al., 2004; Heikkinen et al, Selman et al., 2009; Um et al., 2004) and in flies (Giannakou et al., 2004). Knocking out insulin receptors in fat cells in mice, which leads to significant reduction in fat mass, increases mean and maximum life span (Bluher et al., 2003). Replacing CCAAT/enhancer binding protein-α (C/EBPα), an adipogenic transcription factor, with C/EBPβ results in increased free fatty acid (FFA) oxidation, reduced fat mass, and increased mean and maximum life span (Chiu et al., 2004). Mutations of genes that encode proteins in the insulin/IGF-1/mTOR/S6 kinase-1 pathways and in pathways regulating hormonal and mitogenic signals increase life span and delay onset of age-related diseases (Fontana, 2009). These pathways are implicated in regulating metabolic function and fat tissue development (Fontana, 2009). Rapamycin, which inhibits mTOR signaling and prevents diet-induced obesity (Chang et al., 2009), has been found to increase maximum life span when administration was initiated in middle age (Harrison et al., 2009). mTOR signaling is nutritionally regulated, is involved in control of fat tissue development, and influences insulin/IGF-1 signaling, inflammation, and stress responses (Wullschleger et al., 2006). Furthermore, in mice, deletion of ribosomal S6 kinase-1, a downstream target of mTOR, increases mean and maximum life span (Selman et al., 2009) and prevents obesity (Um et al., 2004).

FAT TISSUE

Fat Tissue Composition

Fat tissue is a specialized connective tissue that functions as the major energy storage site. Fat tissue exists in mammals in two forms: white adipose tissue and brown adipose tissue. White adipose tissue comprises many different cell types. Preadipocytes are fat cell progenitors that are present in adipose tissue throughout life and are capable of replication or differentiation into mature fat cells (Kirkland & Dobson, 1997). Other cell types present in fat tissue include multipotent mesenchymal progenitors, various lymphocyte subsets, mast cells, macrophages, and endothelial and other blood vessel cells, among others (Ailhaud et al., 1992; Bourlier & Bouloumie, 2009; Feuerer et al., 2009; Kirkland et al., 1994; Liu et al., 2009; Nishimura et al., 2009; Weisberg et al., 2003; Winer et al., 2009; Xu et al., 2003).

Fat Tissue Function

Adipose tissue has many functions beyond its pivotal role in energy storage. It has important immune, endocrine, and homeostatic, thermal, and mechanical actions (Bray & Ryan, 2006). Furthermore, subcutaneous, mesenteric, and omental fat depots act in distinct ways (Table 5.1; Leff & Granneman, 2010). They have different metabolic roles, accumulate fat in different ways, and have distinct endocrine and paracrine secretory profiles. With regard to energy storage, subcutaneous fat tissue acts as a long-term caloric reserve. It can expand outward, having less anatomical constraint to enlargement than visceral fat, which has limited space for expansion. Subcutaneous fat is more abundant in women. This could have evolved to support caloric needs during pregnancy and lactation (Leff & Granneman, 2010). Consistent with their long-term storage function, subcutaneous depots signal how much fat they contain to the brain via leptin production. Subcutaneous fat tissue is the main source of circulating leptin, a product of adipocytes that regulates long-term energy storage by acting on the satiety center in the hypothalamus (Van Harmelen et al., 1998; Zhang et al., 1999). Leptin has autocrine effects on insulin-mediated glucose uptake by adipocytes, also regulating lipid storage (Zhang et al., 1999). Visceral fat produces little if any leptin (Hube

Table 5.1 Functional differences between subcutaneous and visceral fat depots

FAT DEPOT CHARACTERISTIC	SUBCUTANEOUS	VISCERAL
Metabolic function	Long-term storage; protection against lipotoxicity	Rapid fatty acid release to the liver
Lipid accumulation	Fat cell proliferation	Increases in fat cell size
Preadipocytes	Increased replication and differentiation	Decreased replication and differentiation
Endocrine/paracrine signaling	Signals long-term stores	Injury response, inflammatory peptides
Age-related changes	Loss of this depot occurs with aging; progenitors have shorter telomeres	Relative increase compared to subcutaneous

Subcutaneous fat, which acts as a long-term energy storage depot, has a progenitor pool with greater capacity for replication and differentiation than visceral fat. In old age, the ratio of visceral to subcutaneous fat is increased, partly because of a relatively greater loss of subcutaneous fat.

et al., 1996; Lefebvre et al., 1998; Samaras et al., 2009; Van Harmelen et al., 1998; Wang et al., 2001; Zha et al., 2009). This indicates that visceral fat expansion does not induce satiety through leptin signaling to the brain. Additionally, leptin influences fatty acid oxidation and immune function, acts like a proinflammatory cytokine, and affects production of other hormones (e.g., those regulating puberty; Ahima et al., 1996; Lago et al., 2008; Wang et al., 2001).

Visceral fat tissue serves as a short-term fuel storage site, rapidly releasing calories. Relatively greater visceral fat stores may have evolved in males to provide bursts of energy during such activities as hunting (Kissebah & Krakower, 1994; Leff & Granneman, 2010). Visceral fat tissue has accelerated turnover of lipids, with higher rates of synthesis and mobilization than subcutaneous fat. Some of the released fatty acids flow through the portal system to the liver where they are metabolized rapidly. Increased visceral fat lipid turnover is associated with higher blood flow in visceral than in subcutaneous fat, as well as a higher density of lipolytic β_3-adrenoreceptors coupled with extensive sympathetic innervation (Kissebah & Krakower, 1994).

Fat tissue, especially visceral fat, provides protection against bacterial and fungal infections as well as tissue injury and blood loss by producing complement components, cytokines, and chemokines together with hemostatic factors, such as plasminogen-activated inhibitor 1 (Bray & Ryan, 2006; Leff & Granneman, 2010). Indeed, undifferentiated preadipocytes have a gene expression profile closer to that of macrophages than to fat cells (Charriere et al., 2003, 2006; Cousin et al., 1999). Like macrophages, preadipocytes are motile, express Toll-like receptors, and respond to bacterial lipopolysaccharide and inflammatory cytokines by releasing proinflammatory chemokines and cytokines (Poulain-Godefroy & Froguel, 2007; Vitseva et al., 2008). These chemokines and cytokines are also increased in obesity, in which they attract inflammatory T-lymphocyte subsets, mast cells, and macrophages and reduce abundance of anti-inflammatory immune cell types, including regulatory T-lymphocytes (Feuerer et al., 2009; Liu et al., 2009; Nishimura et al., 2009; Winer et al., 2009). Changes in intracellular pH in adipocytes, especially during lipolysis, reflect local fatty acid concentrations and suggest that local FFA concentrations are high. Such high adipose tissue FFA concentrations are sufficient to be toxic to most pathogenic bacteria and fungi, as well as to many nonadipose cell types (Akaki et al., 2000; Civelek et al., 1996; Petschow et al., 1996). The high fatty acid microenvironment of fat tissue, together with its innate and adaptive immune responses and phagocytosis by preadipocytes and fat tissue immune cells, could explain why infections and metastases are so uncommon in fat tissue.

Different fat depots have specialized roles in preventing infection. Subcutaneous fat prevents infections from entering through the skin (Mandell, 2009). The omentum acts like a medicated bandage. It can wrap around inflamed organs and wall off infection due to ruptured viscera and, in addition to inflammatory mediators, secrete attachment, pressor, and hemostatic factors that act to prevent mortality from intraabdominal crisis (Karagiannides et al., 2006a; Leff & Granneman, 2010; Uzunkoy et al., 2009). Mesenteric fat also helps prevent dissemination of enteral infections. It responds to inflammation by growing around inflamed viscera ("creeping fat"), preventing spread of infection throughout the abdominal cavity (Karagiannides et al., 2006a; Leff & Granneman, 2010).

Subcutaneous fat around the joints, palms, and soles and fat around the heart and the kidney, along with retro-orbital fat, have a mechanically protective function. Fat tissue can grow in response to mechanical stress, consistent with the close links of preadipocytes to osteoblasts, which also respond to mechanical stress and share a common mesenchymal progenitor (Birk et al., 2006; Knippenberg et al., 2005; Skillington et al., 2002).

White fat has an insulating effect, preventing heat loss, while brown fat tissue generates heat. Brown fat is interspersed with white fat in humans, is concentrated around the great vessels in infants (Leff & Granneman, 2010), and is chiefly located in the neck and upper chest in adults (Cypess et al., 2009).

Fat cells and preadipocytes influence the function of adjacent cell types. For example, bone marrow fat cells produce paracrine factors that influence myeloid and lymphoid lineage differentiation and proliferation (Corre et al., 2004; Yokota et al., 2003). In this way, fat tissue paracrine factors can have an endocrine-like function, as fat tissue grows into target organs and is not restricted to releasing regulatory molecules from distant sites.

AGING, LIPOTOXICITY, AND FAT TISSUE

Aging and Fat Redistribution

Different fat depots undergo changes at different rates with aging, leading to fat redistribution from subcutaneous to visceral depots (Cartwright et al., 2007). Abdominal fat increases, while subcutaneous fat, especially from the lower body, decreases, and fat accumulates in or around the heart, skeletal muscle, and bone marrow, leading to development of an age-related lipodystrophic state (Kotani et al., 1994; Kyle et al., 2001; Meunier et al., 1971; Rabkin, 2007; Slawik & Vidal-Puig, 2006). The percentage of fat from meals stored in subcutaneous fat is lower in older than in younger subjects (mean ages 69 and 23, respectively; Koutsari et al., 2009). A longitudinal study in adult women showed that abdominal circumference, which correlates closely with visceral fat content, increases by 4.0 cm every 9 years (Hughes et al., 2004). Age-related lipodystrophy is associated with metabolic complications, as occurs in genetic or acquired lipodystrophic syndromes, all of which are characterized by selective loss of fat tissue (Garg & Agarwal, 2009), indicating that depot-specific changes in fat tissue function may contribute to age-related metabolic dysfunction. Increased visceral fat is independently associated with mortality, insulin resistance and diabetes, cardiovascular disease, cerebrovascular disease, heart failure, cognitive impairment, Alzheimer disease, and disability in the elderly. The combination of glucose intolerance, insulin resistance, central obesity, dyslipidemia, and hypertension constitutes the metabolic syndrome, which is associated with increased cardiovascular and all-cause mortality, cognitive impairment, and accelerated functional decline in the elderly (Morley, 2004).

Complications arise from the inability of subcutaneous adipocytes to store lipid (Kuk et al., 2009; Uranga et al., 2005). Loss of subcutaneous fat may contribute to reduced leptin-mediated β-oxidation of fatty acids, since leptin is produced principally by subcutaneous fat, normally the largest depot in humans, with little production by visceral fat (Kirkland & Dobson, 1997; Lefebvre et al., 1998; Samaras et al., 2009; Wang et al., 2001; Zha et al., 2009). Reduced leptin-mediated β-oxidation of fatty acids together with reduced capacity of subcutaneous fat to store triglycerides could contribute to increased circulating FFAs in old age and lipodystrophic syndromes. Increased circulating FFAs lead to redistribution of fat to visceral depots, fat deposition in ectopic sites (including bone marrow, pancreatic β-cells, cardiac and skeletal muscle, and liver), and systemic lipotoxicity. High FFA levels lead to lipotoxicity, with cellular stress response activation and apoptosis (Eckel et al., 2005). The redistribution of excess circulating FFAs may contribute to insulin resistance and be a central mechanism of the metabolic syndrome (Eckel et al., 2005). In the pancreas, FFAs lead to insulinopenia, as they are highly toxic to pancreatic β-cells (Bays et al., 2008). FFAs generate endothelial dysfunction and impair vasodilatation, contributing to hypertension together with insulin resistance (Bays et al., 2008). Most tissues do not have the integrated, high-capacity systems existing in fat cells (lipoprotein lipase, fatty acid binding protein-4, glycerol-3-phosphate dehydrogenase, etc.) required to metabolize or sequester high concentrations of fatty acids as less toxic triglycerides (Kirkland et al., 2002). In old age, even preadipocytes become susceptible to lipotoxicity. At fatty acid concentrations at which preadipocytes from young animals remain viable, cells from older subjects accumulate lipid in small lipid droplets, characteristic of lipotoxicity, and die because of apoptosis (Guo et al., 2007a).

Ectopic Fat Accumulation

Liver fat accumulation (hepatic steatosis) is more closely linked to lipid accumulation in other ectopic sites and visceral fat depots than generalized obesity is (Banerji et al., 1995; Machann et al., 2005). Intrahepatocellular lipid increases with advancing age (Akahoshi et al., 2001; Cree et al., 2004). The prevalence of hepatic steatosis is lower among women than among men, which is perhaps related to the greater capacity of subcutaneous fat to store lipid in women (Browning et al., 2004). Fatty liver is associated with impaired hepatic glucose metabolism and reduced insulin clearance, with increased gluconeogenesis and hyperinsulinemia (Kelley et al., 2003). Insulin resistance, dyslipidemia, and hypertension are related to increased liver fat content independently of age and body mass index (Akahoshi et al., 2001; Tiikkainen et al., 2002). Hepatic steatosis is also correlated with coronary risk in the elderly (Akahoshi et al., 2001). The association between metabolic complications and liver fat accumulation appears to be independent of visceral fat depot size, suggesting that liver fat not only is a marker of, but actively contributes to, metabolic dysfunction (Adiels et al., 2006; Seppala-Lindroos et al., 2002; Tiikkainen et al., 2002).

There are positive correlations among intramuscular lipid content, aging, and obesity (Cree et al., 2004; Kelley et al., 1999). Increased intramuscular fat is associated with reduced muscle strength and mobility in elderly subjects, independently of obesity, even after adjustment for muscle cross-sectional area (Goodpaster et al., 2001). Moreover, ectopic fat deposition in muscle is thought to be an important factor in development of insulin resistance and the metabolic syndrome at the level of the myocyte (Eckel et al., 2005; Kelley et al., 1999). Excess circulating FFAs promote increases in substrate availability and malonyl coenzyme A, which inhibits carnitine palmitoyl transferase-1 and thus entry of long-chain fatty acids into mitochondria for oxidation. As a result, extramitochondrial metabolism of long-chain fatty acids is increased and lipotoxic pathways are activated (Cree et al., 2004; Eckel et al., 2005; Kelley et al., 1999). FFAs also impair activation of protein kinase Cλ and protein kinase Cζ (Eckel et al., 2005), as does the reduced adiponectin production related to fat redistribution in old age.

Brown Adipose Tissue

Previously, brown adipose tissue was considered to be almost nonexistent and without physiologic relevance in adult humans (Cannon & Nedergaard, 2004). The development of new diagnostic modalities permitted the identification of quite abundant brown adipose tissue in the cervical–supraclavicular depot in adult humans (Cypess et al., 2009). This finding is of considerable importance, since brown adipose tissue promotes energy expenditure by thermogenesis mediated by uncoupling protein-1 (Cypess et al., 2009). Interestingly, a negative correlation between the prevalence of detectable brown adipose tissue and body mass index was found in a study by Cypess et al. (2009). This negative correlation was stronger among subjects in the top third for age (Cypess et al., 2009). Aging is associated with loss of brown fat tissue and dysfunction of brown fat preadipocytes in rodents (McDonald & Horwitz, 1999). Furthermore, in transgenic mice, ablation of brown adipose tissue was associated with obesity and related complications in the absence of hyperphagia, while regeneration of brown fat reversed this phenotype (Lowell et al., 1993). Increased brown adipose tissue is associated with increased insulin sensitivity, reinforcing the importance of brown fat in metabolic homeostasis (Yang et al., 2003). Brown fat could alleviate or even reverse systemic lipotoxicity by increasing utilization of excess circulating FFA (Langin, 2009). Therefore, recruitment and activation of brown adipose tissue, or conversion of white into brown adipocytes, as has been accomplished by several groups, might constitute attractive therapeutic interventions in the future (Langin, 2009).

OBESITY AND AGING

There has been a marked increase in the prevalence of overweight and obese individuals in developed countries, affecting principally younger and middle-aged adults. Currently, 66% of U.S. adults are either overweight or obese (Wang et al., 2007). Obesity is an independent risk factor for cardiovascular morbidity and mortality. Obesity contributes to the development of hypertension, dyslipidemia, insulin resistance and type 2 diabetes, cancers (breast, prostate, liver, kidney, colon, ovarian, and endometrial), cognitive impairment, and Alzheimer disease, among other morbidities (Bjorntorp, 1990; Colditz et al., 1995; Denke et al., 1993, 1994; Lean, 2000). Furthermore, increased abdominal compared to subcutaneous fat is a bigger risk factor for mortality than generalized obesity and this relation is even stronger in lean subjects (Pischon et al., 2008; Wannamethee et al., 2007).

As in obesity, aging is associated with an increased prevalence of insulin resistance, type 2 diabetes, metabolic syndrome, cancers, and cognitive impairment. However, the metabolic complications associated with aging cannot be fully explained by concurrent obesity. Fat mass peaks in early or middle old age, but declines thereafter (Cartwright et al., 2007; Gallagher et al., 2000; Kuk et al., 2009; Raguso et al., 2006; Visser et al., 2003, 2005), as opposed to the decreases in insulin sensitivity during the later years of life (Wu et al., 2007). Because there are similarities in the metabolic dysregulation associated with aging and obesity, there has been increasing interest in cellular pathways shared by both conditions.

Inflammation

Both aging and obesity are associated with elevated levels of circulating inflammatory factors, including increased TNF-α, interleukin-6 (IL-6), and C-reactive protein (CRP; Bruunsgaard et al., 2001; Chavey et al., 2003; Fried et al., 1998; Hotamisligil et al., 1993; Matsuzawa et al., 2003; Morin et al., 1997b; Samad et al., 1999; Serrano et al., 2009; Xu et al., 2003). Inflammatory cytokines are upregulated in white adipose tissue with aging (Fried et al., 1998; Morin et al., 1997b; Wu et al., 2007). Since adipose tissue is one of the largest organs in the body, adipose tissue may be an important source for the increased levels of circulating inflammatory cytokines that occur with aging, with potentially substantial systemic effects (Flower et al., 2003; Fried et al., 1998; Harkins et al., 2004; Wu et al., 2007). These proinflammatory cytokines may play a role in diseases such as Alzheimer disease, Parkinson disease, atherosclerosis, type 2 diabetes, sarcopenia, and osteoporosis (Bruunsgaard et al., 2001). Some inflammatory cytokines, including IL-6 and CRP, are associated with increased cardiovascular and all-cause mortality (Harris et al., 1999). Since preadipocytes and macrophages are closely related (Charriere et al., 2003, 2006), it not surprising that preadipocytes appear to be a much more important source of circulating inflammatory peptides than generally realized (Mack et al., 2009; Rudin & Barzilai, 2005; Wu et al., 2007). This may be especially the case with aging. Indeed, preadipocyte production of TNF-α and IL-6 increase with aging (Tchkonia et al., 2007b). Fat tissue redistribution might also contribute to the development of a chronic proinflammatory state with aging. Different fat depots make distinct contributions to the production of inflammatory peptides (Sepe et al., 2010). As an example, fragments isolated from omental fat were shown to release two to three times more IL-6 than subcutaneous fat. Furthermore, depot differences in secretion of inflammatory peptides such as IL-6 might contribute to regional differences in fat tissue metabolism (Fried et al., 1998).

As in aging, inflammation plays a key role in the insulin resistance and type 2 diabetes related to obesity (Xu et al., 2003). Compared to adipose tissue from lean individuals, adipose tissue from obese individuals secretes larger amounts of proinflammatory proteins including TNF-α, IL-6, inducible nitric oxide synthase (iNOS), TGF-β1, C-reactive protein, soluble ICAM, and monocyte chemoattractant protein-1 (MCP-1; Weisberg et al., 2003; Xu et al., 2003). These factors not only are markers of inflammatory activity, but also appear to have a causal role in the metabolic complications of obesity. TNF-α increases in obesity and appears to function as a feedback inhibitor of adiposity by inducing cellular insulin resistance (Fried et al., 1998). It decreases insulin receptor tyrosine phosphorylation, downregulates several steps in the insulin signaling pathway, and promotes lipolysis (Rudin & Barzilai, 2005). Furthermore, neutralization of TNF-α enhances insulin sensitivity in obese, insulin-resistant rodents (Hotamisligil et al., 1993; Hotamisligil & Spiegelman, 1994). However, TNF-α receptor knockout mice on a high-fat diet accumulate more fat and are more insulin resistant than wild-type animals, suggesting this receptor is not required for a high-fat diet to cause insulin resistance. Knocking down the TNF-α receptor increases obesity but protects against inflammation; however, obesity-induced insulin resistance is not reduced (Pamir et al., 2009). IL-6, which is released by adipocytes, also increases lipolysis and has been implicated in the hypertriglyceridemia and elevated serum FFA levels associated with obesity and insulin resistance (Fried et al., 1998; Weisberg et al., 2003). Knocking out IL-6 receptors leads to increased obesity and reduced inflammation. However, unlike knocking out TNF-α receptors, removing IL-6 receptors seems to protect against insulin resistance (Pamir et al., 2009). MCP-1, which is increased both in obesity and with aging in fat tissue, recruits macrophages and can induce insulin resistance in fat and skeletal muscle (Sell & Eckel, 2007). In summary, proinflammatory changes that occur with obesity may prevent further increases in fat mass, at the same time contributing to insulin resistance. However, reversal of these changes might not be sufficient to reverse the metabolic complications of obesity.

Based on the similar morbidity and inflammatory profiles of aging and obesity, the argument has been made that obesity is an accelerated form of fat tissue aging (Ahima, 2009). However, the conditions differ in a number of respects. Adipose tissue macrophage content correlates with extent of obesity (Weisberg et al., 2003; Xu et al., 2003). These macrophages express high levels of inflammatory genes and are thought to contribute to increased fat tissue TNF-α, iNOS, and IL-6 in obesity. The percentage of macrophages in adipose tissue ranges from under 10% in lean mice and humans to over 40% in obese mice and humans (Weisberg et al., 2003). However, there is little change in macrophage number in visceral adipose tissue with aging and they appear to have much less of a role in the visceral fat tissue inflammation associated with aging than with obesity (Harris et al., 1999; Wu et al., 2007). Furthermore, macrophage capacity to become activated and release inflammatory cytokines declines with aging (Sebastian et al., 2009), suggesting that preadipocytes could be more important in initiating and sustaining fat tissue inflammation in aging than in obesity. Indeed, preadipocytes, and not macrophages, have been shown to be responsible for the bulk of the increased IL-6, TNF-α, and prostaglandin E2 in old mice. In this study only visceral fat was analyzed, a depot that, compared to subcutaneous fat, undergoes limited age-related changes (Cartwright et al., 2007; Wang et al., 1989). While macrophages do not accumulate with aging in visceral fat, they do in subcutaneous fat (Jerschow et al., 2007), but this increase is not as impressive as in obesity. Even in obesity, macrophages may be farther down the causal chain of events leading to cytokine generation than generally appreciated (Feuerer et al., 2009; Gustafson et al., 2009; Liu et al., 2009; Nishimura et al., 2009; Winer et al., 2009). Preadipocytes and endothelial cells, which can constitute up to 50% of the cells in fat tissue, might be farther up this causal chain of events, inducing changes in T-lymphocyte subsets and mast cells that subsequently lead to macrophage infiltration and activation.

Cellular Senescence

The aging phenotype and age-associated diseases have been linked to cellular senescence (Campisi, 2005; Jeyapalan & Sedivy, 2008), including in fat tissue (Baker et al., 2008; Minamino et al., 2009; Tchkonia et al., 2009). Cellular senescence is defined as irreversible cell cycle arrest driven by a variety of mechanisms, including telomere shortening, other forms of genotoxic stress, or mitogens or inflammatory cytokines, that culminate in the activation of the p53 tumor suppressor and/or the cyclin-dependent kinase inhibitor p16. Increased p16 expression and cellular senescence may have important roles in the loss of subcutaneous fat tissue with aging (Baker et al., 2008). Aging and cellular senescence are both closely associated with inflammation, possibly contributing to the elevated morbidity and mortality risk in the elderly (Bruunsgaard et al., 2001; Campisi, 2005). Genetically obese mice have higher levels of reactive oxygen species (ROS) and DNA damage than lean mice, both of which induce cellular senescence. Adipose tissues of obese mice, rats, and humans exhibit features of cellular senescence, including increased expression of senescence associated β-galactosidase, γ-histone-2-AX, p53, p16, and cyclin-dependent kinase inhibitor 1 (Baker et al., 2008; Minamino et al., 2009; Tchkonia et al., 2009). Obesity leads to telomere shortening. White blood cell telomeres are 240 bp shorter in obese than in lean age-matched women (Valdes et al., 2005). As in cellular senescence (Coppe et al., 2008) and with aging (Morin et al., 1997b; Wu et al., 2007), proinflammatory cytokines and extracellular matrix modifying proteins, such as matrix metalloproteinases (MMPs), are increased in fat tissue in obesity (Chavey et al., 2003; Hotamisligil et al., 1993; Hotamisligil & Spiegelman, 1994; Maquoi et al., 2002; Matsuzawa et al., 2003; Morin et al., 1997a; Traurig et al., 2006; Xu et al., 2003). Therefore, it has been hypothesized that obesity, with its generation of ROS, accelerated DNA damage, and telomere shortening (Valdes et al., 2005), may activate p53 and/or p16, promoting inflammatory responses that lead to metabolic complications both locally and systemically. Indeed obese transgenic mice that are p53 or p16 deficient have lower expression of inflammatory markers and improved insulin sensitivity (Ahima, 2009; Baker et al., 2008; Minamino et al., 2009). Interventions that delay the aging phenotype and prolong life span also reduce oxidative stress and inflammation.

Caloric Restriction

Caloric restriction extends life span in many species (Masoro, 2006). Caloric restriction also slows the functional decline associated with aging in various organ systems and delays the onset of age-related diseases (Csiszar et al., 2009; Masoro, 2006). It appears to normalize cholesterol, triglycerides, fasting glucose, and insulin blood levels and to reduce blood pressure, contributing to reduced morbidity in older individuals (Holloszy & Fontana, 2007). Reduction of fat tissue may be one of several mechanisms that caloric restriction works through. Indeed, total fat tissue, and especially visceral fat tissue (Carr et al., 2004), decreases significantly with this intervention, as does insulin production (Masoro, 2000), which may decrease preadipocyte and fat cell turnover. Furthermore, with long-term caloric restriction, adipogenesis and energy metabolism genes are increased, potentially counteracting age-related decreases in adipogenesis and lipolysis (Higami et al., 2004, 2006). Caloric restriction also ameliorates the chronic inflammation related to aging, as reflected by significantly lower circulating levels of CRP and TNF-α (Fontana et al., 2004). Numerous studies of caloric restriction show that oxidative stress (De Cabo et al., 2004; Hyun et al., 2006; Kim et al., 2008) and inflammatory gene expression (Higami et al., 2006; Lee et al., 2002) are attenuated in a variety of tissues. Caloric restriction decreases oxidative stress and improves endothelial function in arteries of aged rats by reducing NF-κB activity and downregulating NF-κB-dependent genes (Csiszar et al., 2009). It also reduces TNF-α and TNF-α-induced ROS production, with mitochondrial production of ROS being reduced in parallel (Lambert & Merry, 2004). In one study, caloric restriction for a 9-month period decreased expression of 104 of 109 genes involved in inflammation and 55 of 56 related to the extracellular matrix or angiogenesis in epididymal fat of 10- to 11-month-old mice. MCP-1 mRNA declined by 95%, MCP-1 receptor by 69%, lipopolysaccharide binding protein by 47%, and MMP12 by 94%. Although no macrophages were seen in control mouse fat, macrophage markers decreased after caloric restriction (Higami et al., 2006). Caloric restriction promotes significant loss of fat mass and might impact preadipocyte and fat cell function.

FAT CELL FUNCTION AND TURNOVER

The principal function of adipose tissue is to store energy, with lipid accumulation being a highly efficient way of doing so. Triglycerides in fat cells can

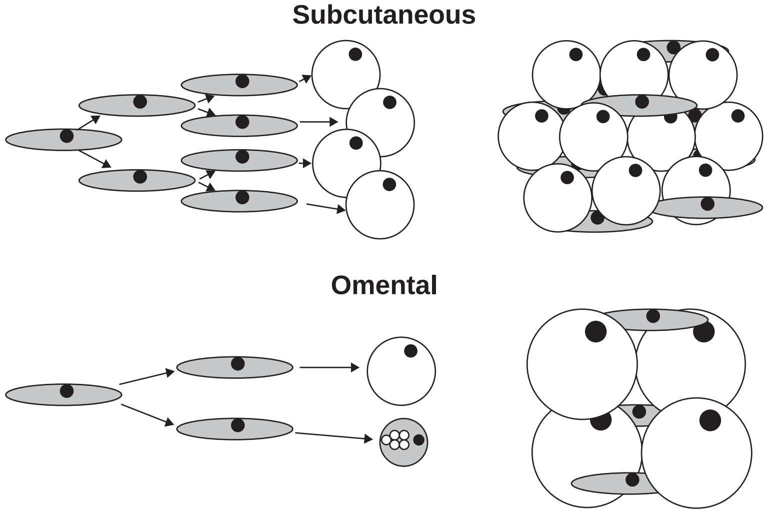

Figure 5.2 Development of subcutaneous and omental fat tissue. During fat tissue expansion, subcutaneous fat grows through increases in fat cell number due to preadipocyte replication and differentiation. Omental preadipocytes have lower capacities for replication and differentiation than subcutaneous cells. Omental fat expands principally through increasing fat cell size, rather than number. The greater proclivity of subcutaneous progenitors to proliferate could lead to replicative stress and cellular senescence, contributing to the disproportionate loss of this fat depot with aging.

arise from de novo lipogenesis (through synthesis from nonlipid precursors) or by uptake of exogenous lipid, with hydrolysis of circulating lipids, uptake as FFAs, and reesterification into triglycerides. Esterification, rather than de novo synthesis, accounts for the vast bulk of triglycerides in adipose tissue in mammals. Intracellular triglycerides can be rapidly hydrolyzed by lipases into glycerol and FFAs that are then released and transported to other tissues to be oxidized in mitochondria.

In times of nutrient excess, fat tissue expands by sequestering FFAs and converting them into less toxic triglycerides. Acute increases in the capacity of fat tissue to store lipids occur initially through increases in fat cell size. However, fat cell enlargement is limited, and if they become too large, fat cells become susceptible to breakage and necrosis with ensuing inflammation (Cinti et al., 2005). Therefore, the most efficient long-term strategy for fat tissue expansion is through increases in fat cell number, as occurs in subcutaneous fat in times of sustained nutrient availability. Visceral fat, with its short-term lipid storage function, changes in bulk principally through increases or decreases in fat cell size, rather than changes in fat cell number (Figure 5.2). To allow for fat tissue expansion, the pool of preadipocytes in fat tissue is large. Indeed, preadipocytes account for 15 to 50% of cells in fat tissue, comprising one of the largest progenitor pools in the body (Kirkland et al., 1994).

Fat tissue turns over regularly, with fat cells having a half-life estimated to be anywhere from a few months to several years (Spalding et al., 2008; Strawford et al., 2004) and with new fat cells being formed throughout the life span. Indeed, fat cell numbers can actually increase in some fat depots between middle and old age (Bertrand et al., 1978, 1980). However, preadipocyte capacities for replication, differentiation, and, thus, generation of new functional fat cells decline with aging (Figure 5.3; Cartwright et al., 2007; Kirkland et al., 1990, 2002). Decreased capacity for differentiation with increased age is at least partly due to decreased expression of key transcription factors involved in adipogenesis.

The capacity for lipid accumulation is impaired in old age, as indicated by reduced fat cell size despite stable or increased circulating lipids, paralleling declines in capacity for adipogenesis with aging. These processes appear to be linked, since acquisition of capacities for both de novo lipogenesis and esterification of circulating FFAs into triglycerides depends on expression of differentiation-dependent genes regulated by adipogenic transcription factors (Kirkland et al., 1993a, 2002). Partly as a result of decreased expression of adipogenic transcription factors that orchestrate differentiation of preadipocytes into fat cells, preadipocytes from older rodents and humans lose the ability to differentiate fully into fat cells and accumulate a large, central lipid droplet. This may contribute to the increased abundance of small, insulin-resistant, dysfunctional fat cells with aging. Fat cells themselves in extraabdominal (i.e., outside the peritoneum) fat of very old rats, monkeys, and humans appear to have decreased expression of these adipogenic transcription factors, while in middle age, they can be increased compared to young adult

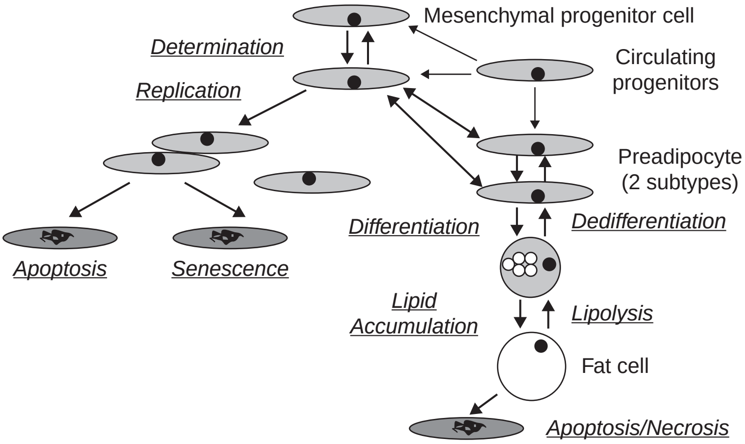

Figure 5.3 Fat cell lineage progression and aging. Cell dynamic properties that have been reported to change with aging are denoted by underlined italics. Committed preadipocytes arise from multipotent, slowly replicating mesenchymal progenitor cells and possibly circulating progenitors (Hong et al., 2005; Crossno et al., 2006). These appear to lose developmental plasticity with aging. Preadipocytes can replicate in response to IGF-1 and other mitogens, reversibly switch into a slowly replicating subtype (Kirkland et al., 1993; Tchkonia et al., 2005), be removed through apoptosis (against which IGF-1 protects preadipocytes), or differentiate into fat cells, losing replicative capacity as they do so. Enlargement of fat cells (lipid accumulation) and maintenance of insulin responsiveness are tied to processes initiated during differentiation, including adipogenic transcription factor expression. Considerable additional work needs to be done on age-related changes in the fat cell lineage.

animals, potentially contributing to expansion of fat mass in middle age (Hotta et al., 1999; Karagiannides et al., 2001; Miard et al., 2009). There is lower expression of these adipogenic transcription factors and less change with aging in visceral fat.

In old age, declines in fat depot size are related to decreases in fat cell size (Kirkland et al., 2002). Lipolysis decreases in parallel, suggesting that impaired capacity for lipid accumulation accounts for decreased fat cell size, rather than increased lipid mobilization. Impaired lipolysis with aging is due to several processes. Lipolytic responses to particular stimuli, for example β-adrenergic agonists, decrease with aging, while responses to others, such as forskolin, which act downstream of receptor activation and transmembrane signaling, appear to be intact (Dax et al., 1981; Gregerman, 1994; Kirkland & Dax, 1984; Kirkland & Dobson, 1997; Kirkland et al., 1987). This implies that alterations in receptors, coupling, G proteins, and membrane fluidity contribute to decreased hormone-responsive lipolysis with aging, and each has been implicated. Fat tissue insulin responsiveness declines with aging (Larkin et al., 2001). Insulin normally inhibits lipolysis, but the age-related decrease in insulin responsiveness is not sufficient to overcome the age-related decrease in lipolysis. Decreased insulin responsiveness would be anticipated, together with decreased hormone-sensitive lipolysis, to reduce the dynamic range of the lipolytic response and contribute to fat tissue dysfunction with aging. Decreased capacity to activate lipoprotein lipase may contribute to an inability to maintain energy homeostasis in response to stressful stimuli in old mice (Araki et al., 2004; Kirkland et al., 2002).

If adipogenesis is impaired and intracellular adipocyte hydrolysis of triglycerides exceeds intracellular free fatty acid esterification, there is net release of free fatty acids into the circulation. This, if extensive enough, could cause lipotoxicity and contribute to systemic metabolic dysfunction. Several mechanisms are probably responsible for the cytotoxicity associated with lipid accumulation in tissues other than fat. These include detergent effects on membranes, increased lipolysis or reduced ability to suppress lipolysis in adjacent lipid-containing cells, generation of ROS and lipid peroxides, effects on protein kinase B and C activity, ceramide generation, stimulation of apoptotic or inhibition of antiapoptotic pathways, necrosis, and promotion of inflammatory cytokine release (Bray & Ryan, 2006; Chiu et al., 2001; Schrauwen & Hesselink, 2004). Fatty acids may cause cellular senescence as well. Saturated fatty acids, including palmitate and stearate, are more lipotoxic than unsaturated fatty acids, such as palmitoleate, oleate, or linoleate, possibly because of the ceramide generated by saturated fatty acids (Aronis et al., 2005; Hardy et al., 2000). Palmitate, oleate, and linoleate are the most common fatty acids in the human diet as well as in human and animal fat tissue. Plasma palmitate levels are higher in elderly than in younger human subjects (Koutsari et al., 2009).

In the absence of inducers of preadipocyte differentiation, palmitate induces stress responses and proapoptotic transcription factor expression in preadipocytes (Guo et al., 2007b). Treatment with oleate or linoleate reverses palmitate-induced apoptosis. Furthermore, saturated fatty acids are involved in fat tissue inflammation. Several in vitro studies (Permana et al., 2006; Suganami et al., 2005, 2007; Takahashi et al., 2008) indicated that adipocytes preloaded with saturated fatty acids, but not with unsaturated fatty acids, exhibit oxidative stress and expression of proinflammatory cytokines and chemokines, including MCP-1, TNF-α, and IL-6 (Takahashi et al., 2008), as occurs in cellular senescence. Moreover, exogenously administered TNF-α enhances MCP-1 release from adipocytes, as does coculture of adipocytes with macrophages (Suganami et al., 2005, 2007). Saturated fatty acids, including palmitate and laurate, induce TNF-α expression in macrophages, which, in turn, promotes lipolysis and augments adipocyte MCP-1 expression, further attracting macrophages and initiating a vicious cycle. Saturated fatty acids may be especially deleterious to fat tissue function in old age.

Defenses against lipotoxicity are highly developed in adipose tissue from young individuals. Nonadipose tissues have very limited capacity to store lipids (Listenberger et al., 2003), while fat cells defend against lipotoxicity as they sequester excess fatty acids as neutral triglycerides for energy storage. Preadipocytes are resistant to levels of fatty acids that would destroy other cell types (Bray & Ryan, 2006). However, with aging, even cells in fat tissue become susceptible to lipotoxicity. Preadipocytes from old rats accumulate lipids in multiple, small droplets that are more characteristic of lipotoxicity than of differentiation. They express markers of apoptosis and are more likely to die than cells from young rats exposed to the same conditions (Guo et al., 2007a). Aging could predispose to a cycle of lipotoxicity in fat tissue, with fatty acids inducing preadipocyte dysfunction (apoptosis and inflammatory cytokine generation), impeding adipogenesis, leading to failure to store fatty acids as triglycerides, causing further increases in circulating fatty acids, proinflammatory changes, increased ectopic deposition of lipids, insulin resistance, and ultimately the metabolic syndrome with its complications (Figure 5.4).

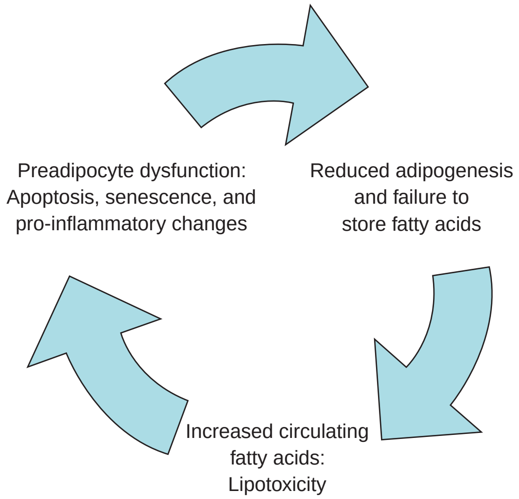

Figure 5.4 Interplay among aging, preadipocyte development, cellular senescence, fatty acids, and lipotoxicity. With aging, preadipocytes become dysfunctional and susceptible to lipotoxicity. Decreased capacity to store lipids leads to increased circulating free fatty acids that induce lipotoxicity and inflammation, leading to further fat tissue dysfunction.

PREADIPOCYTES

Preadipocytes are fat cell precursors and are an important cell type in their own right. They comprise a significant proportion of the cells in fat tissue, often outnumbering fat cells, endothelial cells, and macrophages combined. They actively produce paracrine factors, hormones, and metabolic signals in a manner distinct from adipocytes (Leff & Granneman, 2010; Tchkonia et al., 2007a). Committed preadipocytes arise from multipotent, slowly replicating mesenchymal progenitor cells and possibly circulating progenitors (Crossno et al., 2006; Hong et al., 2005).

Preadipocytes have important immune, proinflammatory, and hemostatic functions. For example, most of the tissue-type plasminogen activator made by adipose tissue appears to be produced by preadipocytes rather than fat cells (Seki et al., 2001). They also have a gene expression profile closer to that of macrophages than that of fat cells, and they appear to be able to convert into macrophage-like cells themselves (Charriere et al., 2003, 2006). Like macrophages, preadipocytes can become activated, assuming a proinflammatory state, and express many of the markers once thought to be specific for macrophages. 3T3-L1 preadipocytes injected into the peritoneum do this (Serrano et al., 2009). Thus, activated preadipocytes can be confused with macrophages originating from blood monocytes.

Preadipocytes replicate in response to IGF-1 and other mitogens (Sekimoto et al., 2005; Siddals et al., 2002). They can reversibly switch into a slowly replicating subtype (Kirkland et al., 1993b; Tchkonia et al., 2005), be removed through apoptosis (which IGF-1 protects preadipocytes against), or differentiate into fat cells, losing replicative capacity as they do so. With initiation of differentiation, IGF-1 switches from promoting replication to initiating a cascade of changes in the expression of transcription factors that result in acquisition and maintenance of the fat cell phenotype (Lowell, 1999; Rosen, 2005). Undifferentiated preadipocytes are responsive to IGF-1 but not insulin. As adipogenesis proceeds, they begin to express insulin receptors and acquire insulin responsiveness (Smith et al., 1988).

The main function of preadipocytes is to differentiate into fat cells. Preadipocyte differentiation is driven by a combination of intra- and extracellular stimuli that culminate in the activation of a cascade of transcription factors, including members of the C/EBP family, as well as peroxisome proliferator activating receptor γ (PPARγ). These transcription factors act sequentially to alter expression of over 2500 genes, causing the acquisition of the fat cell phenotype (Tchkonia et al., 2007a). C/EBPβ and δ are expressed first, followed by C/EBPα and PPARγ. Once C/EBPα is expressed, it supports expression of PPARγ and that of C/EBPα itself (Karagiannides et al., 2001; Tang et al., 2004). The process of preadipocyte differentiation is restrained by antiadipogenic factors, including inhibitory C/EBP family members, such as C/EBPβ liver inhibitory protein (C/EBPβ-LIP) and C/EBP homologous protein (CHOP). They form heterodimers with adipogenic C/EBP family members or prevent adipogenic C/EBP family members from binding to differentiation-dependent promoters, inhibiting adipogenesis. CUG triplet-repeat RNA binding protein (CUGBP) binds to C/EBPβ mRNA, resulting in switching from translation of the adipogenic C/EBPβ-LAP (liver activating protein) to the antiadipogenic C/EBPβ-LIP isoform. Interestingly, the same transcription factors that regulate the preadipocyte differentiation program also control expression of key proteins in pathways responsible for lipid accumulation (Kirkland et al., 1993a, 2002).

Decreased capacity for lipid accumulation and differentiation-dependent gene expression with aging parallel decreases in C/EBPα and PPARγ. Capacity for adipogenesis is restored when C/EBPα expression is increased (Karagiannides et al., 2001). Therefore, mechanisms contributing to decreased preadipocyte differentiation with aging include events at or before increases in adipogenic transcription factor expression during differentiation (Karagiannides et al., 2001).

Antiadipogenic factors also increase with aging in preadipocytes. C/EBPβ-LIP increases in tandem with CUGBP (Karagiannides et al., 2006b). Inhibiting CUGBP in preadipocytes from old animals leads to decreased C/EBPβ-LIP and enhanced lipid accumulation. TNF-α is another antiadipogenic factor that is secreted to a greater extent by preadipocytes and fat tissue from old than from young animals (Morin et al., 1997b; Tchkonia et al., 2007b). TNF-α increases preadipocyte CUGBP activity (Karagiannides et al., 2006b). It also increases CHOP, another antiadipogenic C/EBP family member (Tchkonia et al., 2007b). By inhibiting either CUGBP or TNF-α, lipid accumulation can be partially restored in preadipocytes from old animals.

Preadipocytes appear to develop from multipotent mesenchymal progenitors resident in fat tissue (also referred to as adipose-derived stem cells) that are able to differentiate along several lineages. They have osteogenic, myogenic, and chondrogenic as well as adipogenic capacity. However, their developmental plasticity appears to become constricted with aging. Osteogenic capacity is impaired with aging, while adipogenic potential remains intact (Zhu et al., 2009).

Since new fat cells appear throughout adulthood, regionally distinct cell dynamic properties of preadipocytes could contribute to the fat redistribution that occurs with aging. Preadipocyte capacity for adipogenesis varies among fat depots (Adams et al., 1997; Hauner & Entenmann, 1991; Tchkonia et al., 2002, 2006), with abdominal subcutaneous preadipocytes having greater capacity for adipogenesis than omental cells (Tchkonia et al., 2005). Replicative potential of preadipocytes also varies among depots, with human abdominal subcutaneous preadipocytes being capable of more extensive replication than omental cells (Kirkland et al., 1990; Tchkonia et al., 2005, 2006; Van Harmelen et al., 2004). The greater replicative capacity of subcutaneous fat cell progenitors may lead to increased progenitor utilization, potentially contributing to the disproportionate loss of this fat depot with aging. Consistent with this, telomeres are shorter in human subcutaneous compared to omental cells isolated from the same subjects, indicating that the subcutaneous preadipocytes may have a more extensive replicative history in vivo (Tchkonia et al., 2006). The greater replicative capacity of subcutaneous preadipocytes may be related to the long-term lipid storage function of subcutaneous fat.

Preadipocytes from human subcutaneous, mesenteric, and omental fat depots exhibit distinct patterns of gene expression, further indicating that depot-specific characteristics inherent to preadipocytes may contribute to regional differences in function (Tchkonia et al., 2007a). The same is true in mice and rats (Gesta et al., 2006). Regional differences in gene expression, replicative potential, and capacity for adipogenesis persist despite maintenance under identical culture conditions for many weeks in colonies derived from single preadipocytes (Djian et al., 1983, 1985; Kirkland et al., 1990; Tchkonia et al., 2002). Indeed, human preadipocyte strains made by stably expressing telomerase in single preadipocytes from different fat depots retain depot-specific patterns of replication, adipogenesis, apoptosis, and developmental gene expression profiles for over 40 population doublings (Tchkonia et al., 2007a). These fundamental differences among progenitors may make a major contribution to differences in the nature, timing, and extent of changes in preadipocyte and therefore fat tissue function with aging (Cartwright et al., 2010; Sepe et al., 2010).

Since preadipocyte capacities for replication, differentiation, and susceptibility to apoptosis vary depending on the fat depot the cells are isolated from, and since IGF-1 is important in regulating each of these cell dynamic processes, differences in IGF-1 responses among depots could contribute to variation in the extent of fat tissue progenitor utilization over time and regional variation in age-related dysfunction. Consistent with this, preadipocytes from different human fat depots vary in responsiveness to IGF-1 (Cleveland-Donovan et al., in press). Furthermore, in mice with IGF-1 deficiency due to knocking out growth hormone receptors and binding protein (GHRKO mice), subcutaneous fat is preserved with aging (Berryman et al., 2004). Additionally, overexpressing growth hormone, which also causes increased fat tissue and liver IGF-1 generation, leads to greater loss of subcutaneous than of visceral fat, at least in male mice (Palmer et al., 2009). These findings are consistent with the notion that IGF-1-driven preadipocyte utilization might, over time, contribute to greater loss of subcutaneous relative to visceral fat. This loss of subcutaneous fat leads to a relative increase in visceral fat that could contribute to metabolic dysfunction. Indeed, removal of visceral fat in rodents increases median and maximal life span (Muzumdar et al., 2008).

CONCLUSIONS AND FUTURE AREAS OF RESEARCH

Aging is associated with fat tissue redistribution from subcutaneous to visceral depots. This redistribution is correlated with and may cause insulin resistance and metabolic complications, as well as functional decline and mortality in the elderly. As subcutaneous fat loses its ability to store lipids, circulating fatty acids are elevated, with fatty acids being deposited ectopically, further contributing to metabolic dysfunction. Circulating FFAs are toxic to most cell types, including aged preadipocytes, potentially exacerbating fat tissue dysfunction and establishing a vicious cycle. Thus, when the main function of fat tissue, lipid storage, which requires fat cells to be able to increase in size and preadipocytes to differentiate into new fat cells, becomes dysregulated, the other functions of adipose tissue are disrupted, including immune, hormonal/paracrine regulation, and mechanical protection.

Aging and obesity exert similar effects on metabolic function and share clinical consequences. Aging and obesity are both associated with increased inflammatory cytokines in adipose tissue and systemically. These cytokines, together with reduced anti-inflammatory factor generation (e.g., adiponectin, IL-10), may have a causal role in the generation of insulin resistance, impaired glucose metabolism, and systemic inflammation and are potential targets for future therapeutic interventions. However, there are notable differences between the cellular and the molecular mechanisms of fat tissue dysfunction in aging and obesity, including differences in extent of macrophage infiltration, fat cell size, and gene expression profiles.

The fat tissue dysfunction that occurs with both aging and obesity may be caused partly by reduced capacity of preadipocytes to differentiate. The same transcription factors that control differentiation regulate lipid accumulation, and with aging and obesity, fat cells lose their ability to accumulate lipids efficiently. Furthermore, adipose tissue redistribution in the elderly is associated with regional differences in preadipocyte and adipocyte cell dynamics. As fat cells continue to be replaced throughout life, inherent properties of the preadipocytes from which they arise may contribute to differences in fat depot function. Very little is known about the effects of aging on several key cell dynamic properties of preadipocytes, including switching between the slowly and the rapidly replicating subtypes, dedifferentiation of fat cells back into preadipocytes, fat cell apoptosis/necrosis, or the biology of fat tissue multipotent mesenchymal progenitors. Even less is known about age-related changes in fat tissue macrophage or endothelial cell function, or how or whether changes with aging in T-lymphocyte subpopulations, mast cells, dendritic cells, or other cell types in fat tissue contribute to age-related fat tissue redistribution and dysfunction, systemic disease, and longevity. A great deal of work needs to be done on cellular senescence in preadipocytes, as they are among the most abundant progenitors in humans, and a preadipocyte proinflammatory secretory phenotype could have profound systemic implications.

To date, many interventions that effectively prolong life span, including caloric restriction, act on cell replication, growth, nutritional, and inflammatory pathways, some involving IGF-1 signaling. Most of these have significant impact on fat tissue function and distribution, and directly manipulating fat tissue has profound effects on metabolic function and maximum life span. It appears that fat tissue function is at the nexus of processes contributing to age-related metabolic disease and mediating maximum life span. Potentially, interventions aimed at fat tissue could have a profound impact on health span and age-related functional decline.

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Chapter | 6 |

Aging of Stem Cells: Intrinsic Changes and Environmental Influences

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CHAPTER CONTENTS

Introduction **141**
  Stem Cells 141
    Embryonic Stem Cells and Induced Pluripotent Stem Cells 141
    Adult, or "Tissue-Specific," Stem Cells 143
  Stem Cell Niche 144
Aging **144**
  Cellular Aging 145
  Stem Cells and Aging 146
    Aging of Muscle Stem Cells 147
    Aging of Hematopoietic Stem Cells 149
    Aging of Intestinal Epithelial Stem Cells (ISCs) 152
    Aging of Neural Stem Cells (NSCs) 154
  Stem Cells and Longevity 156
Concluding Remarks **156**
References **156**

INTRODUCTION

The consideration of stem cells in the context of organismal aging and longevity requires an understanding of how stem cells themselves manifest the process of aging and how those changes contribute to the phenotypes of aged tissues and organs and the entire organism. To approach this broad subject, we first examine some key distinctions and characteristics of stem cells. We then present general issues of aging that are relevant to stem cells. Finally we summarize the findings and concepts of stem cell aging in the context of various adult stem cells that have been studied in various systems.

Stem Cells

Current research in stem cells attracts broad interest in part because of the therapeutic potential of stem cell biology as applied to the treatment of both genetic and sporadic diseases. Most of the work on stem cell aging has focused on adult, tissue-specific stem cells. In this chapter, we focus on this population, but some of the broad issues extend to, and involve, pluripotent stem cells and the study of the stem cell niche. Therefore, we consider each of these aspects of stem cell biology individually.

Embryonic Stem Cells and Induced Pluripotent Stem Cells

Stem cells have garnered a lot of attention not only in scientific publications but also in the major media because of the controversy associated with the use of human embryonic stem cells (ESCs) in laboratory research. ESCs and adult somatic stem cells are two types of naturally occurring stem cells, and clear distinctions should be made between the two. Usually ESCs are derived from the inner cell mass of blastocysts (Figure 6.1), the 3- to 5-day-old embryos (Yu & Thomson, 2008). These cells are pluripotent and, during development, give rise to the billions of specialized cells that comprise the entire body. Under the right conditions, ESCs can be propagated indefinitely in vitro and still retain their ability to differentiate into various specialized cell types in the body. This feature has made ESCs invaluable in tissue engineering, understanding tissue morphogenesis, and disease modeling. However, engraftment of ESCs or ESC-derived cells into mammalian tissues can lead

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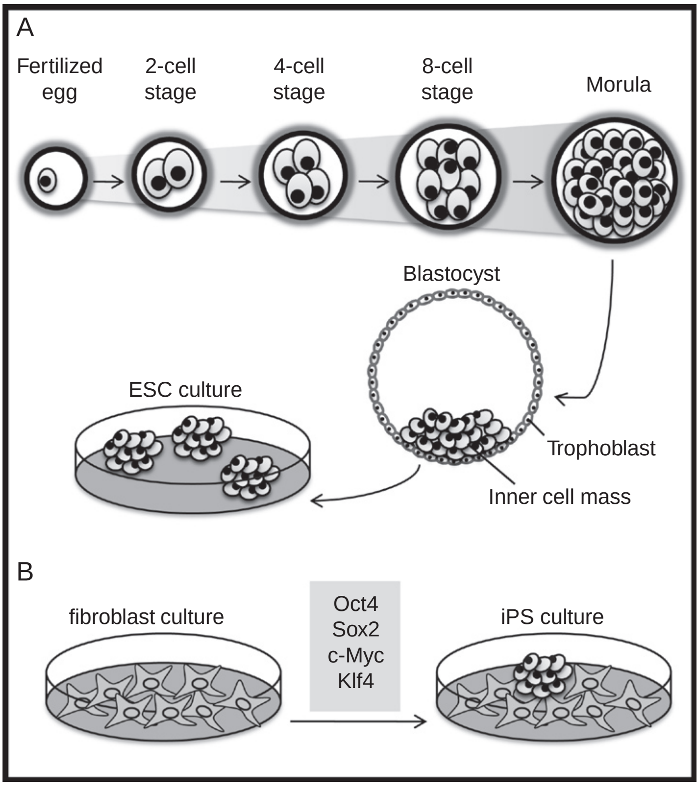

Figure 6.1 In vitro culture of mouse ESCs and iPS cells. (A) ESCs are pluripotent stem cells derived from the inner cell mass of a 3- to 5-day-old embryo, which is termed a blastocyst. At this stage, the blastocyst usually consists of 50–150 cells. (B) iPS cells are derived from differentiated cells by enforced expression of the transcription regulators Oct4, Sox2, c-Myc, and Klf4.

to immunological rejection or the development of teratomas. Such risks, along with the ethical issues related to ESC research, have hindered the therapeutic use of ESCs.

Breakthroughs in somatic cell reprogramming may provide an alternative that avoids many of the practical problems associated with ESC-based therapies. In 2006, it was demonstrated for the first time that mature murine fibroblasts can regain pluripotency in vitro by genetic reprogramming. By retroviral transduction of only four transcription regulators, Oct3/4, Sox2, c-Myc, and Klf4, Yamanaka and colleagues were able to derive induced pluripotent stem (iPS) cells from fibroblasts isolated from murine skin (Takahashi & Yamanaka, 2006). The first human iPS cells were reported in 2007 by Thompson's group, using a similar approach with the transcription regulators Oct4, Sox2, Nanog, and Lin28 (Yu et al., 2007). Early in 2008, Jaenisch's research team reported complete reprogramming of terminally differentiated B cells to pluripotency with additional suppression of transcription factors that maintain their differentiated identity (Hanna et al., 2008). These iPS cells express markers specific to ESCs and are capable of differentiating into cells found in all three germ layers during development. Despite the low efficiency, the generation of iPS cells makes it possible to produce patient-specific and genetically corrected ESC-like cells for cell-based therapy. Because these cells are derived directly from the patient, they will be less likely to cause problems related to immunological rejection following transplantation back into that same individual, although the risk of teratoma formation from these iPS cells still remains.

Mammalian development can be viewed as a programmed and orchestrated differentiation process in which a single zygote generates a small number of ESCs, which then divide and differentiate into the vast array of specialized cells of the mature animal. Development of an individual continues after birth until reproductive maturity is reached. In adulthood, cellular differentiation continues as tissue-specific stem cells give rise to more specialized cells during tissue maintenance and regeneration. The successful generation of iPS cells clearly demonstrates that cellular differentiation is not associated with permanent changes in the genetic information. The profound implication is that different types of terminally differentiated cells in the body differ from their progenitors or from each other only at the epigenetic level. The fact that the reprogramming of mature B cells requires suppression of B-cell-identity maintenance genes suggests that terminal differentiation requires active maintenance at the transcriptional

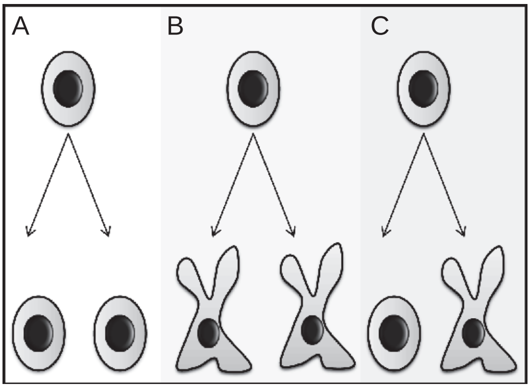

Figure 6.2 Stem cells can undergo three types of division that generate daughter cells of different fates: (A) symmetric division that gives rise to two identical daughter cells that both retain a stem cell fate, (B) symmetric division that generates two lineage-determined daughter cells, and (C) asymmetric division in which one daughter cell remains a stem cell and the other differentiates into a specialized cell type.

level. If the developmental process, once considered unidirectional and irreversible, can be rewound, it is conceivable that the process of aging, also generally considered unidirectional and irreversible, could be "reversed." It is clear that the aged phenotype is due at least in part to epigenetic changes, and an important area of investigation is the contribution of epigenetic mechanisms to the aging of postmitotic cells as well as adult stem cells, a topic that is considered in more detail below.

Adult, or "Tissue-Specific," Stem Cells

After embryonic development, the responsibility for tissue homeostasis falls upon adult somatic stem cells that persist for the lifetime of an individual (Fuchs & Segre, 2000). Many definitions of stem cells, based on essential properties, have been proposed, but among the most central are the ability to self-renew and the ability to generate more differentiated progeny. Often, these two properties occur simultaneously when a stem cell divides asymmetrically to yield one daughter that retains the stem cell identity and another daughter that acquires a more differentiated phenotype (Figure 6.2). As there is no evidence that the longevity of an organism is limited by the capacity of its stem cells, it is clear that either the various adult stem cell populations possess a replicative capacity that exceeds the needs of the individual's maximum life span or there is a mechanism to replenish the stem cells from another source.

The fruit fly *Drosophila* has been very useful in studying adult stem cells, particularly germ-line stem cells, because of the simple tissue composition (Pearson et al., 2009). By contrast, the complexities of mature mammalian tissues, coupled with the rarity of stem cells in those tissues, have made the accurate identification and characterization of mammalian adult stem cells challenging. Traditional histological methods do not reliably distinguish an adult stem cell from its neighboring cells. The technique of preferential bromodeoxyuridine (BrdU) label retention based on an assumed tendency for stem cells to divide more slowly than their progeny is often insufficient and inaccurate, as has been demonstrated in intestinal epithelial stem cells (Marshman et al., 2002; van der Flier & Clevers, 2009). There are few examples of specific stem-cell-specific markers in one tissue being readily useful for the identification of stem cells in another tissue. Although a few common surface molecules, including CD34 and Sca-1 (Hombach-Klonisch et al., 2008), have been found to be expressed by various types of adult stem cells, they often are insufficient by themselves to identify stem cells accurately in a tissue composed of multiple types of cells.

Despite all these challenges, advances in mouse genetics and improved imaging and cell isolation technology have facilitated the identification of adult somatic stem cells in many tissues, including the bone marrow, skeletal muscle, skin, gut, brain, liver, ovary, testis, dental pulp, and adipose tissue. Unlike pluripotent embryonic stem cells, adult stem cells have a more restricted ability to form various types of cells (multipotency) or may even form only one type of cell (unipotency). For example, hematopoietic stem cells are multipotent and can give rise to all types of mature blood cells, whereas spermatogonial stem cells are unipotent and can form only sperm cells.

The first type of adult stem cells discovered in mammals were hematopoietic stem cells (HSCs) in the bone marrow (Becker et al., 1963; Siminovitch et al., 1963; Till & McCullogh, 1963). Transplanted into lethally irradiated mice, HSCs are capable of reconstituting all types of cells in the hematopoietic system. In addition, HSCs that are transplanted into the peripheral circulation are capable of homing to the bone marrow. Transplantation experiments have thus become the gold standard to demonstrate the exceptional regenerative ability of stem cells that distinguishes them from their more differentiated progeny. In the case of skeletal muscle, transplantation of differentiated progenitors, termed myoblasts, results in further myogenic differentiation and fusion with endogenous muscle fibers but does not reconstitute the muscle stem cell pool. However, transplanted muscle stem cells are able to home to their anatomical niche under the basal laminae of mature myofibers and return to their quiescent state. Clearly stem cells, even after removal from the body, have intrinsic abilities of homing and other processes that allow reconstitution of the endogenous stem cell pool and that distinguish them from their progeny.

What determines stem cell identity remains largely unknown. Much evidence has accumulated to suggest that stem cell identity is determined at the level of the epigenome (Hemberger et al., 2009; Sang et al., 2009). Genome-wide chromatin maps have been generated for mouse ESCs, neural progenitor cells (NPCs), and mouse embryonic fibroblasts (MEFs) using the state-of-the-art technologies ChIP–chip and ChIP–seq (Mikkelsen et al., 2007; Pan et al., 2007). The core components of chromatin, histones, carry various posttranslational modifications that regulate gene expression (Berger, 2007). It has been well established that the enrichment of trimethylated histone 3 lysine 4 (H3K4Me3) at the promoter region of a gene is a marker of active transcription, whereas the enrichment of H3K27Me3 often reflects stable repression of transcription. Interestingly, promoters in a large number of genes in ESCs carry both H3K4Me3 and H3K27Me3. This combination of two seemingly opposing histone marks has been termed a "bivalent" chromatin domain. Histone maps of stem cell populations have revealed the presence of "bivalent domains" in the promoter region of genes that are associated with multipotency. These bivalent domains often resolve to monovalent states (i.e., with either marks of activation or marks of repression, but not both) in progenitor cells derived from ESCs. For example, genes involved in regulation or specialized functions in hematopoietic or epithelial lineages generally resolve to H3K27Me3 or carry neither mark in either NPCs or MEFs. Genes related to adipogenesis and chondro/osteogenesis often remain bivalent in MEFs, but resolve to the repressing H3K27Me3 in NPCs. By contrast, genes related to gliogenesis and neurogenesis often resolve to H3K4Me3 or remain bivalent in NPCs, but resolve to H3K27Me3 alone in the MEFs. These epigenetic profiling studies have provided compelling evidence that the multipotency and differentiability of stem cells are controlled at the epigenetic level, in general, and by the bivalent chromatin domains in particular. Although it will be an enormous undertaking to generate maps of chromatin states from all types of stem cells of various stages of development, such chromatin maps are our ultimate answers to what fundamentally defines stem cells and how they acquire "stemness" during organismal development.

Stem Cell Niche

Despite their tremendous ability to divide and differentiate, adult stem cells reside in tissues in a state of quiescence, a steady G_0 state, until they are needed to maintain tissue homeostasis or repair. The stable microenvironment that houses stem cells and influences their behavior was termed the "niche" by Schofield in 1978. The predominant component of a stem cell niche is typically a subset of differentiated cells that anatomically colocalize with the stem cells. Not only do these cells play a very active role in customizing and maintaining the stem cell niches by intercellular signaling to the stem cells, they often are also responsible for creating the extracellular matrix in which the stem cells reside.

The stem cell niche was proposed as a theoretical concept and had remained so for many years. Although the observation that transplanted stem cells survive and propagate only in particular tissue locations strongly suggested the existence of a niche, the experimental identification of stem cell niches has also proven to be technically very challenging (Spradling et al., 2001). When a subset of stem cells has been localized, evidence that they reside in a common microenvironment is sought by the characterization of their neighboring cells, expression patterns of signaling molecules, and local environmental factors such as extracellular matrix proteins. This characterization allows the perturbation of individual niche elements to elucidate which factors are responsible for which aspects of stem cell behavior. Ultimately, demonstrating that a niche exists requires that stem cells be removed and added back. A niche should persist, at least for a short period of time, in the absence of resident stem cells and support the stemness of competent, exogenous transplants. Examples of well-characterized stem cell niches in mouse are summarized in Table 6.1.

How stem cells interact with their niche is of particular relevance in the discussion of aging and stem cells. A niche does not afford stem cells protection against systemic environmental changes in the body; rather, it is a buffer zone between the stem cells and the systemic environment. The basement membranes and extracellular matrices provide structural support to the stem cells and may restrict the permeativity of circulating and paracrine factors, but do not isolate stem cells from the influences of local and systemic factors. Cells comprising the niche play a dynamic role in transmitting environmental cues to their neighboring stem cells. The active role of niche cells in regulating stem cell activity is well illustrated in breast development when mammary stem cells proliferate and drive ductal growth in response to ovarian hormones. Mammary stem cells do not express estrogen receptors themselves. Estrogens act on luminal sensory cells in the niche to induce the expression of growth-promoting factors that act on mammary stem cells directly or indirectly through stromal cells (Asselin-Labat et al., 2006; Brisken & Duss, 2007).

AGING

It would be impossible to summarize briefly the broad aspects of the biology of aging that pertain to

Table 6.1 Characterized stem cell niches in mouse

TYPE OF STEM CELL	LOCATION	CELLS IN CONTACT	SUPPORTING SIGNAL	RECENT REVIEWS
Hematopoietic stem cells	Endosteum, perivascular	Mesenchymal progenitors, reticular cells, osteoblasts, and osteoclasts	CXCL12, SCF, SHH, Ang1, Tpo	Eliasson & Jonsson, 2010; Wilson & Trumpp, 2006; Kiel & Morrison, 2006
Muscle stem cells (satellite cells)	Under basal lamina adjacent to myofibers	Multinucleated myofibers	Notch, Wnt, CXCL12, HGF	Gopinath & Rando, 2008; Sambasivan & Tajbakhsh, 2007; Zammit et al., 2006
Intestinal epithelial stem cells	Base of crypts	Paneth cells, fibroblasts?	Wnt, Notch, BMP	Haegebarth & Clevers, 2009; Walker & Stappenbeck, 2008; Humphries & Wright, 2008
Neural stem cells	Lateral ventricle subventricular zone and subgranular zone of dentate gyrus	Endothelial cells, ependymal cells?	SHH, Notch, Wnt, FGF, VEGF, TGF-α	Suh et.al., 2009; Miller & Gauthier-Fisher, 2009; Basak & Taylor, 2009
Hair follicle stem cells	Hair follicle bulge	Vascular cells?	Wnt, BMP, TGFβ	Robinson & Fisher, 2009; Zouboulis et al., 2008; Blanpain & Fuchs, 2006
Interfollicular epidermal stem cells	Basal layer of the skin	Dermal cells	Notch, Wnt	Yan & Owens, 2008; Jones et al., 2007; Braun & Prowse, 2006
Spermatogonial stem cells	Basal layer of seminiferous tubules	Leydig cells, Sertoli cells, vascular cells	BMP4, BMP8, SCF, FGF, GDNF	de Rooij, 2009; Oatley & Brinster, 2008; Yoshida et al., 2007
Mammary gland stem cells	Inguinal mammary gland	Stroma cells	Wnt, SHH, FGF, TGFβ	Visvader, 2009; Brisken & Duss, 2007; LaBarge et al., 2007

stem cells, and these topics are covered in detail in other chapters. Here we briefly discuss how aging is manifested at the cellular level and how stem cell function may change during aging. Last, we highlight studies of the aging of specific adult stem cell populations from various tissues.

Cellular Aging

Aging of an organism is characterized by a progressive decline in cellular maintenance, defense, and repair processes, resulting in the gradual loss of homeostasis and functionality of tissues and organs over time. At the cellular level, aging is often manifested as structural and functional changes in cellular constituents such as DNAs, proteins, and lipids. Reactive oxygen species (ROS) are among the most prevalent contributors to damage of cellular components (Bertram & Hass, 2008; Blagosklonny, 2008; Lu & Finkel, 2008). The consequence of ROS-induced damage of macromolecules may be different in post-mitotic somatic cells and stem cells that retain the ability to divide. For example, ROS were shown to have an adverse effect on the integrity of the nuclear pore complex by oxidizing the subunits that form the transport channels (D'Angelo et al., 2009), resulting in leakage across the nuclear membrane. Because the nuclear pore complex is an extremely stable structure and subunit exchange takes place only during cell division when the nuclear member is dissembled, damage to the complex may have a lasting detrimental effect on postmitotic cells such as motor neurons during aging. Conversely, in stem cells, subunits of the complex are replaced every time they divide and thus ROS-induced damage is reparable and would not result in persistently dysregulated nuclear transport. ROS can also damage macromolecules in a way that is not repaired even in dividing cells. For example, oxidation of DNA bases can lead to cumulative mutations in the genomic sequence, and protein oxidation can lead to aggregates that are not cleared by cellular proteolytic machinery. Both of these persist in nondividing cells and are heritable in dividing cells and may thus accumulate in self-renewing stem cells.

The cellular DNA repair machinery obviously plays a critical role in guarding the integrity of the genome (Jackson & Bartek, 2009). Failure in DNA repair may result in the accumulation of mutations in the genome, leading to various possible outcomes, including malignant transformation, apoptosis, and senescence (Lou & Chen, 2006). Entering senescence requires an active signaling cascade involving many cell cycle check-point control proteins, including p53 (Figure 6.3). Senescence can also be caused by overly shortened telomeres due to successive rounds of DNA replication (Hornsby, 2007; Kaszubowska, 2008). This type of senescence is sometimes termed replicative senescence. Before 2009, there was little evidence that cellular senescence occurred in vivo and was associated with the aging process. However, studies have revealed an increase in senescent cells in various tissues in aging organisms (Grimes & Chandra, 2009). Senescence, like apoptosis, is actually considered a protective mechanism against aberrant cell growth, which may result in cancer. Paradoxically, senescent cells may also create a local environment that promotes the development of cancer by secreting angiogenic factors and proinflammatory cytokines (Coppe et al., 2006, 2010; Rodier et al., 2009).

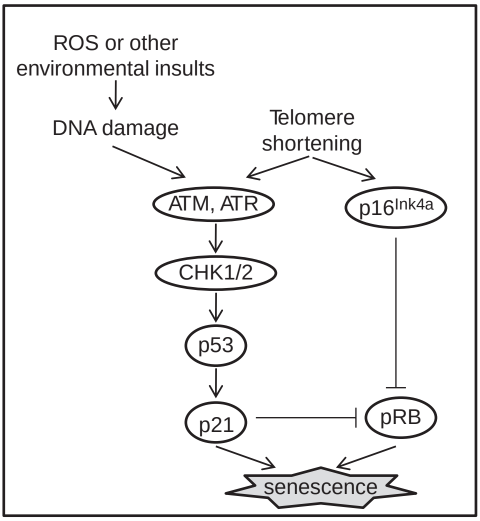

Figure 6.3 Cellular senescence mediated by the DNA repair pathways. DNA damage caused by ROS or other types of environmental insults initiates a cellular DNA damage response, resulting in the sequential activation of ataxia telangiectasia mutated (ATM) and/or ataxia telangiectasia- and Rad3-related (ATR) and downstream kinases CHK1 and CHK2. Activation of CHK1 and/or CHK2 leads to phosphorylation of the tumor suppressor p53. Phosphorylated p53 transcriptionally upregulates genes that mediate cellular senescence, including the cell cycle inhibitor p21. Progressive telomere shortening can activate p53 by a similar mechanism or activate another cell cycle inhibitor, p16^{Ink4A}, in a p53-independent manner. Activation of p16^{Ink4A} inhibits phosphorylation of the RB protein that ultimately leads to cellular senescence.

Stem Cells and Aging

Aging is accompanied by the accumulation of deleterious changes that range from the whole organism down to the molecular level, and stem cells are not protected from those changes. Over time, various kinds of mutations can occur in genomic and mitochondrial DNA in stem cells that result in irreversible age-related changes. In addition, alterations can occur at the epigenetic level during the aging process, also resulting in intrinsic, but reversible, changes that render aged stem cells less effective (Blasco, 2007). Alternatively, extrinsic factors can also contribute to stem cell aging. An aged niche may fail to support normal stem cell homeostasis and lead to the loss of stem cell functionality. Likewise, systemic factors in the aged organism can suppress stem cell function. The extent to which loss of stem cell functionality is mediated by intrinsic irreversible changes, intrinsic reversible changes, or extrinsic influences is of fundamental interest and has very different implications for stem-cell-based therapy.

Stem cell senescence, if it occurs, could have a profound effect on tissue homeostasis and regeneration if there is a resulting depletion of the stem cell pool of sufficient magnitude. To date, there is no direct evidence that stem cell senescence occurs during normal aging. Senescent stem cells have been reported in aged mice deficient in DNA repair (d'Adda di Fagagna et al., 2003; Van Nguyen et al., 2007). Such mice are often proposed to be models of accelerated aging,

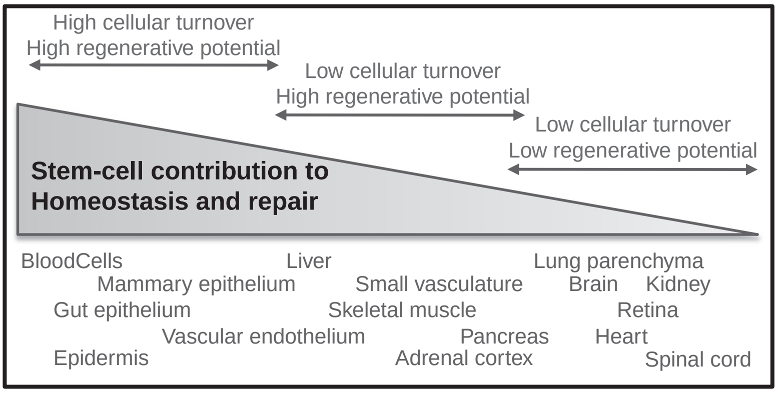

Figure 6.4 Categorization of mammalian tissues based on turnover rate and regenerative potential. The extent to which the effects of aging on the resident stem cells determine the phenotype of an aged tissue is likely to correlate with the extent to which stem cells are responsible for normal tissue homeostasis and repair. Along this spectrum, tissues generally fall into one of three categories. First, tissues with high turnover (such as blood, skin, and gut) have a prominent stem cell compartment and, by definition, have high regenerative capacity. Second, tissues with low turnover but high regenerative potential might use different strategies to ensure effective repair in the setting of acute injury. Third, tissues with low turnover and low regenerative potential might have stem cells that mediate only limited tissue repair. (*Image adapted from Rando, 2006*)

but it is unclear whether the phenotype is truly reflective of normal aging or simply progressive cellular and tissue dysfunction due to the DNA repair defect (Park et al., 2003; Ito et al., 2004; Oguro et al., 2006; Ju et al., 2007; Rossi et al., 2007).

The activity of adult stem cells is determined by the need of the tissue in which they reside. Stem cells in tissues with high cellular turnover, such as blood, skin, and gut, often exhibit high levels of replicative activity. In tissues with low cellular turnover, such as skeletal muscle and most of the central nervous system, there is very low replicative activity (Figure 6.4). As such, the characteristic of stem cell aging might be very tissue-specific. For this reason, stem cells from selected tissues are discussed separately, and the discussions focus on how they change during aging and what can account for these changes.

Aging of Muscle Stem Cells

Skeletal muscle is a highly specialized tissue composed of organized myofiber bundles. Each myofiber, formed from the fusion of mononucleated muscle progenitor cells termed myoblasts, is a postmitotic and multinucleated cell. Skeletal muscle has an extremely low turnover rate of its myonuclei that is almost undetectable under resting conditions (Morgan & Partridge, 2003). In sharp contrast to this slow turnover rate is the remarkable regenerative capacity of this tissue. In adult mammals, new functional muscles can form within weeks after severe and extensive injuries. This rapid regeneration is attributed to muscle stem cells, or "satellite cells" (SCs), that reside adjacent to myofibers, underneath the fibers' basal laminae. SCs were discovered in 1961 by electronic microscopy and named satellite cells for their peripheral location relative to the myofibers (Mauro, 1961) (Figure 6.5). SCs express the transcription factor Pax7, are quiescent in resting muscles, and are activated to self-renew, proliferate, and generate committed myogenic progeny in response to various physiological or pathological stresses, such as intense exercise or degenerative muscular dystrophies (Seale et al., 2000). SCs have been recognized as an excellent model for studying stem cell biology because of the well-defined anatomical location, the availability of markers for both imaging and isolation, and the development of both in vivo and ex vivo methods to study activation, self-renewal, and differentiation. Individual single myofibers can be isolated with the associated SCs and cultured in vitro for days (Bischoff, 1986, 1989), thus providing a good model system for studying stem cell–niche interaction.

Deterioration of muscle integrity and function with age is a common health concern. The elderly often develop sarcopenia, a condition characterized by loss of muscle mass and a component of the frailty syndrome. Replacement of myofibers by adipose and fibrous connective tissue has also been reported in individuals with sarcopenia (Thompson, 2009). In addition to this gradual atrophic process, there is also a marked decline in muscle regenerative potential with age, leading to a delay in recovery following muscle injury (Conboy et al., 2003). Older animals require more time to regenerate after injury, the regenerated myofibers are smaller in diameter, and fibrosis becomes obvious in the interstitial regions, compared to young animals. These age-related phenotypes indicate that there is a decline in muscle regeneration with age.

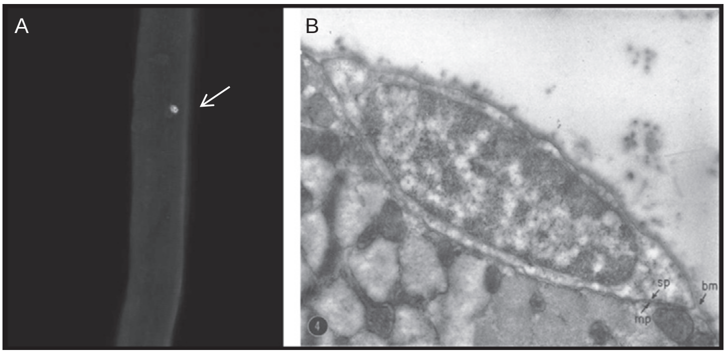

Figure 6.5 Localization of SCs in the muscle. (A) Identification of a SC (arrow) by Pax7 immunofluorescent staining on a single myofiber. (B) The peripheral localization of SCs in relation to myofibers demonstrated by electron microscopy.

(Image adapted from Mauro, 1961)

Depending on the methods, species, and muscles used, estimates of SC numbers during aging have ranged from a decrease, to a negligible change, to a slight increase (Brack & Rando, 2007). However, even the largest estimates of reductions in SC number with age could not account for the age-related muscle decline in regenerative potential, since very few SCs are needed to restore muscle following injury. Transplantation has been widely applied to demonstrate the regenerative ability of SCs. It has been demonstrated that intramuscular injection of a limited number of freshly isolated SCs is sufficient to regenerate myotoxin-injured limb muscles fully in irradiated, immunodeficient mice (Sacco et al., 2008). These transplanted SCs were able to home to their niche and self-renew in the recipients. They were also capable of regenerating injured limb muscle in a subsequent serial transplantation. With such a remarkable regenerative potential, it is clear that the main determinant of the aging phenotype in skeletal muscles must be a decline in the SC functionality rather than in the cell number.

There is currently no experimental evidence that a decline in SC number leads to age-related loss of mass. As with studies of regeneration, the functional consequences of any changes in SC number during aging will need to be tested, not as a correlation, but as direct tests in which SCs are eliminated or SC numbers are enhanced to determine the effect on changes in fiber size with age.

In contrast to the rapid turnover rate of cells in some tissues such as peripheral blood and intestine, skeletal muscle is a very stable tissue that does not require frequent SC cycling for homeostasis. Therefore it is unlikely that SCs age from replicative senescence because of critically shortened telomeres. This notion has been confirmed by comparing the telomere lengths in SCs from individuals of 5 to 86 years of age without finding significant differences (Decary et al., 1997; Ponsot et al., 2008). However, because SCs (or their self-renewing progeny) reside for a lifetime in a tissue with high metabolic activity, they are subject to ROS-induced damage. As such, it is possible that the decline in SC functionality is due to the accumulation of damage in the form of DNA mutations and nondegradable protein depositions. Mutations in mitochondrial DNA, increased mitochondrial permeability, and increased protein aggregates have been found in skeletal muscles from aged rodents and humans (Wanagat et al., 2001; Combaret et al., 2009). Increased levels of some cell cycle inhibitors, including p21, p27, and p53, were also found in the nuclei of a crude mononuclear cell preparation from aged rodent limb muscle (Machida & Booth, 2004). However, in all of these studies, no distinction was made between SCs and myofibers or between SCs and other types of mononucleated cells residing in skeletal muscles. Therefore, direct evidence as to whether these age-related changes, some of them irreversible, occur in SCs during aging has yet to be obtained.

Several reports have revealed intrinsic functional changes in SCs with age. Age-related differences in gene expression profiles have been reported in the progeny of human SCs (Bortoli et al., 2003). Isolated SCs from old animals give rise to far fewer progeny during proliferative expansion in vitro compared to SCs from young animals (Schultz & Lipton, 1982). However, direct tests of the functionality of SCs from aged animals suggest that age-related changes are in fact largely reversible if the cells are given the appropriate conditions for self-renewal and proliferation. SCs from old rats were fully competent in regeneration when they were transplanted into young rats as whole muscle grafts (Carlson & Faulkner, 1989). It is even more striking that there was no distinguishable difference in the regenerative capacity between old and young muscle grafts when the recipients were young. In parabiosis experiments in which young and old mice were surgically connected and developed a single, shared circulatory system, the regenerative capacity of the SCs in the old animal was also remarkably restored (Conboy et al., 2005). These results indicate that the changes in SC function with age are not all permanent and may be readily influenced by the environment in which the cells reside. While the aging niche may have an impact on the activity of SCs, the systemic environment sits on top of this hierarchy as demonstrated by the parabiosis experiments. These findings support the hypothesis that the age-related decline in SC function occurs largely at the epigenetic level. The old systemic milieu may affect the chromatin state of SCs, resulting in epigenetic repression or expression of genes that impair cellular function. Therefore, exposing SCs that reside in aged individuals to the right stimulus can potentially reverse the epigenetic changes and invigorate the cellular regenerative capacity.

The Notch signaling pathway has been demonstrated to be a key determinant of SC activation and cell fate determination, both of which are less effective and reliable in aged animals (Conboy & Rando, 2002; Conboy et al., 2003). Upon muscle injury, the Notch ligand Delta-like 1 (Dll1) is upregulated within the SC niche, leading to activation of the Notch pathway in SCs, a process that is essential for their proliferative expansion. Aged muscle shows a deficit in Dll1 expression following injury and thus a less effective proliferative response of the myogenic progenitors. Forced activation of the Notch pathway in old muscle restores the regenerative capacity of the resident SCs and, reciprocally, inhibition of the Notch pathway in young muscle results in impaired regeneration. The membrane-bound Notch ligand Dll1 activates SCs directly from the myofiber niche, and its expression is influenced by environmental factors, as demonstrated by the restoration of Dll1 induction upon injury in old muscle in a heterochronic parabiosis experiment in which an old mouse was paired with a young one (Conboy et al., 2005). These studies have definitively established an indispensible role for the Notch pathway in normal muscle regeneration, but the systemic factors that influence the induction of this pathway in young and aged mice remain to be elucidated. Muscle is a highly vascular tissue and SCs are known to respond to growth factors produced by endothelial cells. It has been documented that some of these growth factors, vascular endothelial growth factor as one example, are reduced in aged muscle (Croley et al., 2005). In this regard, studying the growth factor production profile of endothelial cells may help to identify the local and systemic factors that influence muscle regenerative capacity and may account for age-related declines in that capacity.

The Wnt pathway has also been shown to play an important role in muscle regeneration and to be altered with age. Using the Wnt reporter mouse, it was demonstrated that Wnt signaling increased during the regenerative response following injury (Brack et al., 2007, 2008). The activation of the canonical Wnt signaling pathway promoted differentiation of SC progeny to become fusion-competent myoblasts. The temporal control of this pathway appears to be critical to achieve optimal regeneration. In aged mice, the Wnt pathway appears to be active even in resting SCs. This "premature" Wnt signaling inhibited the expansion of SC progeny and skewed the lineage specificity of SCs toward a fibrogenic fate. The resulting insufficient supply of myogenic progeny contributed not only to poor muscle regeneration but also to increased fibrosis in old animals, perhaps as a result of the increased generation of fibrogenic cells. The lineage potential of stem cells is believed to be controlled at the epigenetic level. As mentioned above, lineage determination genes have been shown to possess bivalent domains in their promoter regions, and these domains resolve to either an activated or a repressed state as stem cells differentiate into lineage-specific progenitors. The ability of SCs to adopt nonmyogenic fates, such as adipogenic or osteogenic progenitors or as fibrogenic progenitors during aging, has been documented (Taylor-Jones et al., 2002; Shefer et al., 2004; Brack et al., 2007), suggesting that the chromatin of SCs is at a permissive state for various mesenchymal lineages. Current evidence suggests that during normal regeneration, once the lineage specificity of SCs is determined, i.e., in the transiently amplifying myogenic progeny, determinant genes for other cell fates are no longer in an accessible state on the chromatin. In aged animals, it is possible that the untimely activation of Wnt takes advantage of the permissive chromatin in a very narrow window of time before myogenic lineage determination occurs and drives the SCs to adopt a nonmyogenic fate. This hypothesis, while plausible, can be tested only when a detailed characterization of the epigenome of SCs is available, as well as a characterization of how it changes during lineage progression during aging.

Aging of Hematopoietic Stem Cells

HSCs give rise to all cells in the blood that carry out life-sustaining functions such as oxygen transport, blood coagulation, and immune defense (Figure 6.6). Aging is correlated with a decline in immune functions. The elderly suffer increased morbidity and mortality associated with infection, have reduced capacity to generate high-affinity antibodies in response to vaccination, and are more likely to develop select cancers and autoimmune disorders (Larbi et al., 2008; Grubeck-Loebenstein et al., 2009). Studies of the blood components from healthy elderly humans or older laboratory animals compared to their young counterparts have revealed a number of changes associated with advanced age (Rink et al., 1998). A chronic hyperinflammatory state was found in old individuals exemplified by elevated circulating levels of proinflammatory cytokines such as IL-6, IL-1β, and TNF-α. While microbial infection rapidly induces the expression of these proinflammatory cytokines in young and healthy individuals, their production in response to acute stimulation by lipopolysaccharide, a major component of the outer membrane of gram-negative bacteria, is significantly impaired in innate

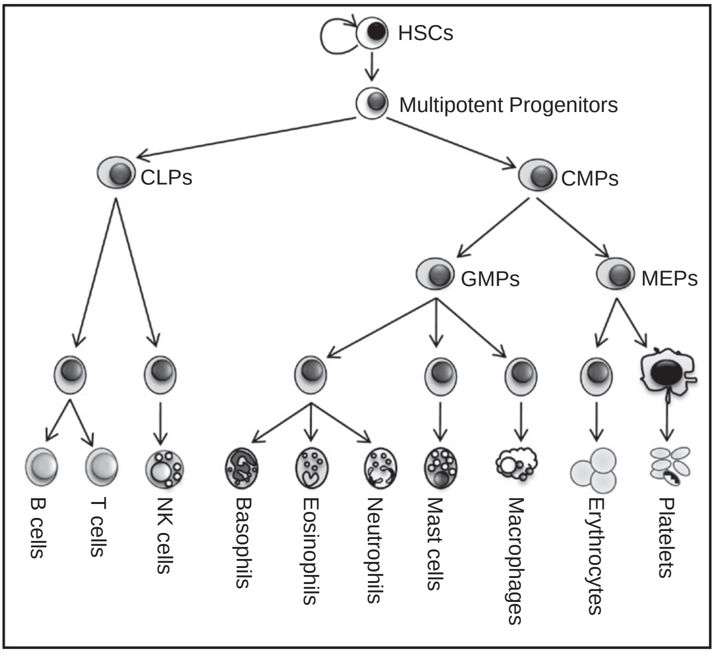

Figure 6.6 Mammalian hematopoiesis mediated by HSCs. CLPs, common lymphoid progenitors; CMPs, common myeloid progenitors; GMPs, granulocyte–macrophage progenitors; MEPs, megakaryocyte–erythrocyte progenitors.

immune cells, including macrophages/monocytes and natural killer cells, isolated from old individuals. The elderly also have a skewed ratio of various adaptive immune cells, evidenced by a decreased number of memory B cells and naïve T cells (Linton & Dorshkind, 2004; Min et al., 2004). Because mature blood cells turn over every few days and the hematopoietic system is constantly regenerating throughout the lifetime of an individual, the question arises as to whether age-related functional changes in cells in the blood are a reflection of changes that have occurred in the HSC compartment over time.

An age-related decline in hematopoietic function could be caused by the gradual loss of HSCs during aging. However, despite the gradual decline in immune function, there is little evidence that HSCs are depleted from the pool with the passage of time. On the contrary, FACS analyses have revealed that HSCs are much more frequent among bone marrow-derived cells from aged mice than from young mice in certain strains (Morrison et al., 1996; Sudo et al., 2000; Chambers et al., 2007). While the vast majority of HSCs in young mice were found to be in a quiescent state, a large portion of HSCs in old, but otherwise healthy, mice were in the cell cycle. Although it is unclear whether the higher frequency of HSCs in old bone marrow is due to an actual increase in the absolute HSC number or simply a decrease in other nonstem cells in the compartment, these studies provide strong evidence against the notion that depletion of HSCs in older individuals is the cause of the decline in immune function.

To investigate whether the age-related decline in the hematopoietic system is caused by diminishing HSC functionality, Rossi and colleagues isolated HSCs from young and old mice using the same set of surface markers (Rossi et al., 2005). In vitro characterization of these two populations revealed no difference in their abilities to proliferate, to form colonies, or to interact with stromal cells. Furthermore, single reconstituting HSCs obtained by limiting dilution from old and young mice formed the same sized splenic clones in vivo. However, HSCs from old mice were significantly less effective in homing to and engrafting in the bone marrow of irradiated recipients. Their differentiation potential, as a population, was also skewed toward the myeloid lineage in vivo (Rossi et al., 2005; Cho et al., 2008). While such transplantation approaches do not reveal the cause of these defects in old HSCs, they suggest that some intrinsic characteristics of HSCs, whether reversible or irreversible, do change with the passage of time. Indeed, gene expression profiling revealed a concurrent downregulation of lymphoid specification genes and an upregulation of myeloid fate-determining genes in HSCs isolated from aged mice (Rossi et al., 2005). These changes in gene expression correlate well with the previous observation that HSCs from old mice preferentially give rise to myeloid cells in transplantation recipients, reflecting an aged phenotype at the genomic level (Linton & Dorshkind, 2004).

Fundamentally, at any given time, gene expression is determined by the epigenome in a cell. It remains to be determined to what extent age-related changes in HSC function are due to changes in the cellular epigenetic profile. Well-characterized epigenetic changes that affect gene expression include methylation of genomic DNA itself and posttranslational modification of core histones. DNA methylation has been shown to play a critical role in regulating HSC homeostasis (Broske et al., 2009). Self-renewal of HSCs appears to require constitutive DNA methylation. HSCs that express only very low levels of the maintenance DNA methyltransferase DNMT1 have a significant reduction in their ability to reconstitute the hematopoietic system in a competitive transplantation assay compared to wild-type controls. In addition, DNMT1-low HSCs fail to differentiate into lymphoid progeny, whereas myeloid differentiation was unaffected. This is an intriguing correlation with the observation that HSCs from aged mice skew toward myeloid differentiation. Locus-specific loss in DNA methylation has been described in peripheral blood cells during aging, but direct evidence from HSCs is still lacking. It will be interesting to determine whether a reduction in DNA methylation occurs in HSCs during aging either at the global level or in a locus-specific manner and whether such changes could account for the impaired lineage potential of HSCs in old animals. At the histone level, it remains to be determined whether histone modification patterns change with age. However, a few chromatin remodeling enzymes were found to be downregulated in HSCs during aging (Chambers et al., 2007). These enzymes include members of the sirtuin family of histone deacetylases that are involved in chromatin silencing. Although the specific targets of these deacetylases in HSCs are not known, it is possible that an imbalance among chromatin-modifying enzymes could perturb the epigenetic state, including the prevalence of bivalent domains that ensure multipotency. Epigenetic marks are generally reversible and subject to change upon activation of various signaling pathways. Therefore, changes in HSCs at the epigenetic level are likely to reflect the influence of an aging niche or systemic environment upon the cell.

Bone marrow mesenchymal cells form a cellular niche that prevents HSC exhaustion by controlling the balance between HSC self-renewal and differentiation. Age-related changes in the bone marrow and their impact on HSCs have been investigated in telomerase-deficient mice. At 1 year of age, a significantly higher number of mesenchymal cells with critically short telomeres are found in the bone marrow of these mice in comparison to control animals of the same age, concurrent with a decrease in B lymphopoiesis (Ju et al., 2007). These mutant stromal cells fail to support the engraftment and reconstituting ability of healthy wild-type HSCs, demonstrated by a two-way transplantation experiment: young wild-type HSCs into 1-year-old telomerase-deficient mice and 1-year-old telomerase-deficient stromal mesenchymal cells into young wild-type mice. The resemblance between stromal cells bearing shortened telomeres and truly aged stromal cells suggests that a healthy niche is required to maintain HSC functionality. In contrast to the vast literature on HSCs, much less is known about their niche because of current technical limitations. As HSCs reside primarily within the marrow of long bones, the niche is extremely difficult to image and the markers used to isolate HSCs by FACS do not work nearly as well to identify them in situ. Advances in the technologies to image and trace HSCs in vivo should yield exciting discoveries concerning the interaction between HSCs and their niche cells and constituents, how these interactions change with age (Lo Celso et al., 2009; Xie et al., 2009), and how age-related changes in the systemic environment affect HSC–niche cell interaction (Mayack et al., 2010), as has been well established in the muscle stem cell compartment (Conboy et al., 2003, 2005; Brack et al., 2007).

As telomere shortening is associated with cellular aging, the relationships between age, telomere shortening, and replicative life span have been examined in HSCs. Vaziri and colleagues reported that adult human HSCs carry shorter telomeres than umbilical cord blood cells (Vaziri et al., 1994). In addition, hematopoietic progenitor cells that are derived from adult HSCs under specific cytokine selection in vitro have shorter telomeres than their HSC precursors. However, neither of these two experimental systems recapitulates HSC activity during natural aging. The former comparison was between developing and mature HSCs (not aging per se) and the latter addressed telomere length change during lineage differentiation of HSCs. Interestingly, telomere shortening is accelerated in peripheral blood cells in patients who suffer from segmental progerias such as Werner syndrome and ataxia telangiectasia in comparison to healthy individuals of similar chronological age (Metcalfe et al., 1996; Schulz et al., 1996). Shortened telomeres were also reported in HSCs that had a decreased reconstituting potential following successive rounds of serial transplantation (Allsopp et al., 2001). These two observations are consistent with the notion that telomere shortening could limit the replicative potential of HSCs. However, the caveat is that in serial transplantation studies, a very small number of HSCs must repeatedly divide to reconstitute the entire hematopoietic system in lethally irradiated hosts, thus overwhelmingly exaggerating the replicative demand placed upon HSCs during the lifetime of any individual. Unlike most adult somatic cells, HSCs express telomerase (Jaras et al., 2006). This discovery provides evidence that telomere length is not likely to impose a limit on HSC function during normal aging. The importance of telomerase for HSC functionality was demonstrated in studies using telomerase-deficient mice (Allsopp et al., 2003a; Rossi et al., 2007). HSCs from these mice exhibit telomere shortening at twice the rate as those from wild-type animals, and in contrast to normal HSCs that can reconstitute the hematopoietic system typically for four to six rounds of serial transplantation, telomerase-deficient HSCs fail to do so after two rounds of serial transplantation. By 60 weeks of age in secondary transplant recipients, HSCs from telomerase-deficient donors failed to regenerate blood. However, this is not necessarily a model of aging for reasons already mentioned—the nonphysiologic reflection of "aging" by mutant mice that have shortened life span and by serial transplantation. The expression of telomerase in HSCs may limit the age-related phenotype of telomere shortening, but even telomere-deficient HSCs are capable of successfully performing the vital HSC functions during a single lifetime. Furthermore, the fact that a few telomerase-deficient HSCs are capable of reconstituting the blood system through two rounds of serial transplantation clearly demonstrates that telomere shortening is not a limiting process for the life span of the HSCs. In addition, HSCs that overexpress telomerase and preserve telomere length following serial transplantation cannot be serially transplanted more often than wild-type cells (Allsopp et al., 2003b). This clearly demonstrates that telomere shortening is not the determinant of serial transplantation capacity.

The notion that DNA damage results in HSC aging comes primarily from studies using genetically engineered mice that are defective for DNA repair. In these studies, the functionality of HSCs from mice that lack critical proteins for nonhomologous end joining or nucleotide excision repair was compared to that of HSCs from wild-type mice (Nijnik et al., 2007; Rossi et al., 2007). Competitive transplantation assays demonstrated that HSCs from the mutant mice were more than 20-fold less efficient in reconstituting the blood system of recipients, and this difference became even more pronounced with age. While these studies provide convincing evidence for the critical role of DNA repair machinery in maintaining HSC homeostasis, they are not sufficient to conclude that HSCs age because of accumulated DNA damage for several reasons. First, despite increased DNA damage detected in HSCs from the mutant mice compared to those from wild-type mice, the HSC pool was not depleted with age. Second, a decline in the functionality of the DNA repair machinery has not been reported in healthy individuals during aging. On the contrary, although a marked increase in double-strand DNA breaks was detected by staining for γ-histone-2-AX foci staining in HSCs from old wild-type mice compared to young mice, the difference in the number of foci was not observed in their progeny, including both myeloid and lymphoid progenitors (Rossi et al., 2007). This observation suggests that the DNA repair machinery is fully functional in HSCs during aging and that its activity is triggered by cell cycle progression.

DNA abnormalities that necessitate effective repair mechanisms include mutational errors that occur during replication and damage to individual bases and to the double-stranded helix from genotoxic stresses. Among the most widely studied genotoxic agents are ROS, which, as noted above, can cause oxidative damage to individual bases as well as single- and double-strand DNA breaks. The detrimental effect of ROS on HSCs was suggested by studies of mice deficient in the gene ataxia telangiectasia mutated (ATM) or FoxO transcription factors (Ito et al., 2004, 2006; Miyamoto et al., 2007; Tothova et al., 2007). These mice have elevated levels of intracellular ROS and, correlatively, their HSCs display severe functional defects that lead to progressive bone marrow failure, albeit by different mechanisms—ATM-deficient HSCs appear to have a self-renewal defect, whereas FoxO-deficient HSCs exhibit an increased rate of apoptosis. In fact, ATM expression was decreased in FoxO-deficient HSCs. A target gene that is normally suppressed by ATM, the p16^{Ink4a} tumor suppressor, was upregulated in both ATM- and FoxO-deficient HSCs. Given the role of p16^{Ink4a} in promoting cellular senescence, it was proposed from these studies that ROS contributes to HSC aging by inducing the expression of p16^{Ink4a} and functional senescence. However, this hypothesis is not supported by the finding that HSCs from aged wild-type mice do not express p16^{Ink4A} (Attema et al., 2009). The epigenetic repression of the *Ink4A* locus is well maintained in HSCs isolated from old mice, suggesting that p16^{Ink4A}-mediated senescence is unlikely to be an important determinant of HSC aging. Furthermore, HSCs appear to reside selectively in hypoxic regions in the bone marrow (Eliasson & Jonsson, 2010). The level of ROS that HSCs are exposed to during physiological aging remains to be determined.

Another well-characterized tumor suppressor, p53, has also been implicated in HSC aging from studies using mice that express elevated levels of p53 (Dumble et al., 2007). These mice have an increased number of HSCs, resembling the change in HSC number in old wild-type mice, and therefore were considered a potential model of accelerated aging. However, it is unclear whether p53 levels increase in HSCs during normal aging. While all the above mouse models are valuable in unraveling important mechanisms for HSC homeostasis, it remains highly debatable whether they recapitulate the normal aging process and thus conclusively reveal changes that take place in HSCs during aging.

Aging of Intestinal Epithelial Stem Cells (ISCs)

The mammalian intestine is one of the most actively renewing tissues of the body. It is composed of two segments, the small intestine and the large intestine, the last portion of which is the colon. The inner lumen of the intestine is lined with a layer of stratified epithelial cells that is renewed every 4–5 days, and remarkably, this renewal does not seem to diminish throughout the lifetime of an individual (van der Flier & Clevers, 2009). Considerable effort has gone into identifying the stem cells in the intestinal epithelium that are able to fulfill such a high demand and degree of tissue regeneration. The epithelium of the small intestine is organized as vast numbers of invaginations, or "crypts," surrounding the base of projections called "villi." Slow-cycling cells, as candidate ISCs, have long been described to exist at the +4 position relative to the base of the crypts (Figure 6.7) (Booth & Potten, 2000; Marshman et al., 2002; Bjerknes & Cheng, 2006). With the further development of mouse genetic tools, the identity of another putative population of ISCs has been determined (Barker et al., 2007; van der Flier et al., 2009). They express the Wnt target genes Lgr5 and Ascl2 and reside not only in the intestine, but at the crypt base in the colon epithelium and at the gland base at the bottommost region of stomach epithelium as well. Unlike the cells at the +4 position, these Lgr5-positive cells appear to cycle rapidly. Therefore, there may be more than one type of ISC.

In the small intestines of mice, ISCs are responsible for the production of about 250 cells per crypt per day (Booth & Potten, 2000). The rare ISCs give rise to a pool of transit-amplifying cells that differentiate into the four principle epithelial cell types in the gastrointestinal tract—goblet cells, enteroendocrine cells, and Paneth cells, all of which are secretory cells, and absorptive enterocytes (van der Flier & Clevers, 2009). Both the Wnt and the Notch pathways are important for the ISC-mediated intestinal epithelium homeostasis (van der Flier & Clevers, 2009). The Wnt pathway is the master switch of proliferation and differentiation for the transient-amplifying epithelial cells. Active Wnt signaling promotes proliferation, and the diminishment of Wnt pathway activity is necessary to trigger differentiation. While the Notch pathway is required for the maintenance of crypt epithelial cells in the undifferentiated state, it is also essential for the lineage decision between the secretive and the absorptive fates (Fre et al., 2005; Stanger et al., 2005; Riccio et al., 2008). Elimination of the Notch pathway in mice results in the depletion of the transient-amplifying population and the abolishment of their conversion into goblet cells.

Age-related histological changes in the intestinal epithelium have been described (Martin et al., 1998a,b). Villi are generally larger but cellularity is reduced in old animals. The number of crypts also decreases over time. These age-related histological changes appear to be accompanied by functional changes in response to DNA damage. Cells within the crypts in old mice are more susceptible to apoptosis

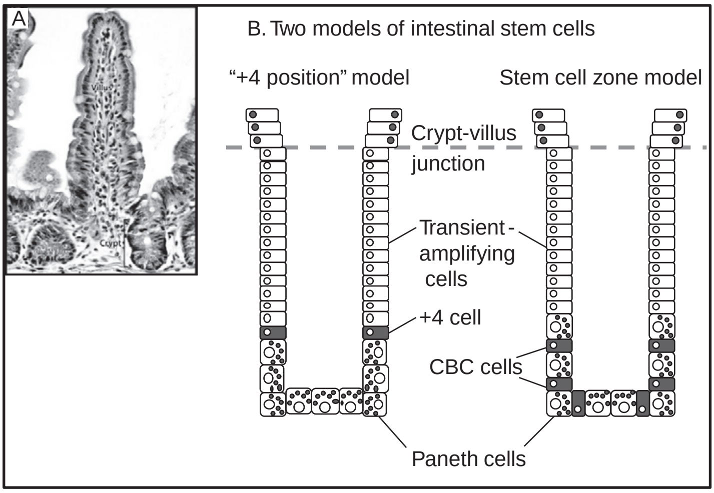

Figure 6.7 Localization of intestinal epithelial stem cells. (A) Hematoxylin and eosin staining of the mouse intestine. The intestine is lined with a single layer of epithelial cells organized into invaginations called the crypts and finger-like protrusions called villi. (Image adapted from van der Flier & Clevers, 2009.) (B) The two models of intestinal epithelial stem cells. The "+4 position" model proposes that the crypt base is exclusively populated by terminally differentiated Paneth cells and the stem cells must therefore be located just above the Paneth cells at the +4 position. This model predicts that the enterocytes, goblet cells, and enteroendocrine cells are derived from +4 cell progeny that differentiate as they migrate out of the crypts onto the villi. In contrast, the Paneth cells differentiate as they migrate down from the +4 position toward the crypt base. The "stem cell zone" model states that intestinal stem cells are small, undifferentiated, cycling cells termed crypt base columnar cells (CBCs) intermingled with the Paneth cells, and that the CBCs are capable of giving rise to all four cell types in the intestinal epithelium.

induced by low-dose γ-irradiation, and regeneration of the gut epithelium is significantly slower in old animals after high-dose irradiation. These observations prompted the hypothesis that ISCs undergo intrinsic aging well before the identification of ISCs themselves (Kirkwood, 2004). However, without a definitive marker of ISCs at the time of analysis, it was not clear whether the increase in apoptosis was specific to ISCs or included their neighboring cells (as part of the niche). Therefore, the possibility that the aged niche and/or environment fails to support the survival of ISCs under genotoxic conditions could not be ruled out.

Aging also appears to induce epigenetic changes in the gut epithelium. Increased DNA methylation was reported in both intestinal and colon crypts from older humans (Yatabe et al., 2001). DNA methylation of CpG islands in the promoter region of genes results in their repression. The extent to which the age-related increase in DNA methylation impacts the transcriptome of gut epithelial cells remains to be determined. Interestingly, quantitative simulation using the experimental DNA methylation data revealed that age-related DNA methylation appeared to increase more rapidly in the colon than in the small intestine, correlating with the faster cell division rate in the colon compared to the small intestine (Kim et al., 2005). This observation suggests that DNA methylation, as one important determinant of the epigenome, may be used as an indicator of the mitotic age of cells.

Studies of intestinal epithelium and ISC aging have often focused on the increased incidence of colon cancer rather than the loss of regeneration potential since otherwise healthy individuals do not die solely from gut epithelium failure and since age is undoubtedly the number 1 risk factor for colon cancer. Mutations that result in the activation of the Wnt pathway initiate the vast majority of colorectal cancer (CRC) (Fodde & Brabletz, 2007). As evident by its elevated activity in old skeletal muscles (Brack et al., 2007), aberrant Wnt pathway activation appears to occur with aging as a result of systemic factors. During aging, abnormal activation of the Wnt pathways in the intestinal epithelium could also be induced by these systemic influences. While the onset of CRC is typically triggered by mutations that perturb the Wnt pathway in the colon epithelium, activation of the Wnt pathway by systemic factors could also contribute to the progression and influence the prognosis of CRC in aged patients. However, it remains to be determined whether this occurs in ISCs and whether it is these cells that give rise to CRC with age.

The cellular origin of age-related colon cancer has been studied using a transgenic *Drosophila* line in which the ISCs are fluorescently labeled (Biteau et al., 2008). ISCs from old flies appeared to have a higher rate of proliferation that resulted in an expanded transient-amplifying population. Normally the Notch ligand Delta is expressed in ISCs and downregulated in their progeny for differentiation to proceed. However, Delta levels remained unexpectedly high in neighboring cells of ISCs in old flies, suggesting a failure of differentiation that results in the accumulation of proliferating ISC progeny. It is interesting that the Notch pathway appears to be altered in distinct ways during aging in ISCs in comparison to muscle stem cells. Aged mammalian muscle exhibits a failure of Delta induction upon injury (Conboy et al., 2005), whereas aged *Drosophila* ISCs appear to exhibit a failure of Delta downregulation (Biteau et al., 2008). While the two distinct patterns may reflect species differences in Notch signaling for regulating stem cell activity in *Drosophila* and mammals, they may also suggest that Notch signaling is altered in different manners during aging in different types of tissue-specific stem cells. The establishment of ISC markers in mammals should allow a more accurate assessment of their changes during aging and even the isolation of these cells for molecular characterization.

Aging of Neural Stem Cells (NSCs)

It had been a long-standing dogma until the 1990s that de novo neurogenesis did not occur in the adult brain. This dogma was challenged in 1992 by the isolation of proliferative cells from the adult murine brain that were capable of differentiating into neurons and astrocytes (Reynolds & Weiss, 1992). Currently, two regions in the adult brain are recognized to exhibit active neurogenesis (Figure 6.8): the subventricular zone (SVZ) of the lateral ventricle and the subgranular zone (SGZ) of the dentate gyrus in the hippocampus (Zhao et al., 2008; Suh et al., 2009). Adult NSCs in the SVZ generate neurons that migrate a significant distance to the olfactory bulb to become granule and periglomerular neurons that are essential for the maintenance of the olfactory bulb, whereas neurons born in the SGZ migrate into the granule cell layer of the adjacent dentate gyrus where they are important for the processes of memory and learning. In addition to giving rise to new neurons, adult NSCs are also capable of generating astrocytes and oligodendrocytes that play essential supportive and protective roles in maintaining proper function of the central nervous system (Taupin & Gage, 2002).

BrdU labeling along with neural markers has been used to provide evidence for neurogenesis and for the existence of NSCs in adult brain. Using this approach, it has been well documented that both the number of NSCs and their proliferative activity decline with age in rodents, and this decline is correlated with a gradual loss of cognitive and olfactory functions (Seki & Arai, 1995; Kuhn et al., 1996; Tropepe et al., 1997; Kempermann et al., 2002; Bondolfi et al., 2004). However, it is unclear whether this attenuated

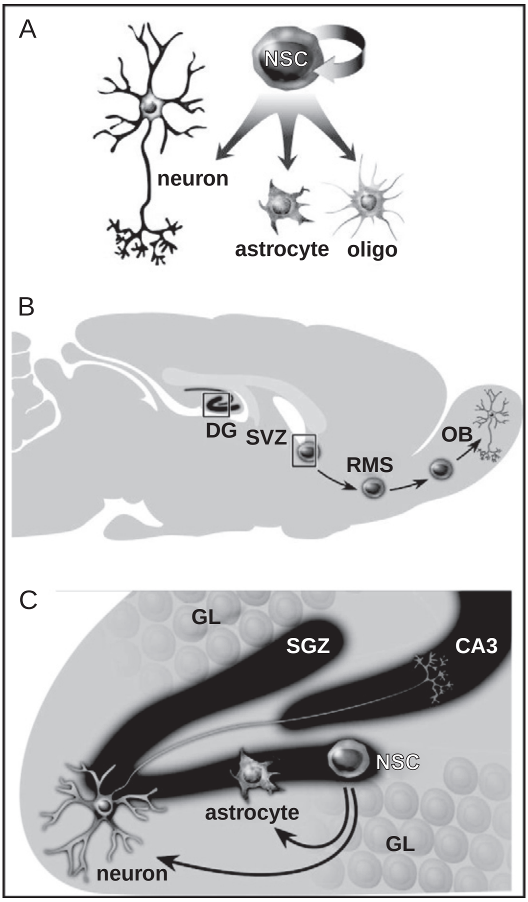

Figure 6.8 Neurogenesis and NSCs of the adult mammalian central nervous system. (A) NSCs are self-renewing, multipotent cells that generate cells of the neuronal lineage: neurons, astrocytes, and oligodendrocytes. (B) Neurogenesis occurs in two areas of the adult CNS: the olfactory bulb (OB) and the dentate gyrus (DG) of the hippocampus. The new neuronal cells in the OB are generated from NSCs of the subventricular zone (SVZ), which is a narrow zone of tissue in the wall of the lateral ventricle in the forebrain. The NSCs of the SVZ migrate to the OB via the rostromigratory stream (RMS), where they differentiate into interneurons of the OB. (C) The new neuronal cells in the adult DG are generated from NSCs of the subgranular zone (SGZ) of the hippocampus and differentiate into neural and glial cells in the granular layer of the DG.
(Images adapted from Taupin & Gage, 2002.)

neurogenesis during aging is due to the aging of NSCs or of the niche. Transplantation of NSCs has been used to assess the effect of the host environment on their neurogenic potential. Purified fetal hippocampal cells that were highly proliferative were transplanted into the ventricles of young and old rats after hippocampal injury (Zaman & Shetty, 2002). The survival of grafted cells in aged hosts was less than 50% of that in young hosts. Interestingly, the diminished survival of cells in aged hosts could be prevented by pretreatment of cells with neurotrophic factors prior to transplantation. Although it remains to be determined whether the production of neurotrophic factors declines during normal aging in older animals without brain injury, this study provides supportive evidence of a failing environment (niche) for NSCs in the brain with advanced age.

One of the major challenges in the study of NSCs and the impact of aging on their function is the absence of any specific markers to label these cells in vivo. Under the right conditions, NSCs can survive in vitro in the presence of neurogenic growth factors. In such cultures, NSCs undergo proliferative expansion to form heterogeneous clusters of cells, termed "neurospheres," that consist mostly of neural progenitors but also of some NSCs. Current studies of NSCs rely heavily on the formation of these neurospheres in culture to evaluate the neurogenic potential of particular populations of NSCs. This allows for molecular studies to identify critical molecules or signaling pathways that regulate the self-renewal of NSCs. Using knockout mice, certain genes have been identified as playing essential roles in the normal homeostasis of the NSC pool. Self-renewal of NSCs was shown to be impaired in mice deficient in the expression of the chromatin-interacting protein HMGA2 (Nishino et al., 2008). HMGA2-deficient mice have reduced numbers of NSCs throughout their central and peripheral nervous systems. It appears that HMGA2 deficiency leads to increased expression of the cell cycle inhibitors $p16^{Ink4a}$ and $p19^{Arf}$, and the double deletion of $p16^{Ink4a}$ and $p19^{Arf}$ partially rescues the self-renewal defect of the $HMGA2^{-/-}$ NSCs. The Forkhead family transcription factor FoxO3 has also been identified as a critical regulator of NSC homeostasis (Renault et al., 2009). A progressive depletion of the NSC pool was observed in FoxO3 knockout mice, and in vitro neurosphere assays suggested a defect in the self-renewal and cell survival of the $FoxO3^{-/-}$ NSCs.

Although mice that lack a vital gene in regulating the normal physiology of stem cells are not necessarily reflective of normal aging, the HMGA2- and FoxO3-deficient mice provide some insight into the maintenance of NSC homeostasis with age because of the link between these two genes and aging. HMGA2 expression decreases in stem cells, including HSCs and NSCs, with age (Nishino et al., 2008). Therefore, given the role of HMGA2 in the normal self-renewal of NSCs, this could provide an explanation for the decreased neurogenesis observed in aged animals. FoxO transcription factors are necessary for the enhanced longevity of mutants of the insulin pathway in invertebrates (Murphy et al., 2003). The association of FoxO3 polymorphism with longevity in humans has been reported, raising the possibility that FoxO3 also regulates life span in mammals

(Willcox et al., 2008; Flachsbart et al., 2009). This link between FoxO3 and longevity in multiple species and the role of this protein in regulating and protecting adult stem cell populations support the hypothesis that genes that enhance longevity may do so in part by ensuring a healthy and youthful pool of adult stem cells.

Stem Cells and Longevity

From the above discussion of tissue-specific stem cells, SCs, HSCs, ISCs, and NSCs, it is clear that stem cells do exhibit intrinsic changes that underlie at least some alterations in their functional potential during aging. However, the functionality of stem cells is tightly integrated with the status of the niches in which they reside. The functionality of a stem cell population is an integration of intrinsic and extrinsic, especially niche, influences. Conversely, stem cells play a critical role in sustaining the integrity of their niche. This cross talk makes it very difficult to definitively determine any specific upstream causes and downstream effects when evaluating stem cell and niche aging. Nevertheless, it is possible to intervene to enhance the functionality of aged stem cells. As demonstrated in both SCs and HSCs, exposing old cells to a healthy youthful environment rejuvenates these stem cells such that their regenerative capacity is nearly that of young stem cells (Conboy et al., 2005; Brack et al., 2008; Dorshkind et al., 2009; Mayack et al., 2010). These observations strongly suggest that age-related changes in stem cell function may be due at least in part to their response to the aged environment and that stem cells both respond to and contribute to organismal aging.

Much attention has been focused on the role of stem cell number and functionality in the determination of tissue aging. However, the relationship between tissue aging and organismal longevity remains elusive. It will be interesting to determine if there is a correlation between stem cell functionality and changes in life span in genetic mutants, particularly long-lived mutants. The greater challenge will be to test whether enhancement of stem cell functionality in individual tissues, or groups of tissues, has any effect on overall organismal life span. Importantly, there is no evidence that declining stem cell number or function determines the maximal life span of any species.

CONCLUDING REMARKS

Stem cells hold the promise of cure of many human diseases, in particular degenerative diseases and diseases of aging, whether by enhancement of endogenous stem cell function or transplantation of exogenous stem cells. In the context of aging, the success of any stem-cell-based therapy will be highly dependent on the status of the niche, appreciating the likelihood that age-related changes may render the niche less able to promote optimal stem cell function. Therefore, while enhancing the functionality of endogenous stem cells or optimizing the functionality of exogenous stem cells will be important for the success of such therapy, so will any treatments that may prime or prepare the niche to support those cells optimally, particularly in the aged host.

The concept that stem cells may have applications to the delay of organismal aging requires the consideration of the universality of the aging process of both the proliferative and the nonproliferative tissues and the profound influence of the systemic milieu on cells within any tissue. Any experimental methods for increasing longevity are more likely to emerge from an understanding of the systemic coordination and regulation of cellular metabolic activity and cellular defenses that characterize the physiological homeostatic mechanisms that are disrupted during aging. However, it is almost certain that any extension of life span will be associated with a reduction in organismal function in some other physiological context, consistent with evolutionary theory (Kirkwood, 2005), and this in principle could be associated with a suppression rather than an enhancement of stem cell functionality.

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Chapter 7

Leukocyte Telomere Dynamics, Human Aging, and Life Span

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CHAPTER CONTENTS

Introduction 163
Human Aging and Longevity, Life Span, and Life Expectancy 164
Telomere Shortening, Replication, and Evolution 165
Comparative Telomere Biology between Mouse and Human 166
Why do Humans Have Relatively Short Telomeres? 166
The End Replication Problem vs Oxidative Stress 167
Telomere Dynamics in Human Leukocytes 167
Leukocyte Telomere Length Dynamics: the Gender and Race Effects 168
Leukocyte Telomere Length and Atherosclerosis 169
Hematopoietic Stem Cell Telomere Dynamics and the Human Life Span 170
Ramifications 170
Conclusion 171
Acknowledgment 171
References 171

INTRODUCTION

The considerable life-span gap between humans and other terrestrial mammals has made it difficult to understand how we live as long as we do compared with, for instance, a mouse. Suppose the biological processes that cause the mouse to age were deciphered and the factors that determine its life span identified. Should we then extrapolate the findings in the mouse to human beings? The average human lives roughly 30 times as long and weighs 3000 times as much as the average laboratory mouse. Could we presume that, because the principles of biology are universal, identical mechanisms cause mouse and human aging and interspecies differences in life span and body size matter little to the aging process? Our present knowledge of aging is insufficient to provide unqualified answers to these questions. That being said, a genetic mouse model has given us important clues as to what the answers might be, not necessarily because it has unraveled the mechanisms that cause the mouse to age, but because it has pointed to a major factor that is likely to contribute to variation in longevity among humans. The factor is the length of telomeres, and the mouse model is that in which telomerase has been rendered inactive by genetic means. In mammals, telomeres consist of thousands of nucleotides with TTAGGG repeats and their binding proteins at the two ends of each chromosome (de Lange, 2008). Telomerase is a RNA-dependent DNA polymerase reverse transcriptase, an enzyme that transcribes single-stranded RNA into TTAGGG repeats and thereby elongates the telomeres (Blackburn, 2005).

The following narrative distills the essence of a large body of research and is by no means comprehensive. For this reason, an apology is extended to all researchers whose meritorious papers are not discussed for reasons of space. In addition, excellent reviews have been published about the molecular biology of aging (Sharpless & DePinho, 2007; Vijg & Campisi, 2008) and the potential role of telomere biology in aging and aging-related diseases (Blasco, 2005; Garcia et al., 2007). Although this chapter takes stock of basic telomere research, it emphasizes telomere

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epidemiology to address the above-mentioned and other yet unresolved questions relating to human aging and longevity. These include questions such as: Does it matter for our survival that we display shorter telomeres than many other mammals do? Are there any biological ramifications of having a long or short leukocyte telomere length (LTL)? What might be the explanation for the longer LTL in women than in men? And then, what does the steadily expanding list of associations between LTL and cardiovascular diseases (CVD) tell us about the biology of our aging and perhaps interindividual variation in human longevity? Again, there are no definitive answers to any of these questions at present, but telomere research has already generated a whole new way of thinking about the intersection of aging with aging-related diseases and longevity among humans.

HUMAN AGING AND LONGEVITY, LIFE SPAN, AND LIFE EXPECTANCY

The terms "longevity," "life span," and "life expectancy" are used, often interchangeably, in the literature on aging; their definitions are as follows: longevity usually means long life, but here longevity is used in the strict sense of the length of life of an individual (an animal or a human) who is still alive. Life span denotes the duration of time that an individual has lived, i.e., it can be determined only after the death of the individual. Life expectancy at birth, in contrast, is an actuarial term that means the average period that an individual may expect to live. Thus, while longevity and life span are accurate biological parameters, life expectancy is an abstract based on demographic data.

Accordingly, the term life expectancy at birth is rarely used in animal research, which usually gauges the effects of the environment and genes on aging by the mean survival or mean life span.

The roles of the environment and genes, of course, also figure in the longevity and life span of humans and to understand these roles researchers have often resorted to animal models, freely and broadly extrapolating from them to the human condition. No doubt, animal models are indispensable for understanding biological principles, and this chapter relies heavily on basic animal research for its overall thesis. However, variations in life span exist both among species and within a given species, and genes that affect the life span of the individual in one environment might not exert the same effect in a drastically different environment. Variant genes that bolster the survival of a mouse in the laboratory may be less relevant for a Masai in East Africa, and those gene variants that ostensibly increase longevity of a Masai may diminish longevity of an Inuit in the Arctic Circle. Adaptive mutations in human mitochondrial DNA to colder climates and their impact on longevity and disease in modern humans exemplify this concept (Ruiz-Pesini et al., 2004).

The first direct evidence that human genes are intertwined with the environment was the discovery more than half a century ago that sickle cell anemia stems from a single mutation that alters the structure of the β-globin gene (Ingram, 1957). This mutation provides a survival advantage to individuals that harbor the sickle-cell trait because it increases resistance to malaria—an endemic disease in regions in which variants of the β-globin gene are common (Williams, 2006). It follows that to understand human longevity fully, one must study humans of different racial extractions and origins under different geographic and demographic circumstances. That is a tall order!

Furthermore, the outliers of human longevity—the centenarians—have been the focus of intensive research in an attempt to gain a better insight into so-called successful human aging (Suh et al., 2008). Centenarians may indeed enlighten us about the upper limit of the human life span, but to understand longevity in the general population, it would be more insightful to study ordinary mortals. Suppose that in a given population 1 in 5000 individuals is a centenarian and that all centenarians display a variant gene X that explains their exceptional longevity. What then are the odds that the same variant gene also explains longevity in the general population? If the gene variant is common enough to explain longevity in general, it is unlikely to account for the exceptional longevity of the centenarians. With regard to environmental effects and exceptional longevity in humans, recent reports suggest that caloric restriction, for instance, improves metabolic and cardiovascular indices and perhaps increases longevity not only of rodents but also of nonhuman primates (Colman et al., 2009) and humans (Holloszy & Fontana, 2007). Still, most individuals who adapt a healthy lifestyle, including curtailing caloric intake, will not defy the odds and live to celebrate their century birth date, not in this generation and perhaps not in the next one.

For multiple reasons, humans now live longer and are healthier than ever before. However, in reality, the human life span is unlikely to increase indefinitely, unless researchers start tinkering with our genetic makeup. The increased life expectancy humans have experienced in the past 2 centuries is indeed remarkable and it stems from multiple factors, all of which relate in one way or another to the environment. However, predictions that in the not-too-distant future, a large segment of modern humans will live for 10 decades or more (Christensen et al., 2009; Oeppen & Vaupel, 2002) might be imprecise. These predictions are based on extrapolations from past trends of life expectancy, but in the past, longevity of most persons did not collide with the life-span boundary of the human species. It is improbable that a laboratory mouse that on average lives 2 years will survive for 4 years, no matter how much we engineer its environment. Jeanne Calment lived to the age of 122 years (Coles, 2004), but Ms. Calment was clearly an extreme outlier that epitomized the maximal human life span. Accordingly, the ultimate question, yet to be answered, is: What is the life-span boundary for most ordinary mortals and what are its determinants? As will be discussed below, from the biological perspective, there must be an ultimate boundary to the life span of all mammals no matter what life expectancy trends of humans during modern times seem to tell us based on purely statistical considerations (Oeppen & Vaupel, 2002). Though this boundary might be constantly modified by evolution, it is very unlikely that the remarkable increase in human life expectancy during the previous 2 centuries is the outcome of natural selection.

A number of hypotheses have attempted to explain the evolutionary origin of aging with a view to understanding variations in life span among species (Ljubuncic & Reznick, 2009). Evolution, however, has repeatedly humbled efforts to make sense of why we age. Still, a worthwhile question that we might ultimately be able to answer is how we age. As mammals are molded by the function of multiple tissues and organs, we need to find out the most common cause of death of a mammalian species, since such a cause might point to the system that largely defines the average life span of that species. The causes of death of most feral mammals are predation, starvation, and other environmental events, but in domesticated mammals the cause of death often depends on deliberate human intervention. The death of pets and zoo animals is often due to true "old age," but the main cause of death of a cow or a chicken is the butcher.

Humans die from a variety of causes, but if they live long enough, their main cause of death stems from CVD, principally atherosclerosis. In 2005, for all ages, death due to heart disease ranked the highest, at 26.6% of the total mortality in the U.S. population; malignant neoplasms came in second at 22.8% (U.S. Census Bureau, 2009). However, when cerebrovascular disease and diabetes, the complications of which primarily affect the heart and the vasculature, are factored into the mortality statistics, in 2005, death in the United States from CVD was 35.5% of total mortality. But these statistics tell only part of the story. Between the ages of 65 and 74 years, heart disease, cerebrovascular diseases, and diabetes accounted for 41.4% of total mortality, and death from malignant neoplasms was 34.7%. In individuals older than 85 years, death from heart disease, cerebrovascular disease, and diabetes combined was 47.2% of all causes, while that from malignant neoplasms was only 11.9%.

Clearly, CVD, and particularly atherosclerosis, is the outstanding cause of death of Americans and, presumably, individuals from other nationalities, provided that they live long enough. Thus, the aging of the cardiovascular system is the factor most likely to curtail the life span of modern humans. What then is the reason for the central role of the cardiovascular system in human aging and presumably life span, and could it relate to telomere biology?

TELOMERE SHORTENING, REPLICATION, AND EVOLUTION

The ability of the DNA molecule to replicate itself with fecundity and high, though never absolute, fidelity is a key feature of evolution. Nuclear DNA has a linear configuration and it cannot completely replicate itself because of the inability of DNA polymerase to synthesize the lagging strand of DNA to its terminus. Replication requires RNA primers, but there is no place for these primers to bind to at the end of the DNA strand. Were it not for telomerase, this phenomenon, referred to as the "end replication problem" (ERP) (Watson, 1972; Olovnikov, 1973), would have stopped evolution as we know it.

The ERP causes a constant loss of telomere repeats to a point at which telomere length reaches a critical threshold, setting off a cell cycle checkpoint that, in turn, stops replication (Hemann et al., 2001; Zou et al., 2004). The resulting permanent arrest of replication, i.e., replicative senescence—also termed the Hayflick limit (Hayflick, 1965)—is the ultimate outcome of telomere shortening in cultured somatic cells.

Telomere length is highly variable among chromosomes, between the p and the q arms of each chromosome, and among cells in the same preparation, even though they are of the same type (Martens et al., 1998). Thus, cultured cells do not experience simultaneously replicative senescence due to telomere shortening. Rather, the number of cells entering replicative senescence gradually increases as the shortest telomeres in different cells attain the critical length that signals cessation of replication.

Though replicative senescence is well documented in vitro, it is not a universal feature of aging in multicellular organisms. For instance, adult insects are largely postmitotic, in that most of their somatic cells do not appear to divide, yet insects do age and die. Moreover, replicative aging is not a major feature of postmitotic tissues such as skeletal muscle in much more complex organisms, including mammals; still, these tissues display an array of aging phenotypes. But does replication play a role in the aging of replicating tissues of mammals? Let us consider this question first by comparing telomere biology in the germ line with that in the soma.

In multicellular organisms that reproduce sexually, the male germ line displays robust telomerase activity and no evidence in sperm of telomere shortening with age (Wright et al., 1996; Fujisawa et al., 1998). In fact, sperm from older men display longer telomeres than those from younger ones (Kimura et al., 2008a; Baird et al., 2006; Allsopp et al., 1992). Little information is available about telomere length in human eggs. But given that oogenesis takes place only during fetal development, it is unlikely that telomeres become shortened with maternal age in human eggs (Keefe & Liu, 2009). In addition, robust telomerase activity is detected during embryogenesis (Wright et al., 1996). Collectively, these findings suggest that telomerase activity in the germ line and in utero offsets the ERP, thereby ensuring the survival of successive generations of multicellular organisms whose telomeres undergo shortening in replicating somatic tissues during extrauterine life. From the evolutionary perspective, the germ line reigns supreme, while the soma is dispensable (Kirkwood & Rose, 1991)—a concept that was indirectly put to the test in the telomerase knockout mouse.

COMPARATIVE TELOMERE BIOLOGY BETWEEN MOUSE AND HUMAN

The mouse is perhaps the most studied mammalian model of telomere biology in vivo. It is hence instructive to compare telomere biology of the mouse with that of humans. All mouse strains examined thus far have much longer telomeres than do humans, although recently domesticated mouse strains tend to have shorter telomeres than other strains that have been propagated for research purposes for approximately a century (Hemann & Greider, 2000). During extrauterine life, while somatic tissues in the mouse generally display telomerase activity, albeit at a level much lower than in utero (Prowse & Greider, 1995; Chadeneau et al., 1995; Gardner et al., 2007), little or no telomerase activity is detected in most human somatic tissues,

Telomere dynamics (birth telomere length and its age-dependent shortening) are probably not a major factor in the life span of wild-type mice, because during the relatively short life span of these animals their telomeres do not become critically short and thereby impede somatic cell replication (Hemann & Greider, 2000). It is noteworthy, however, that this assertion is based on observations that relate to the mean telomere length. Telomere biology might still play a role in the life span of a mouse if its shortest telomere reaches the critical threshold that brings about replicative senescence in subsets of cells. In addition, telomere dynamics may impact aging and life span in the mouse and perhaps other organisms not only through replicative senescence but also by exerting a "telomere position effect." At least in cultured cells, telomere shortening changes the expression of genes in the subtelomeric region, which might affect aging and life span (Baur et al., 2001).

Even though telomere length in somatic tissues of most mice might not be calibrated for a finite number of cell replications during their life span, the telomerase knockout mouse model is proof of the concept that telomere length imposes an ultimate boundary on the life span of perhaps all mammals. Telomerase comprises two major components: the catalytic subunit (TERT) and the RNA component (TR), which in the mouse are designated mTERT and mTR, respectively (Blackburn, 2005; Chan & Blackburn, 2004). A transgenic mouse in which mTR was knocked out from the germ line was generated in the 1990s (Rudolph et al., 1999; Lee et al., 1998; Herrera et al., 1999). Because the telomere length in the original strain used to generate the $mTR^{-/-}$ mouse was long, six generations (G's) of telomerase knockout mice were necessary to establish animals with critically shortened telomeres. Proliferation of somatic cells, including hematopoietic cells, of the G5 and G6 of $mTR^{-/-}$ mice was compromised by cellular growth arrest and apoptosis. These animals also exhibited signs of premature aging and diminished life span. Importantly, the G6 was infertile. The lesson from the $mTR^{-/-}$ is this: whereas telomere shortening in the soma imposes a boundary on the life span of the individual animal, telomere shortening in the germ line would ultimately cause the extinction of the species.

It is noteworthy that both telomere length and telomerase were shown to exert independent effects on health and survival of genetically engineered mice. For instance, CAST/Eij mice have relatively short telomeres (~15 kb) compared to other mouse strains. CAST/Eij $mTR^{+/-}$ mice display diminished survival and characteristics of hematopoietic compromise, expressed in immune senescence (Armanios et al., 2009). Accordingly, in this mouse strain, shortened telomere length rather than the absence of telomerase activity accounts for the aging-related phenotype. In contrast, the constitutive overexpression of TERT in mice that were engineered to be cancer resistant delayed aging-related phenotypes and increased survival (Tomas-Loba et al., 2008). These findings emphasize the importance of overall genetic makeup in the ultimate impact of telomere dynamics on aging and aging-related diseases in the individual animal.

WHY DO HUMANS HAVE RELATIVELY SHORT TELOMERES?

From the biological perspective, an answer to a "why" question is not as straightforward as it may seem. Only evolution could tell us why an organism is endowed with one trait or another. But on many occasions we have failed to figure the evolutionary process correctly. We have already established that, if not for telomerase activity in the germ line, the ERP would have impeded the evolutionary survival of multicellular organisms because of infertility. But can evolutionary pressure foster short telomeres in one species vs long telomeres in another? Large bodies and long lives entail higher numbers of cell replications, which serve not only to build tissues and organs but also to sustain them throughout life. More cellular replications increase the risk of spontaneous (acquired) mutations, which might ultimately trigger cancerous transformation. Short telomeres and suppression of telomerase activity in somatic tissues during extrauterine life could hence function as fail-safe mechanisms that forestall the development of cancer (O'Brien et al., 1986; Wright & Shay, 2002; Seluanov et al., 2008). At the same time, in the presence of inadequate DNA damage response, telomeres that are too short could also bring about "genomic instability," a phenomenon that promotes the development of cancer by mechanisms that circumvent the telomere barrier. These might include telomerase activation. Thus, the anticancer properties of relatively short telomeres might hinge upon a delicate balance between "short" and "too short" telomere length.

As evolutionary forces primarily operate during the reproductive years, by early adulthood, the telomeres of most humans probably do not reach the "critical length" that risks cancerous transformation. But in reality, we do not know what that critical length of telomeres might be. Moreover, as telomere length varies among chromosomes, the vital question is not so much "What is the mean telomere length that causes genomic instability?" as "What is the length of the shortest telomere among all chromosomes that brings about genomic instability?"

THE END REPLICATION PROBLEM VS OXIDATIVE STRESS

Most of the knowledge about telomere shortening with replication has been derived from studies in cultured cells. The artificial conditions of growth media are very different from the in vivo environment. For this reason, we can only surmise the number of nucleotides that are clipped with each cell division in vivo because of the ERP. Theoretical considerations suggest, however, that though all-pervasive, the ERP accounts for only a portion of telomere shortening (perhaps fewer than 15 nucleotides) per replication and that a major influence on this process is exerted by oxidative stress (op den Buijs et al., 2004, 2007; Proctor & Kirkwood, 2002). It is noteworthy that interspecies variation in sensitivity of cultured cells to oxidative stress may account for a constellation of phenotypic differences that distinguish, for instance, the growth in culture of mouse cells compared with that of human cells (Parrinello et al., 2003; Itahana et al., 2004). However, telomeres from all mammals share sensitivity of their G triplets to hydroxyl radicals. Artificial growth media and the high ambient oxygen used to cultivate cells provide a milieu rich in reactive oxygen species. Accordingly, the bulk of telomere shortening in cultured human cells (≈50–200 nucleotides per replication) probably stems from oxidative stress.

As longer telomeres provide a bigger target, oxidative stress should cause telomere shortening that is proportional to telomere length. That is, longer telomeres should shorten more quickly than shorter telomeres. The extent of telomere shortening that is due to oxidative stress in vivo is unknown, but indirect evidence suggests that it is substantial. Consider, for instance, telomere length of the p arm of a given chromosome (P_{chr}) and the mean telomere length of all chromosomes (M_{all}). As the ERP elicits a constant loss of telomere repeats with each replication from the two ends of each chromosome and as telomere length is highly variable among chromosomes, the $P_{\text{chr}}/M_{\text{all}}$ would change with successive replications if telomere shortening were solely the outcome of the ERP. However, the $P_{\text{chr}}/M_{\text{all}}$ is apparently constant in ex vivo leukocytes and sperm donated by individuals of a wide age range (Britt-Compton et al., 2006; Kimura et al., 2007). In addition, longitudinal observations of age-dependent telomere shortening in leukocytes indicate that telomere shortening is proportional to telomere length (Aviv et al., 2009). Taken together, these findings suggest that oxidative stress exerts a major effect on telomere shortening in vivo.

TELOMERE DYNAMICS IN HUMAN LEUKOCYTES

Leukocytes have been the main model of telomere dynamics in clinical and epidemiological settings. The original studies examining telomere parameters in leukocytes probably used these cells because they are readily available. It turns out that despite their heterogeneity, leukocytes might be the most appropriate cells to test hypotheses that link telomere dynamics with human aging, at least the type of aging experienced by humans in modern societies.

Consider, for instance, telomere dynamics in the liver. Apoptosis or necrosis of hepatocytes may stimulate liver regeneration by accelerating the proliferation of precursor cells or mature hepatocytes. As somatic cells of adult humans lack sufficient telomerase activity to prevent completely telomere attrition with replication, relatively short telomere length is observed in tissue specimens from livers that exhibit an increase in the turnover of hepatocytes because of disease or injury, e.g., infection and toxic chemicals (Lechel et al., 2004). If these afflictions entail increased oxidative stress, telomere shortening in hepatocytes might also arise from an increase in the loss of telomere repeats per replication (Sekoguchi et al., 2007). Therefore, telomere shortening in the liver over and above that resulting from "normal" liver aging would provide clues about the disease history of the liver. Whether we consider aging as the integrated outcome of aging-related diseases or an independent process, relatively shortened hepatocyte telomere length might reveal information about the disease/aging status of the liver, but it would tell us nothing about the systemic (general) aging of the individual whose liver is being examined. As will be discussed below, this systemic aging largely stems from the aging of the vasculature.

Oxidative stress and inflammation are at the center of present paradigms of aging-related diseases (Van Bodegom et al., 2007; Hulbert et al., 2007), including CVD (Libby, 2007; Gutierrez et al., 2006). Oxidative stress burden might be recorded by telomere shortening in any replicating somatic cells but the inflammatory burden can be registered only by telomere dynamics of leukocytes. LTL at any age is a measure of the individual's LTL at birth and its shortening afterward. The LTL at birth minus the LTL at a given age, i.e., the amount of telomere shortening since birth, is hence an index of the cumulative burden of oxidative stress and inflammation at the systemic level over the individual's life since birth. This is because chronic inflammation entails a decline in the life of peripheral leukocytes, which would stimulate the replication of hematopoietic progenitor cells (HPCs) and in turn hematopoietic stem cells (HSCs). Increased rate of HSC replication would accelerate LTL shortening, a phenomenon that might explain, in part, the shortened LTL in a host of chronic, aging-related disorders that are marked by an increase in the systemic burden of oxidative stress and inflammation.

In this context, LTL shortening after birth is a record of the replication kinetics of HSCs. During growth and development, HSCs divide symmetrically, one HSC giving rise to two HSCs, to expand the HSC pool. They also divide asymmetrically, one HSC giving rise to another HSC and a more differentiated HPC, to expand the HPC pool (Morrison & Kimble, 2006; Attar & Scadden, 2004). These pool expansions are necessary to keep pace with the growing soma. Consequently, HSC replication rate and, therefore, LTL shortening, are probably proportional to growth rate, slowing down during adulthood (Sidorov et al., 2009).

In the final analysis, the rate of LTL shortening reflects the rate of telomere shortening resulting from HSC replication. An increase in the systemic burden of oxidative stress might enhance telomere shortening in HSCs in several ways, including: (a) increasing telomere loss per division of HSC; (b) diminishing the life of cells down the hematopoietic system hierarchy, thereby increasing the demand on the HPCs and ultimately on the HSCs to divide in order to sustain the HPC pool; and (c) diminishing the life of HSCs themselves by telomere-independent mechanisms, which would stimulate further division of the remaining HSCs. However, oxidative stress is probably one of many variables, including intrinsic factors and local cues in the HSC compartment, that might impact the life span of HSCs (Waterstrat & Van Zant, 2009) and consequently LTL shortening.

Leukocytes comprise cells of different lineages, different behaviors, and different functions. For instance, granulocytes (neutrophils, eosinophils, and basophils) are postmitotic cells with a short biological life. In contrast, lymphocytes are capable of proliferation and their biological life might vary considerably, with clones of memory cells surviving for many decades in the circulation. These leukocyte subsets display relatively small variations in telomere length among themselves compared with those observed among individuals, which amount to as much as 4–6 kb at birth (Okuda et al., 2002; Akkad et al., 2006). Though LTL represents the mean length of telomeres in all leukocyte subsets, its overall dynamics mirror those of telomeres in HSCs (Sidorov et al., 2009). It is this feature of LTL that might account for its associations with aging-related diseases and longevity in humans.

LEUKOCYTE TELOMERE LENGTH DYNAMICS: THE GENDER AND RACE EFFECTS

At birth, LTL is equivalent in boys and girls (Okuda et al., 2002; Akkad et al., 2006) but age-adjusted LTL is longer in adult women than in men (Bekaert et al., 2007; Hunt et al., 2008; Jeanclos et al., 2000; Vasan et al., 2008; Nawrot et al., 2004; Fitzpatrick et al., 2007; Benetos et al., 2001). It follows that with age, the loss of telomere repeats from HSCs of females is less than that of males. Potential explanations for the gender difference include: (i) as women are generally smaller than men, their HSCs would undergo fewer divisions than those of men to expand the HSC and HPC pools during somatic growth (Sidorov et al., 2009); (ii) the promoter region of the catalytic subunit of telomerase harbors an estrogen response element; thus, estrogen might stimulate telomerase (Kyo et al., 1999; Breitschopf et al., 2001; Kang et al., 1999) in HSCs or more differentiated hematopoietic cells to attenuate age-dependent LTL shortening in women; (iii) endogenous estrogen displays antioxidant and anti-inflammatory properties, which might attenuate LTL shortening (Mooradian, 1993; Reckelhoff, 2006; Massafra et al., 2000; Sack et al., 1994). The question then is: might the longer LTL in women explain the approximately 6-year greater life expectancy in women than in men (World Population Data Sheet, 2007; also see Chapter 23) in modern societies? This question lingers unanswered. Interestingly, the gender gap in LTL is about 100–200 nucleotides and the pace of LTL shortening during adulthood is approximately 20–30 nucleotides per year. In telomere-year equivalence, this difference amounts to about 5 years.

African-American and white newborns appear to have the same LTL (Okuda et al., 2002), but by the age of 30 years African Americans show a longer LTL than do whites (Hunt et al., 2008). The following are two potential explanations for this phenomenon. First, individuals of recent African ancestry display "physiological neutropenia" that is attributed to a decrease in the production of neutrophils in the bone marrow, meaning less replication of HSCs (Mayr et al., 2007; Phillips et al., 2000; Haddy et al., 1999; Bain et al., 2000). Second, African Americans rely less than whites on angiotensin II (Price & Fisher, 2003; Sagnella, 2001), a powerful proinflammatory and pro-oxidant, to maintain their blood pressure (Mehta & Griendling, 2006). Exposure to lower levels of angiotensin II might attenuate LTL shortening (Vasan et al., 2008), at least by early adulthood. That being said, LTL shortening during adulthood is faster in African Americans than in whites, so that by the age of 80 years the racial gap in LTL practically disappears (Hunt et al., 2008).

LEUKOCYTE TELOMERE LENGTH AND ATHEROSCLEROSIS

Atherosclerosis is an aging-related disease that is marked by chronic, low-grade inflammation and increased oxidative stress (Libby, 2007; Gutierrez et al., 2006). Individuals with atherosclerosis are more likely to have a shorter LTL than their peers (Benetos et al., 2004; Brouilette et al., 2003, 2007; Cawthon et al., 2003; Samani et al., 2001; van der Harst et al., 2007; O'Donnell et al., 2008, reviewed in Samani & van der Harst, 2008). Likewise, individuals with risk factors for atherosclerosis, including obesity (Hunt et al., 2008; Vasan et al., 2008; O'Donnell et al., 2008; Valdes et al., 2005; Gardner et al., 2005), sedentary lifestyle (Cherkas, 2008), insulin resistance (Gardner et al., 2005; Demissie et al., 2006), and smoking (Vasan et al., 2008; Nawrot et al., 2004; Valdes et al., 2005) also tend to have shorter LTL. It is well established that women, principally premenopausal women, have less atherosclerosis than men. However, less appreciated are the epidemiological data of lower prevalence and severity of coronary atherosclerosis in African Americans compared to Caucasian whites (McClelland et al., 2006; Loria et al., 2007; Tang et al., 1995; Aiyer et al., 2007; Detrano et al., 2008). The longer LTL in women compared to that in men is in line with the lower predilection of women, particularly premenopausal women, to atherosclerosis. But most of the associations between indices of atherosclerosis and LTL are based on observations made in whites and little is known about the LTL–atherosclerosis link in African Americans.

The LTL–atherosclerosis connection has been attributed to the increased demand on the HSCs to divide by inflammation and the greater loss of telomere repeats per division that arises from a chronic increase in oxidative stress. Based on this premise, HSC dynamics should have no active role in the pathogenesis of atherosclerosis. However, LTL is a measure not only of telomere shortening after birth but also of LTL at birth, which is highly variable among newborns (Okuda et al., 2002; Akkad et al., 2006). It is unlikely, therefore, that HSCs of all adults who display shortened LTL experience an accelerated telomere shortening after birth. Other factors might explain the LTL–atherosclerosis connection. One of these relates not to the injurious effect of inflammation and oxidative stress but to the repair that attenuates the progression of atherosclerosis. And this repair apparently depends on the HSC reserves, which are expressed in the absolute value of LTL rather than the rate of LTL shortening.

Atherosclerosis is largely a systemic disease of the vascular endothelium (Ross, 1999) and it starts in utero (Napoli et al., 1997). The vascular endothelium is a systemic organ, because it is part of the vascular network supplying blood throughout the body. For obvious reasons, the hematopoietic system is also a systemic organ. Recent studies have shown that both systems originate from a common precursor, referred to as the hemogenic endothelium (Eilken et al., 2009; Chen et al., 2009; Lancrin et al., 2009; Yoshimoto & Yoder, 2009), which gives rise not only to vascular endothelial cells but also to HSCs. The embryonic connection between HSCs and the vascular endothelium might be crucial in understanding the role of telomeres in the repair of vascular damage inflicted by atherosclerosis. The repair task is relegated to endothelial progenitor cells (EPCs).

EPCs originate from the bone marrow, circulate in the blood, and incorporate themselves into the vascular wall, where they evidently mend the injured endothelium. The numbers of circulating EPCs and their function, including replicative potential—expressed in colony-forming units—are diminished in patients with atherosclerosis (Werner & Nickening, 2007; Schmidt-Lucke et al., 2005; Hill et al., 2003; Werner et al., 2005; Kunz et al., 2006; Fadini et al., 2006a,b). Moreover, the numbers of EPCs, their functions, or both decline with age (Kunz et al., 2006; Fadini et al., 2006a,b; Hoetzer et al., 2007a,b; Heiss et al., 2005; Xiao et al., 2007) and are relatively diminished in men compared to women (Xiao et al., 2007; Hoetzer et al., 2007b) and in individuals at risk for atherosclerosis because of insulin resistance (Cubbon et al., 2007; Fadini et al., 2006b; Murphy et al., 2007), sedentary lifestyle (Van Craenenbroeck et al., 2008; Steiner et al., 2005), and smoking (Michaud et al., 2006; Hoetzer et al., 2007b). These are the individuals and circumstances associated with shortened LTL. Since LTL is a stand-in gauge of telomere length in HSCs, the obvious question is whether telomere dynamics in HSCs might be linked to the number and function of EPCs. The answer is in the affirmative, as recent studies indicate that diminished EPC numbers and function might arise from shortened telomere length (Satoh et al., 2008; Oeseburg et al., 2007; Imanishi et al., 2005). In fact, shortened telomeres might impact HSC reserves (Ju et al., 2007), which are defined as the ability of HSCs to provide enough numbers of adequately functioning cells of various lineages to sustain homeostasis.

The focus on peripheral leukocyte telomere dynamics as a model of HSC kinetics is in large measure due to the fact that leukocytes have nuclei. However, erythrocytes are far more numerous than leukocytes, meaning that erythrocyte homeostasis might also exact a large demand on HSCs. Accordingly, the cardinal sign of exhaustion of HSC reserves due to either genetic or environmental factors that drastically inhibit telomerase activity is aplastic anemia (Calado & Young, 2008). But in the general population, a less profound strain on the HSC reserves might then be expressed by a gradual decline in erythrocytes. A 2006 study suggested that this might be the case, in that it showed a positive correlation between LTL and erythrocyte count in a large cohort of middle-aged persons (Bischoff et al., 2006). However, further research is needed to confirm this finding.

As EPCs originate in the HSC pool, the decline in their number and function with age and atherosclerosis might stem from diminished HSC reserves. Accordingly, the LTL–atherosclerosis connection is probably more complex than initially thought. Shortened LTL may arise from shortened HSC telomere length at birth, an accelerated telomere shortening in HSCs after birth, or both. But regardless of its etiology, shortened telomere length in HSCs, expressed in shortened LTL, suggests diminished HSC reserves. This would be manifested in reduced numbers of EPCs. In addition, EPCs derived from HSCs with shortened LTL might also express diminished replicative potential. In this way, as per findings in cultured somatic cells, telomere length in HSCs is an index of not only the replicative history but also the replicative potential. The ramifications of this tenet, if true, are substantial, as they suggest that telomere dynamics in HSCs play an active role in atherosclerosis. That role might provide a connection between telomeres and the life span of modern humans.

HEMATOPOIETIC STEM CELL TELOMERE DYNAMICS AND THE HUMAN LIFE SPAN

The etiology of aging is as mystifying now as it was several decades past, but in the final analysis, telomere dynamics are algorithms, namely, the tempo of telomere shortening is not random. Rather it follows a set of rules that are fashioned by genes, the impact of which is modified by the environment. The relentless influence of these genes starts at birth, or perhaps conception, and it ultimately leads to an outcome—replicative senescence. By endowing humans with relatively short telomeres and suppressing telomerase in their somatic tissues during extrauterine life, evolution might have made it possible for humans to resist cancer in the early phase of their life at the expense of limiting their life span to approximately 8–10 decades. Accordingly, based on our present knowledge of LTL epidemiology, the following paradigm is proposed for human longevity: in absolute terms, LTL is an index of the HSC reserves at any given time in the human life span. LTL at birth is one of a host of factors that define the oldest age that might be attained by an individual. However, the amount of LTL shortening after birth is an indicator of whether this landmark will ever be reached.

Expressed in an accelerated rate of LTL shortening with age, a chronic increase in oxidative stress and inflammation may tax the HSC reserves—a process that would ultimately hasten the individual's demise. Still, those persons who inherit relatively short telomeres, expressed in shortened LTL at birth, might be destined to a shorter life span even though they experience less oxidative stress and inflammation in the course of their lives than others who are endowed with a longer LTL at birth. This paradigm presupposes, therefore, that LTL would be associated not only with atherosclerosis but also with the life span of those individuals who have survived this disease and other aging-related disorders, namely, the elderly. Earlier studies provided conflicting results on this matter (Cawthon et al., 2003; Bischoff et al., 2006; Martin-Ruiz et al., 2005; Harris et al., 2006; Honig et al., 2006). However, more recent works, examining same-sex elderly twins, have established that indeed the co-twin who had the shorter LTL was more likely to die first (Kimura et al., 2008; Bakaysa et al., 2007). What is more, the shortest telomeres in the LTL distribution were more predictive of mortality than the mean telomere length (Kimura et al., 2008).

RAMIFICATIONS

From the standpoint of HSCs, what then is the role of telomeres in the biology of human aging? Is it passive or active? The answer is probably both, which is why the meaning of associations of LTL with aging-related diseases and longevity—the crux of the debate about telomeres and human aging—is often so hard to figure out. Furthermore, diverse and often conflicting findings have sapped the initial enthusiasm generated by studies that explored the links of LTL with aging-related diseases and life span in humans, e.g., the now resolved controversy regarding the relationship of LTL with survival in the elderly (Cawthon et al., 2003; Bischoff et al., 2006; Martin-Ruiz et al., 2005; Harris et al., 2006; Honig et al., 2006). The conflicting findings probably relate to a poorly designed and inadequately powered subset of these studies, some of which might have employed questionable methods to measure telomere length in epidemiological/clinical settings (Aviv, 2008).

Ultimately, three criteria will determine the value of LTL in medical practice: (i) whether LTL can help in predicting susceptibility to disease or assessing the individual's health status, in general; (ii) whether the knowledge about LTL can guide therapy through drugs or behavioral modifications to improve the individual's health status, e.g., attenuate the progression of atherosclerosis; and (iii) whether LTL can be measured with accuracy and reproducibility and at an affordable cost in clinical settings. At present, there is no consensus on which method of telomere length measurement meets all the requirements of the third criterion. That is the first challenge that needs an immediate resolution.

We might also make use of LTL to identify a subset of variant genes that determine human aging. Given the exceedingly complex nature of aging, there is but a slim chance of discovering these genes in the general population by resorting to the standard and somewhat simplistic recipes that attempt to link phenotypes of aging with genotypes. Phenotypes of aging are many steps removed from the variant genes that contribute to their characteristics, which often are poorly quantifiable and might be influenced by environmental factors. In contrast, telomeres are considerably more proximal to the variant genes than the aging phenotypes and their length can be measured with relative accuracy (at least in research settings). Therefore, using LTL as an intermediate phenotype might facilitate identifying networks of genes that are engaged in telomere dynamics at the interface of the hematopoietic and vascular systems, as these systems develop, mature, and age (Schadt, 2009).

Finally, matters of methodology of telomere length measurements have not curbed the development of a new industry that has sprung up to cash in on the recent advances in telomere biology and epidemiology. For a handsome cost, for-profit entities now offer directly to consumers the service of measuring their LTL and sell them supplements that allegedly attenuate telomere length shortening through the stimulation of telomerase. This trend is unhealthy, as it might subject gullible individuals to useless tests and costly potions. More ominously, compounds in these supplements might be hazardous if they were able to stimulate telomerase, since we know little about the consequences of telomerase activation in humans. Regardless of the ability of these supplements to lengthen telomeres or slow down their attrition (in what cells?), their use may increase cancer risk.

CONCLUSION

As the paradigm laid out in this chapter is based on empirical observations that link telomere biology to the human life span, it unfortunately cannot establish causality. In that light, only genetically engineered mouse models under- or overexpressing telomerase activity have thus far demonstrated a causal link between telomere length and life span. In this link there lurks the answer to what lies ahead for any attempt to reverse aging-related diseases and increase human longevity without factoring telomere biology into the life span equation.

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Chapter 8

An Objective Appraisal of the Free Radical Theory of Aging

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CHAPTER CONTENTS

Introduction 177
Antioxidant Supplementation Extends Life Span Only in Relatively Short-Lived Mouse Strains 178
- Vitamin E 178
- Vitamin C 182
- Mixed Antioxidant Supplementation 183
- Coenzyme Q_{10} 184
- Other Studies Involving Antioxidant Supplementation and Life Span 184
  - α-Lipoic Acid 184
  - Glutathione 185
  - Strawberry Extract and Melatonin 185
  - β-Carotene 185
  - Creatine 185
  - Tetrahydrocurcumin 185
  - Carboxyfullerene Superoxide Dismutase 186
  - PBN 186

Effects of Antioxidant Enzyme Overexpression on Life Span in Mice 186
- Cu,Zn-SOD 186
- Mn-SOD 187
- Catalase 188
- Glutathione Peroxidase 4 189
- Thioredoxin 1 189
- Combined Antioxidant Overexpression Studies 189

The Effect of Antioxidant Enzyme Reduction on Mouse Life Span 189
- Cu,Zn-SOD 190
- Mn-SOD 191
- Catalase, Glutathione Peroxidase 1, and Peroxiredoxins 191
- Phospholipid Glutathione Peroxidase 4 191
- Thioredoxin and Thioredoxin Reductase 192
- Combined Antioxidant Enzyme Knockouts 192

Knockouts of Oxidative Damage Repair Enzymes 192
- Methionine Sulfoxide Reductase 192
- Ogg1 193

Facts and Artifacts of Oxidative Damage Markers and their Relation to the Free Radical Theory of Aging 193
Synopsis, Conclusions, and Perspectives 195
References 196

INTRODUCTION

The free radical theory of aging was proposed by Denham Harman a little over 50 years ago (Harman 1956, 1972). In essence, he proposed that the cumulative damage by oxygen free radicals is the major driver of aging. Since then, a large body of indirect evidence, from phylogenetic studies to examinations of calorically restricted or dwarf mice, has been generated that can be viewed as consistent with the free radical theory of aging. In fact, the theory has

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enjoyed consistent support, despite the fact that very little *direct* evidence actually supports it.

Most investigators would agree that the free radical theory makes two straightforward predictions: (1) increasing antioxidant defense should decrease steady-state levels of oxidative damage, which would then increase life span; and (2) reduced antioxidant defense should increase steady-state levels of oxidative damage, which would then shorten life span. The first point has been tested by initial studies of antioxidant feeding and more recently in mice overexpressing antioxidant enzymes. Regarding the second point, a number of genetic alterations in antioxidant genes have been made in mice, and the effects of these on life span and on aging-related pathology have been considered. In this chapter, we review the current evidence in support of these two predictions with respect to the impact of antioxidant feeding and genetic manipulation of antioxidant enzymes in mouse models of aging. We also provide summaries of a large number of studies evaluating the free radical theory of aging in three detailed tables. In Table 8.1 we have included a summary of the role of antioxidant feeding in life span. Table 8.2 outlines the results of studies measuring the role of transgenic antioxidant enzyme overexpression in life span. Finally, in Table 8.3 we summarize the role of antioxidant enzyme ablation in life span. In this review, we present evidence that supports the idea that the simplest interpretation of the free radical theory is not valid and that oxidative damage (unless very high) has no direct influence on murine life span. However, it may be premature to say that the notion that oxidative damage is not life-span-limiting has not been proven conclusively, given some of the uncertainties of measuring oxidative damage in vivo.

ANTIOXIDANT SUPPLEMENTATION EXTENDS LIFE SPAN ONLY IN RELATIVELY SHORT-LIVED MOUSE STRAINS

Dietary supplementation with exogenously added antioxidants would be expected to support the endogenous antioxidant defense system, to reduce oxidative damage, and potentially, to increase life span. This section will evaluate the effects of various antioxidants, such as vitamin E, vitamin C, coenzyme Q_{10}, β-carotene, α-lipoic acid, creatine, glutathione, strawberry extract, melatonin, tetrahydrocurcumin, and carboxyfullerene superoxide dismutase, and *N-tert*-butyl-α-phenylnitrone (PBN) on life span in mammalian systems. While a large number of these antioxidant feeding studies have been conducted, it is frequently unclear whether the antioxidant under study really resulted in reduction of in vivo oxidative stress. This is especially a concern given what is now known about the pro-oxidant effects of most antioxidants when supplied at high doses. Table 8.1 provides a review of the studies used to examine the role of antioxidant supplementation on life span.

Vitamin E

Vitamin E (α-tocopherol) was discovered in 1922 by Evans & Bishop (1922) as a necessary dietary factor for reproduction in rats. Since then, vitamin E has been shown to be the major lipid-soluble, peroxyl radical-scavenging antioxidant in human plasma (Ingold et al., 1987) and is important in maintaining the integrity of long-chain polyunsaturated fatty acids in the membranes of cells (Traber & Atkinson, 2007). Vitamin E supplementation has been reported not to improve, or to have no effect on, health outcomes and life span, in humans. In patients age 55 years or older with vascular disease or diabetes mellitus, long-term vitamin E supplementation did not prevent cancer or major cardiovascular events (the mean follow-up time was 7 years) and was shown to have the potential to increase the risk for heart failure (Lonn et al., 2005). Serum levels of vitamin E and markers of oxidative stress or damage were not measured by Lonn and colleagues (2005). Similarly, vitamin E given twice a day to women did not reduce the risk of cardiovascular disease, cancer, or mortality (Lee et al., 2005). Again, serum vitamin E and markers of oxidative damage levels were not measured, making the association between vitamin E intake and health outcomes difficult. Roberts and colleagues (2007) have shown that vitamin E supplementation in excess of 1600 IU/day is necessary to reduce levels of plasma oxidative stress. It is important to note that the studies of both Lonn and colleagues (2005) and Lee and colleagues (2005) used 400 IU/day and 600 IU twice daily, respectively, which may explain the absence of a positive effect on health outcomes. Longitudinal assessment of the effect of vitamin E supplementation on human life span is impossible. Thus, rodent models are useful for answering this question.

Vitamin E is the most studied exogenously added dietary antioxidant with respect to life span in rodents. Wistar rats fed vitamin E were found to have a 24% increase in average, but not maximal, life span (Porta et al., 1980). It is important to note that an increase in mean or median life span has been proposed to result from a delayed onset of disease, whereas an increase in maximal life span may be related to changes in the rate of aging (Wang et al., 2004). The serum vitamin E concentration was found to be ~2-fold elevated, relative to the controls, for the duration of the life span. Unfortunately, markers of oxidative stress and damage were not measured.

Table 8.1 Antioxidant supplementation and life span in mice

ANTIOXIDANT, DOSE	INCREASE IN BLOOD OR TISSUE (ANTIOXIDANT)?	REDUCTION OF OXIDATIVE STRESS OR DAMAGE?	INCREASED HEALTH SPAN OR LIFE SPAN?	SURVIVAL CURVE (*P*)	RODENT STRAIN OR HUMAN	REFERENCE
Vitamin E						
2 mg/g food	Yes	NM	Yes	<0.05	Wistar rat	Porta et al., 1980
5 mg/g food	Yes	Yes	Yes	<0.001	CD-1/UCadiz	Navarro et al., 2005
2.5 mg/g food	Yes	Yes	No	NS, NR	C3H/He and LAF_1	Blackett, 1981a,b
0.02, 0.4, 4 mg/g food	Yes	NM	No	NS, NR	Balb/c	Morley & Trainor, 2001
0.5 mg/g food (=17 × control dose)	Yes	NM	No	NS, NR	C57BL/6	Lipman et al., 1998
1.65 mg/g	Yes	No	NM	NM	C57BL/6	Sumien et al., 2003
400 IU (268 μg/day)	NM	NM	Inc. risk of heart failure	NM	Human	Lonn et al., 2005
600 IU (402 μg/day)	NM	NM	No	NM	Human	Lee et al., 2005
Vitamin C						
10 mg/g food	NM	NM	Yes	$0.01 < P < 0.05$	C57BL/6	Massie et al., 1984
NM	Yes	NM	Yes	Increasing [C], decreased mortality	Human	Enstrom et al., 1992
Mixed antioxidant						
Vitamin C, vitamin E, β-carotene, etc.	Yes	NM	No	NS, NR	Long Evans rats	Holloszy, 1998
Vitamin C, vitamin E, etc.	NM	NM	Yes	<0.002	C57BL/6 × SJL	Lemon et al., 2005
Vitamin C, vitamin E, etc.	NM	NM	Yes	<0.05	C57BL/6	Bezlepkin et al., 1996

(*Continued*)

Table 8.1 (Continued)

ANTIOXIDANT, DOSE	INCREASE IN BLOOD OR TISSUE (ANTIOXIDANT)?	REDUCTION OF OXIDATIVE STRESS OR DAMAGE?	INCREASED HEALTH SPAN OR LIFE SPAN?	SURVIVAL CURVE (*P*)	RODENT STRAIN OR HUMAN	REFERENCE
Royal jelly (0.005, 0.050, 0.5 mg/g food)	NM	Yes	Yes	<0.05	C3H/HeJ	Inoue et al., 2003
Coenzyme Q_{10}						
10 mg/kg bw	Yes (plasma)	NM	No	0.073 for rat; 0.24 for mouse	Sprague–Dawley and in C57BL/6	Lönnrot et al., 1998
100 mg/kg bw	NM	NM	No	0.086 (for a decreased life span)	C57BL/6 × C3H (B6C3F1)	Lee et al., 2004
93, 371 mg/kg bw	Yes	No	No	>0.065	C57BL/6	Sohal et al., 2006
α-Lipoic acid						
600 mg/kg bw	No	NM	No	NS, NR	C57BL/6 × C3H (B6C3F1)	Lee et al., 2004
Glutathione (GSH)						
5 mg/g food	No	No	No	NS, NR	C57BL/6	Lipman et al., 1998
GSH (5 mg/g food) + vitamin E (0.5 mg/g food)	Yes (vitamin E)	NM	No	NS, NR	C57BL/6	Lipman et al., 1998

Strawberry extract						
10 mg/g food	NM	NM	No	NS, NR	C57BL/6	Lipman et al., 1998
Melatonin						
0.011 mg/g food	Yes	NM	No	NS, NR	C57BL/6	Lipman et al., 1998
β-Carotene						
5 mg/g food	Yes	NM	No	>0.05	C57BL/6	Massie et al., 1986
Creatine						
10 mg/g food	Yes	Trend for less	Yes	<0.05	C57BL/6	Becker et al., 2008
Tetrahydrocurcumin						
2 mg/g food	NM	NM	Yes	<0.01	C57BL/6	Kitani et al., 2007
Carboxyfullerene SOD						
10 mg/kg bw/day	NM	Yes	Yes	<0.001	C57BL/6	Quick et al., 2008
PBN						
30 mg/kg/day	NM	NM	Yes	NR	SAM	Edamatsu et al., 1995
38 mg/kg/day	NM	NM	Yes	<0.01	C57BL/6	Saito et al., 1998
53 mg/kg/day	NM	NM	No	NS, NR	UM-HET3	Miller et al., 2007

Abbreviations: NS, not significant; NR, not reported; NM, not measured.

To date, no follow-up studies in Wistar rats have confirmed these data, but multiple studies in mice have examined the effect of vitamin E supplementation on life span. For example, mice of the CD-1/UCadiz strain were supplemented with vitamin E beginning at 7 months of age for the duration of their life span in a study by Navarro and colleagues (2005). Both male and female mice that were supplemented with vitamin E showed significant increases in median life span, but only males were found to have an increased maximal life span (Navarro et al., 2005). In addition, the maximal life span of the female mice used by Navarro and colleagues (2005) was ~17% greater than the maximal life span of the male mice, regardless of the presence or absence of vitamin E supplementation. The vitamin E content of brain and liver isolated from CD-1/UCadiz mice was reported to be 2.5- and 7-fold increased in the vitamin E-supplemented group, respectively. Age-related increases in protein and lipid oxidative damage in both brain and liver were significantly reduced in the vitamin E-supplemented group, relative to controls. These data are in agreement with the free radical theory of aging, as addition of the antioxidant vitamin E reduced oxidative damage and extended life span. However, mice from the CD-1/UCadiz strain are short-lived (average life span 15 months), relative to other mouse strains (C3H/He mice, 22 months, Inoue et al., 2003; LAF1 mice, 27 months, Blackett & Hall, 1981a; C57BL/6, 31 months, Perez et al., 2009a,b). Although these data show that oxidative stress can be life-span-limiting in a relatively short-lived strain of mouse, we believe the strongest evidence in support of the oxidative stress theory of aging would be if antioxidant supplementation could extend the life span of long-lived animals.

Mice of the C3H/He and LAF1 strains are both longer lived than mice of the CD-1/UCadiz strain (Blackett & Hall, 1981a; Inoue et al., 2003). C3H/He and LAF1 mice were each supplemented with vitamin E for the duration of the life span in a study by Blackett & Hall (1981a). A significant reduction in mortality was shown during the first 24 months of life for both vitamin E-supplemented groups, but no significant difference in mean or maximal life span was found compared with controls. The concentration of vitamin E found in the liver was increased by 3- to 4-fold in the vitamin E-supplemented groups, relative to controls. In addition, levels of lipofuscin (the intracellular accumulation of insoluble, highly oxidized, and cross-linked proteins; Jung et al., 2007) and lipid and carbohydrate degradation (Terman & Brunk, 2004) products were significantly reduced in heart isolated from both vitamin E-supplemented mouse strains, relative to the controls (Blackett & Hall, 1981b).

Balb/c mice have been shown to have an average life span similar to that of C57BL/6 mice, approximately 31 months (Festing & Blackmore, 1971; Goodrick, 1975). Three groups of female Balb/c mice were exposed from the time of conception until death to 20× and 200× vitamin E, relative the amount of vitamin E in the control diet. Life span was not different in either of the vitamin E-supplemented groups (Morley & Trainor, 2001). Vitamin E levels in plasma were ~1.6-fold increased, but only for the 200× vitamin E-supplemented group. Unfortunately, tissue levels of vitamin E and oxidative damage were not measured. Nonetheless, it can be concluded that an increase in the plasma vitamin E concentration is not associated with changes in life span. Similarly, Lipman and colleagues (1998) supplemented C57BL/6 mice with vitamin E, starting at 18 months of age. Vitamin E-fed mice had significantly greater levels of circulating vitamin E than controls, but no effect on average or maximal life span was found. In a separate study, Sumien and colleagues (2003) fed vitamin E or the base diet (NIH-31) to C57BL/6 mice for 13 weeks, beginning at 21 months of age. Vitamin E concentrations increased 3- to 5-fold in plasma and in tissue homogenates and 2- to 3-fold in mitochondria isolated from liver, skeletal muscle, and heart. However, these increases in the vitamin E concentration were insufficient to reduce rate of heart mitochondrial H_2O_2 generation nor lipid peroxidation (thiobarbituric acid-reactive substances) or protein oxidation (protein carbonyls). Collectively, these data show that an increase in the vitamin E concentration is insufficient to reduce oxidative damage or to extend life span in Balb/c or in C57BL/6 mice.

In summary, vitamin E supplementation has been shown to increase life span in Wistar rats and in mice that are genetically short-lived (CD1-UCadiz). In both studies, blood levels of vitamin E were increased. CD1-UCadiz mice supplemented with vitamin E were shown to have reduced oxidative damage and an increased life span, as the free radical theory of aging would predict. Plasma and tissue levels of vitamin E have been found to be increased in longer-lived mouse strains, but this has been insufficient to reduce oxidative stress and/or damage (with the exception of Blackett & Hall, 1981b), and as a result, life span was not affected. Nonetheless, from evidence in studies using long-lived strains of mice supplemented with vitamin E, we conclude that elevations in both plasma and tissue concentrations of vitamin E are not a good predictor of longevity.

Vitamin C

In contrast to the lipophilic antioxidant properties of vitamin E, vitamin C is a powerful water-soluble antioxidant (Carr & Frei, 1999), but has also been shown to act as both an antioxidant and a pro-oxidant (Podmore et al., 1998). Supplementation of vitamin C in humans has been shown to reduce one form of DNA damage (8-oxoguanine), but levels of another form of DNA oxidative damage (8-oxoadenine) were increased (Podmore et al., 1998). Nonetheless, a large body of evidence supports the role of vitamin C as an antioxidant, as addition of vitamin C has been shown to reduce various forms of oxidative damage (Amer, 2002; Carty et al., 2000; Huang et al., 2002). Therefore, longitudinal analysis involving the addition of vitamin C to the diet should provide a means for investigating the role of oxidative damage on life span.

It is important to note that unlike humans and other primates, rodents are capable of endogenous vitamin C synthesis (Scheer, 1948), which may complicate interpretation of dietary supplementation studies. Unfortunately, only one study has investigated the role of lifelong vitamin C supplementation on life span in rodents. Massie and colleagues (1984) found significant increases in average (9–20%) and maximal (3%) life span in C57BL/6 mice fed vitamin C for the duration of their life span. However, it should be noted that the vitamin C-supplemented group weighed 6–7% less than the control group up until 27 months of age, leaving open the possibility that inadvertent caloric restriction may have contributed to the life-span extension. Unfortunately, levels of oxidative damage were not measured.

Although no further reports on the role of vitamin C in life span in relatively long-lived rodent strains have been performed, Fletcher and colleagues (2003) found an association between a decrease in mortality risk and an elevated plasma concentration of vitamin C in humans ages 75–84 years (with an average follow-up time of 4.4 years). Those in the lowest quartile of vitamin C concentration (<17 μmol/L) had the highest mortality risk, whereas those in the highest quartile (>66 μmol/L) had a mortality risk nearly half that value. There was no evidence for an influence of vitamin E, β-carotene, or retinol on total mortality. In addition, dietary antioxidant content measured by the food-frequency questionnaire was found not to be associated with all-cause or cardiovascular disease mortality. This is important because Enstrom and colleagues (1992) found all-cause mortality to be strongly inversely related to vitamin C intake for males and weakly inversely related for females, using food-frequency and 24-h questionnaires. However, the serum vitamin C concentration was not measured in this study, nor were markers of oxidative stress or damage. The importance of measuring plasma levels of vitamin C can be illustrated by a study performed in female Swiss Webster mice, in which six levels of dietary vitamin C (0, 0.076, 0.5, 1, 5, and 8%) were used. The plasma vitamin C concentration rose as dietary vitamin C intake increased from 1 to 8%. Mice fed a diet with 1, 5, or 8% vitamin C were found to have significantly higher tissue levels of vitamin C. However, mice fed a diet with either 0.076 or 0.5% vitamin C had statistically significant lower tissue levels of vitamin C, compared with controls (Tsao et al., 1987). These data indicate another potential flaw in studies that investigate the role of antioxidant supplementation on life span, i.e., it is critical to choose the proper dose. These data also suggest that additional studies using vitamin C supplementation in rodents should be performed.

Mixed Antioxidant Supplementation

Various investigators have used mixed antioxidant supplementation as a means of influencing life span in rodents, with variable results. Long Evans rats that were supplemented (starting from 3 months of age) with an antioxidant mixture consisting of vitamin E, vitamin C, β-carotene, butylated hydroxytoluene, and menadione sodium bisulfite complex had measured increases in the concentrations of serum vitamin E (approximately twofold elevated) and vitamin C (~40% elevated). The increase in serum concentrations of vitamin C and vitamin E, in addition to the presence of the other antioxidants in the diet, was not sufficient to extend life span in Long Evans rats (Holloszy, 1998). Unfortunately, markers of oxidative damage were not measured. As mentioned earlier, Wistar rats supplemented with vitamin E were shown to have approximately twofold elevated blood levels of vitamin E and an increased life span (Porta et al., 1980). However, the twofold elevation in serum vitamin E found in Long Evans rats was insufficient to affect life span. The average life span of Long Evans rats has been reported to be 32 months (Holloszy et al., 1985), while the average life span of Wistar rats has been reported as 25 months (Hoffman, 1978). These data indicate that vitamin E may be effective in extending life span in Wistar rats because they are genetically shorter lived, relative to Long Evans rats.

Conversely, an antioxidant supplement containing vitamin C, vitamin E, β-carotene, ginger, garlic, and *Ginkgo biloba* (among others, see Table 1 in Lemon et al., 2005) given to C57BL/6 × SJL hybrid mice was shown to increase average life span significantly, by 11%, relative to controls. Body weight did not differ between the two groups, evidence that reduces the possibility that a mild calorie restriction was responsible for the increased life span. Unfortunately, serum and tissue antioxidant concentrations and markers of oxidative damage were not measured. It should also be noted that the life span of the control strain was 23 months, and one interpretation of the data is that addition of the antioxidant mixture extended life span in a mouse strain that was relatively short-lived. In a separate study (Bezlepkin et al., 1996), an increase in life span was also found when an antioxidant mixture consisting of β-carotene, vitamin E, vitamin C, rutin, selenium, and zinc was given to C57BL/6 mice starting at 2, 9, 16, and 23 months of age. The age at which mice began to die was significantly delayed in the antioxidant-supplemented group that began at 2 months of age. Increases in average and maximal survival were also found when the antioxidant mixture was initiated at both 2 and 9 months of age, but not when started later in life (at 16 and 23 months). Average life span for the four groups ranged from 22 to 26 months. Again, it could be argued that the life span-extending effect of the antioxidant mixture was due to the control strain being relatively short-lived, as others have reported an average life span of 31 months for C57BL/6 mice (Perez et al., 2009a,b).

Royal jelly (RJ), a principal food of the honeybee queen, is produced by the hypopharyngeal and mandibular glands of worker honeybees (Inoue et al., 2003). RJ comprises water (50 to 60%), proteins (18%), carbohydrates (15%), lipids (3 to 6%), mineral salts (1.5%), and vitamins (Nagai & Inoue, 2004). RJ has been shown to have antioxidant capacity against superoxide and hydroxyl radicals (Nagai & Inoue, 2004; Nagai et al., 2006). Inoue and colleagues (2003) fed three separate groups of 2-month-old C3H/HeJ mice a low, intermediate, or high concentration of RJ for 16 weeks and found a significant reduction in oxidative damage to kidney DNA (8-hydroxy-2-deoxyguanosine, or 8-OHdG) in the RJ-supplemented groups. Mean life span in the intermediate- and high-dose RJ-fed mice was significantly increased, relative to controls. No significant difference in the mean life span for low-dose RJ-fed mice was found, compared with controls. Maximal life span was unaffected by any dose of RJ supplementation. The mean life span of the C3H/He mice used in this study (22 months) is in agreement with that previously published (Inoue et al., 2003). However, use of RJ to extend life span in C3H/He mice (Inoue et al., 2003) further supports the role of antioxidants to improve longevity only in relatively short-lived strains of mice.

Coenzyme Q_{10}

Coenzyme Q_{10} is well known to carry electrons and to translocate protons during mitochondrial respiration (Crane, 1989). Coenzyme Q_{10} has also been shown to act as an antioxidant (James et al., 2004; Takayanagi et al., 1980) and is involved in the reduction of lipid peroxidation (Forsmark et al., 1991). Because of its unique role in the mitochondrial electron transport chain as an electron acceptor (but also as an electron donor to O_2, thereby forming superoxide; James et al., 2004), various investigators have examined the role of coenzyme Q_{10} supplementation on life span.

Lifelong oral supplementation with coenzyme Q_{10} (ubiquinone) in Sprague–Dawley rats and in C57BL/6 mice was not sufficient to affect life span (Lönnrot et al., 1998), despite significant elevations in the plasma and liver ubiquinone concentration. However, tissue levels of coenzyme Q_{10} were not affected by supplementation. Unfortunately, markers of oxidative damage were not measured by Lönnrot and colleagues (1998). This result was verified by Lee and colleagues (2004), who supplemented (C57BL/6 × C3)F1 mice with coenzyme Q_{10} starting from 14 months of age, but saw no significant effect on mean or maximal life span, relative to the life span of controls. In fact, a trend ($P = 0.086$) was observed for a decreased life span in the coenzyme Q_{10}-supplemented group. It is important to note that the average and maximal life span of the control mice used in this study was 31 and 41 months, respectively, which further illustrates the absence of an effect of antioxidant supplementation in relatively long-lived strains of mice. The failure of lifelong coenzyme Q_{10} supplementation to extend life span was also shown by Sohal and colleagues (2006). C57BL/6 mice were fed three different diets providing coenzyme Q_{10}, starting at 3.5 months of age. Amounts of coenzyme Q_{10}, measured after 3.5 or 17.5 months of intake, in tissue homogenates and in isolated mitochondria were increased with the dosage and duration of coenzyme Q_{10} supplementation in all tissues except brain. Administration of coenzyme Q_{10} did not affect antioxidant defense, as determined via measurement of the enzymatic activity of superoxide dismutase, catalase, and glutathione peroxidase (measured at 19 or 25 months of age). Rates of mitochondrial superoxide generation by submitochondrial particles isolated from heart, brain, and skeletal muscle in 25-month-old coenzyme Q_{10}-supplemented mice were not different compared with the corresponding values in controls, nor was the glutathione redox state of liver, kidney, heart, or brain. The amount of protein oxidative damage was not different in liver, kidney, heart, or brain isolated from 25-month-old mice in any of the coenzyme Q_{10}-supplemented mice, relative to controls. The average and maximal life span of control mice was reported as 32 and 42 months, respectively, and further illustrates that antioxidant supplementation in a relatively long-lived strain of mouse is insufficient to affect oxidative stress or damage, or to increase longevity.

Other Studies Involving Antioxidant Supplementation and Life Span

α-Lipoic Acid

α-Lipoic acid (LA) is an antioxidant that acts as a cofactor for the mitochondrial enzymes pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Packer et al., 1997). LA has two thiol groups that when reduced are involved in scavenging reactive oxygen (and nitrogen) species (see Table 4 in Shay et al., 2009). (C57BL/6 × C3H)F1 mice fed LA starting at 2 months of age for the duration of the life span did not show changes in mean or maximal life span, relative to control-fed mice (Lee et al., 2004). It is important to note that average and life span of the controls used in this study were 31 and 41 months, respectively, and is additional evidence that antioxidant supplementation in a relatively long-lived strain of mouse is without benefit.

Glutathione

Glutathione (GSH) is a tripeptide (glutamine–glycine–cysteine) that has a free thiol group, allowing GSH to act as an antioxidant. The reduced:oxidized glutathione ratio is considered to be the primary intracellular determinant of the redox state, because it is three- to fourfold more abundant than the other redox couples (Rebrin & Sohal, 2008). C57BL/6 mice supplemented with GSH starting at 18 months of age did not show differences in mean or maximal life span compared with controls (Lipman et al., 1998). Unfortunately, the average levels of liver GSH were not different from those of control-fed mice, evidence that suggests the amount of GSH supplemented in the diet was insufficient or that the exogenously added GSH was degraded into its constituent amino acids by the gastrointestinal tract prior to its absorption into the bloodstream. Supplementation of a combination of GSH and vitamin E in mice starting at 18 months of age also failed to affect life span. The circulating vitamin E concentration was elevated in the vitamin E plus GSH-supplemented group, data that again confirm that an elevated serum vitamin E status is not positively associated with longevity.

Strawberry Extract and Melatonin

Lipman and colleagues (1998) supplemented C57BL/6 mice with either strawberry extract or melatonin, starting at 18 months of age, for the duration of the life span. Strawberries have been shown to have a high antioxidant capacity (Wu et al., 2004). Melatonin has been shown to have moderate antioxidant activity against selected oxidant species (Marshall et al., 1996). Strawberry-supplemented mice did not show changes in life span relative to controls (Lipman et al., 1998). Although serum melatonin levels were significantly elevated in melatonin-supplemented mice, no effect on life span was found, relative to controls (Lipman et al., 1998). That melatonin levels were elevated in melatonin-fed mice is important because Ebihara and colleagues (1986) found that detectable levels of melatonin were not present in the pineal gland of C57BL/6 mice, providing evidence that this strain of mouse does not endogenously synthesize melatonin.

β-Carotene

β-Carotene is an antioxidant that has greater efficacy against the reactive oxygen species singlet oxygen ($^1O_2$) compared with vitamin E and vitamin C (Sies et al., 1992). Feeding β-carotene in the diet of C57BL/6J mice beginning at 1 month or at 20 months of age for the duration of the life span increased the serum concentration of β-carotene by 60% but did not change the β-carotene content of heart, liver, or kidney and did not significantly affect either mean or maximal life span (Massie et al., 1986).

Creatine

Creatine is a nitrogenous organic acid that is produced from the amino acids arginine, glycine, and methionine and has been extensively used as an ergogenic aid (Kraemer & Volek, 1999). Creatine has been shown to have antioxidant capability against superoxide and peroxynitrite radicals, but limited capability against hydrogen peroxide or lipid peroxides (Lawler et al., 2002). To evaluate the effect of supplementation on life span, Becker and colleagues (2008) fed C57BL/6 mice creatine beginning at 12 months of age and found significant increases in both mean (9%) and maximal (3.5%) life span. Serum creatine levels were significantly elevated in the creatine-supplemented group. A nonsignificant trend ($P = 0.06$) for decreased oxidative damage was found in aged mice that were given creatine, as reduced lipofuscin autofluorescence was found in the CA2 region of the hippocampus. Oxidative damage to DNA, measured as 8-OHdG, also trended ($P = 0.08$) to be lower in the brain of creatine-fed mice. However, mitochondrial DNA deletions were not significantly different in brain or muscle isolated from creatine-fed 24-month-old mice, compared with controls. It is important to note that the mean (~19 months) and maximal (~23 months) life spans of controls were relatively short, data that again indicate antioxidant efficacy only when mice are shorter lived.

Tetrahydrocurcumin

Curcuminoids are the main yellow pigments found in turmeric (*Curcuma longa*), the traditional Indian spice. Tetrahydrocurcumin (TC) is a curcuminoid metabolite and has been shown to act as an antioxidant (Osawa et al., 1995). Kitani and colleagues (2007) fed TC to C57BL/6 mice starting at 13 months of age for the duration of the life span and found increases in both average (12%) and maximal life span (6.5%). In contrast, in mice that started to receive TC in their 19th month of life no significant difference in either mean or maximal life span was found. Unfortunately, the life-span-extending effect in TC-supplemented mice may be ascribed to TC-fed animals having a significantly lower body weight (suggesting they may be caloric restricted) during the first 6 months of TC feeding. Body weight of the mice on the TC and the control diets was not significantly different from 19 months onward (Kitani et al., 2007).

Carboxyfullerene Superoxide Dismutase

Quick and colleagues (2008) fed C57BL/6 mice a mitochondrially located (Ali et al., 2004) superoxide dismutase (SOD) mimetic, the *C3*-tris malonyl-C60 fullerene derivative (*C3*), and found significant increases in mean (11%) and maximal (10%) life span relative to the life span of controls. Mice were supplemented with *C3* beginning at 12 months of age. No significant difference in body weight or food consumption was found, compared with controls. Brain oxidative stress was reduced in *C3*-treated mice, as detected indirectly via dihydroethidine oxidation and directly in isolated mitochondria using electron paramagnetic resonance. Mitochondria isolated from brains of *C3*-treated 24-month-old mice were found to produce less superoxide than mitochondria isolated from brains of age-matched, control-fed mice and were found to produce a similar amount of superoxide relative to mitochondria isolated from brains of young, control-fed 3-month-old mice. The mean and maximal life span of the control strain was 25 and 32 months, respectively, and the use of *C3* further illustrates the ability of exogenously added antioxidants to extend the life span of relatively short-lived strains of mice. C57BL/6 mice have previously been reported to have an average and maximal life span of 31 and 41 months, respectively (Perez et al., 2009a,b).

PBN

N-tert-Butyl-α-phenylnitrone is a nitrone-based spin trap agent that acts by capturing free radicals, thereby potentially reducing their concentration. PBN has been proposed to function as an antioxidant, although its mechanism of action is not well understood (Saito et al., 1998). PBN when supplemented in the diet has been shown to significantly increase mean and maximal life span by 33 and ~70% (Edamatsu et al., 1995), respectively, in the senescence-accelerated mouse (SAM), an animal model that has been reported to be genetically short-lived (mean life span 18 months, maximal life span 23 months; Rebrin & Sohal, 2004). The SAM model has been shown to have elevated endogenous oxidative stress, relative to C57BL/6 mice (Rebrin & Sohal, 2004). PBN administration starting at 24.5 months of age in C57BL/6 mice has also been found to significantly increase both mean (4%) and maximal (5%) life span, relative to controls (Saito et al., 1998). Conversely, addition of 4-OH-PBN (the principal metabolite of PBN) to the diet of mice with a heterogeneous genetic background (four-way cross, UM-HET3) failed to extend life span (Miller et al., 2007). Nonetheless, the evidence provided by Rebrin & Sohal (2004) suggests that oxidative stress may be sufficient to limit life span, especially when the animal is short-lived, either genetically or because of exposure to suboptimal husbandry conditions.

EFFECTS OF ANTIOXIDANT ENZYME OVEREXPRESSION ON LIFE SPAN IN MICE

One straightforward approach to testing the oxidative stress theory is through the overexpression of antioxidant enzymes, which will potentially increase antioxidant protection and decrease oxidative damage. Over the past 15 years, investigators have used this approach to overexpress a number of antioxidant defense genes, including *Sod1*, *Sod2*, *Cat*, *Gpx4*, and *Trx1*, to test this hypothesis in mice. The results have not been dramatic. Sometimes a small benefit is seen, but most often no effect on life span is observed. In a few instances, the effect of antioxidant enzyme overexpression is even pathological (Rando & Epstein, 1999; Raineri et al., 2001). In the following paragraphs, and in Table 8.2, we review the results of several studies on the role of antioxidant enzyme overexpression on life span. We have also included a schematic diagram (Figure 8.1) illustrating the compartmentalization of antioxidant enzymes referred to in the next sections.

Cu,Zn-SOD

Cu,Zn-SOD (*Sod1*) is the cytosolic and mitochondrial intermembrane space-localized isoform of superoxide dismutase (Weisiger & Fridovich, 1973) and is responsible for the dismutation of superoxide radicals ($O_2^{\bullet -}$) into hydrogen peroxide (H_2O_2). Mice on a CD1 background overexpressing human *Sod1* (two- to fivefold; Huang et al., 2000) have been shown to have increased resistance to focal cerebral ischemia, pulmonary oxygen toxicity, and reactive oxygen species such as peroxynitrite. However, no significant difference in life span (mean 20 months, maximum 31 months) was found comparing nontransgenic, hemizygous, and homozygous *Sod1* transgenic mice (Huang et al., 2000), and a trend ($P = 0.086$) for decreased survival was found in mice with the highest expression of Cu,Zn-SOD. The absence of a life-span-extending effect for *Sod1* overexpression was confirmed by Perez and colleagues (2009b), who found that the mean, median, and maximal life spans of Cu,Zn-SOD transgenic mice on a C57BL/6 background were not different from the corresponding values found in controls. Thus, it can be concluded that overexpression of

Table 8.2 Antioxidant enzyme overexpression and life span

GENOTYPE (TRANSGENIC)	DECREASED OXIDATIVE DAMAGE?	INCREASED MEAN OR MAXIMAL LIFE SPAN?	SURVIVAL CURVE (*P*)	STRAIN	REFERENCE (LIFE SPAN)
WT				CD1	Huang et al., 2000
Sod1	NM	No	0.086	CD1	Huang et al., 2000
WT				C57BL/6	Perez et al., 2009b
Sod1	NM	No	0.27	C57BL/6	Perez et al., 2009b
WT				C57BL/6 × C3H	Hu et al., 2007
Sod2	NM	Yes	NP	C57BL/6 × C3H	Hu et al., 2007
WT				C57BL/6	Jang et al., 2009
Sod2	Yes	No	0.48	C57BL/6	Jang et al., 2009
WT				C57BL/6 × C3H	Schriner et al., 2005
MCAT4033	Yes	Yes	<0.001	C57BL/6 × C3H	Schriner et al., 2005
MCAT4403	Yes	Yes	<0.001	C57BL/6 × C3H	Schriner et al., 2005
PCAT2058	No	No	NS, NR	(C57BL/6-C3H × DBA2-C57BL/6) F2	Schriner et al., 2005
PCAT2088	No	Yes (median)	0.02		Schriner et al., 2005
NCAT885	No	No	NS, NR	F2	Schriner et al., 2005
NCAT900	No	No	NS, NR	F2	Schriner et al., 2005
WT		No		C57BL/6	Perez et al., 2009b
CAT	NM	No	0.87	C57BL/6	Perez et al., 2009b
WT				C57BL/6	Perez et al., 2009a
Gpx4	No	No	0.84	C57BL/6	Perez et al., 2009a
WT				C57BL/6	Perez et al., 2009a
Trx1	NM	Yes	0.039	C57BL/6	Perez et al., 2009a
WT				F2 × CD1	Schriner et al., 2005
PCAT × *Sod1*	NM	Yes (median)	<0.001	F2 × CD1	Schriner et al., 2005
WT				C57BL/6	Perez et al., 2009b
CAT × *Sod1*	NM	No	0.75	C57BL/6	Perez et al., 2009b
Sod1 × *Sod2*	NM	No	0.41	C57BL/6	Perez et al., 2009b

Abbreviations: NS, not significant; NR, not reported; NM, not measured.

Cu,Zn-SOD on two different genetic backgrounds does not extend the life span of mice. Although these studies are not supportive of the oxidative stress theory, one should note that no parameter of oxidative damage was actually measured. Therefore, it is not clear if the increased expression of Cu,Zn-SOD had any effect on the age-related accumulation of oxidative damage in tissues isolated from the transgenic mice.

Mn-SOD

The mitochondrial matrix-localized Mn-SOD (*Sod2*) plays a critical role in protecting the mitochondria from oxidant stress by enzymatically scavenging superoxide anions that are produced as a by-product of the respiratory chain. Transgenic mice overexpressing Mn-SOD have been generated (Raineri et al., 2001; Jang et al., 2009). Twofold overexpression of Mn-SOD throughout the life span increased aconitase activity in both young and old Mn-SOD transgenic mice, relative to the corresponding values found in controls. An increase in aconitase activity is indicative of a reduction in mitochondrial matrix superoxide content (Gardner et al., 1994; Gardner, 2002). However, Mn-SOD overexpression did not prevent the age-related increased rate of mitochondrial H_2O_2 production found in mitochondria isolated from old wild-type mice. Overexpression of Mn-SOD did not reduce protein oxidation (protein carbonyl content) in either young or old mice, but was sufficient to reduce lipid peroxidation (F_2-isoprostanes) in old

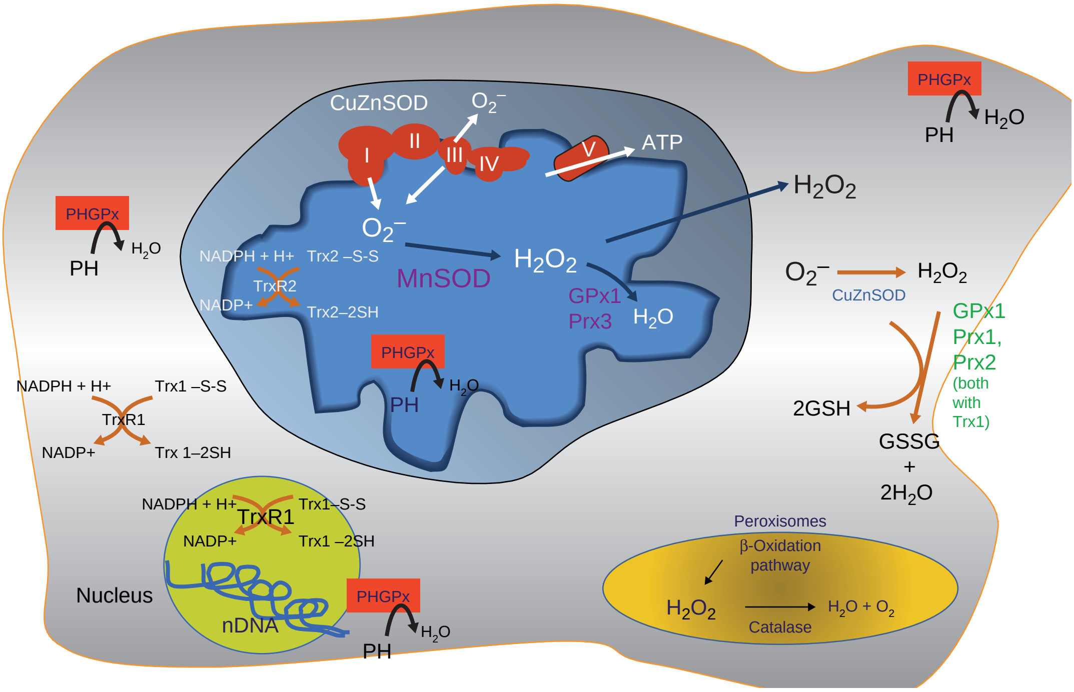

Figure 8.1 Schematic diagram of major antioxidant defense enzymes.

Sod2 transgenic mice, compared with controls. These data argue against the mitochondrial oxidative stress theory of aging, as reductions in oxidative stress and damage did not lead to an increased life span.

Conversely, Hu and colleagues (2007) reported that approximately threefold overexpression of the human *Sod2* gene significantly increased mean life span by 4%, relative to controls. Moreover, 18% of the *Sod2* transgenic mice lived longer than 40 months, while the maximal life span of the longest-lived wild type was 36 months of age (Hu et al., 2007). When the life span found by Jang and colleagues (2009) is compared with that found by Hu and colleagues (2007), the maximum life spans of *Sod2* transgenic mice are similar (1245 vs 1290 days), but the life spans of wild-type controls are notably different (1260 in Jang et al., 2009; 1095 days in Hu et al., 2007). It is possible that the disparity between the survival curves of the two studies is because of differences in the genetic background (C57BL/6 in Jang et al., 2009; C57BL/6 × C3H in Hu et al., 2007) used and the different regulatory elements used to drive transgene expression. Nonetheless, ectopically increasing Mn-SOD content throughout the life span to augment mitochondrial antioxidant defense is equivocal in terms of life span. It could be also be argued that the life-span-extending effect of *Sod2* overexpression in Hu et al. (2007) occurs because the life span of the control mice is shorter, relative to the life span of the control strain used in Jang and colleagues (2009).

Catalase

Catalase is an enzyme normally localized to peroxisomes, where it is responsible for the degradation of H_2O_2. Transgenic mice that overexpress catalase have shown conflicting results with respect to life span. Mice overexpressing catalase targeted to peroxisomes (PCAT), nuclei (NCAT), or mitochondria (MCAT) were backcrossed into a C57BL/6 genetic background by Schriner et al. (2005). MCAT mice were shown to have significant increases (20%) in both median and maximal life span. Fiftyfold higher levels of catalase in heart were found in MCAT mice, relative to controls. The rate of mitochondrial H_2O_2 production and the oxidative inactivation of aconitase were reduced in mitochondria isolated from hearts of MCAT mice. DNA oxidation and levels of mitochondrial deletions were also reduced in skeletal muscle, relative to controls. Cardiac pathology and cataract development were delayed in MCAT transgenic mice (Schriner et al., 2005). A reduction in age-associated pathologies such as malignant nonhematopoietic tumors and cardiac lesions has also been reported (Treuting et al., 2008). That catalase overexpression targeted to mitochondria increases life span makes awaiting overexpression studies involving other mitochondrially localized H_2O_2-degrading proteins (i.e., Prx3) very exciting.

Data from MCAT mice undoubtedly support the mitochondrial theory of aging in that a reduction in mitochondrial oxidative stress and damage is associated with an increase in life span. However, Schriner and colleagues (2005) failed to observe an extended life span when MCAT mice were examined in F1 crosses of C57BL/6 with C3H or Balb/c mice (personal communication). In addition, it is important to note that control mice used by Schriner and colleagues (2005) were reported to have an average life span of ≈28 months, which is shorter than the average life span of 31 months commonly reported for mice on a C57BL/6 background (Perez et al., 2009a,b).

Life-span analysis was conducted in two separate lines of PCAT mice (PCAT 2058 and PCAT 2088) and a 10% increase in median life span was found, but this effect was significant for only PCAT 2088. No significant difference in maximal life span was found for either PCAT line. Conversely, Perez and colleagues (2009b) demonstrated that when catalase is approximately twofold overexpressed in all tissues, and in its natural location in the peroxisome, no difference in mean, median, or maximal life span was observed. Control C57BL/6 mice in Perez and colleagues (2009b) had an average and maximal life span of greater than 31 and 41 months, respectively, data that support the notion that antioxidant enzyme overexpression in a long-lived strain of mouse under good animal husbandry conditions does not lead to life-span extension.

Two separate lines of NCAT mice (NCAT 885 and NCAT 900) did not show changes in life span, relative to controls. In addition, oxidative damage to DNA was not reduced in PCAT or NCAT mice nor was in vivo mutation frequency, as measured by the LacZ technique (Schriner et al., 2000).

Glutathione Peroxidase 4

Glutathione peroxidase 4 (*Gpx4*) is a member of the glutathione peroxidase family (*Gpx1*, *Gpx2*, *Gpx3*, *Gpx4*), which is involved in the detoxification of lipid peroxides (Ursini & Bindoli, 1987). While all glutathione peroxidases degrade hydrogen peroxide, alkyl peroxide, and fatty acid hydroperoxides, only *Gpx4* can degrade hydroperoxides found in lipoproteins and complex lipids such as those derived from cholesterol, cholesteryl esters, and phospholipids (Brigelius-Flohé, 1999). The mean, median, and maximal life span of *Gpx4* transgenic mice (on a C57BL/6 background) was not significantly different from that of controls (Perez et al., 2009a). *Gpx4*-overexpressing transgenic mice on a C57BL/6 background were shown to have a greater than twofold elevation in Gpx4 protein content, but lipid oxidative damage (F2-isoprostanes) was not reduced (Ran et al., 2004).

Thioredoxin 1

Thioredoxins are a family of small (approximately 12 kDa) redox-active proteins that undergo NADPH-dependent reduction by thioredoxin reductase, thereby allowing for the reduction of protein disulfides. Thioredoxin acts with peroxiredoxins to degrade peroxides into water plus oxygen, and in this regard, thioredoxin exists within the framework of antioxidant defense (Yoshida et al., 2003). Thioredoxins exist as the cytosolic and nuclear form thioredoxin-1 (Trx1) and the mitochondrial isoform thioredoxin-2 (Trx2). Thioredoxin-1 is involved in providing reducing equivalents to thioredoxin peroxidases and ribonucleotide reductase, the regulation of transcription factor activity, and is involved in the regulation of enzyme activity. Thioredoxin-1 has also been shown to be involved in the stimulation of cell growth and to act as an inhibitor of apoptosis (Powis & Montfort, 2001). Median and maximal life spans were both significantly increased in *Trx1* transgenic mice (on a C57BL/6 background) by 35 and 22%, respectively, compared with controls (Mitsui et al., 2002). *Trx1*-overexpressing mice have been shown to have approximately six- and twofold increased thioredoxin activity in brain and heart, respectively (Mitsui et al., 2002). Unfortunately, markers of oxidative damage were not measured. It is important to note that the life-span data reported for the control strain used in Mitsui and colleagues (2002) was significantly shorter than is commonly reported for C57BL/6 mice (by Mitsui et al., 2002; mean ~21 months, maximal ~27 months), making it difficult to assess whether the life-span-extending effect of *Trx1* overexpression was because of suboptimal animal husbandry conditions.

Combined Antioxidant Overexpression Studies

Combined overexpression of both *Sod1* and *Sod2* does not increase life span, relative to the life span of controls (Perez et al., 2009b). The combined overexpression of both *PCAT* and *Sod1* was found to increase median life span significantly (Schriner et al., 2005), compared with the life span of controls. Conversely, the simultaneous overexpression of both *Sod1* and *CAT* does not result in increased life span, relative to controls (Perez et al., 2009b). It is important to note that *Sod1*/*Sod2* and *Sod1*/*CAT* transgenic mice were bred on a C57BL/6 genetic background, while *PCAT*/*Sod1* mice were bred on an F2 × CD1 background. In addition, the reported average and maximal life span of 31 and 41 months for control mice by Perez and colleagues (2009b) was not exceeded by *PCAT*/*Sod1* mice.

THE EFFECT OF ANTIOXIDANT ENZYME REDUCTION ON MOUSE LIFE SPAN

During the first decade of the 21st century, various knockout mouse models were generated that have

Table 8.3 Antioxidant and oxidative damage repair enzyme knockout and life span

GENOTYPE	INCREASED OXIDATIVE DAMAGE?	DECREASED MEAN OR MAXIMAL LIFE SPAN?	SURVIVAL CURVE (*P*)	STRAIN	REFERENCE (LIFE SPAN)
WT				C57BL/6	Perez et al., 2009a
Sod1$^{-/-}$	Yes	Yes	<0.001	C57BL/6	Perez et al., 2009a
WT				C57BL/6	Van Remmen et al., 2003
Sod2$^{+/-}$	Yes	No	0.12	C57BL/6	Van Remmen et al., 2003
WT				C57BL/6	Zhang et al., 2009
Gpx1$^{-/-}$	NM	No	0.70	C57BL/6	Zhang et al., 2009
CAT$^{-/-}$	NM	NM	NM	C57BL/6	Ran et al., 2007
Prx1$^{-/-}$	Yes	Yes	0.05	C57BL/6 × 129	Neumann et al., 2003
Prx2$^{-/-}$	Yes	NM	NM	C57BL/6	Lee et al., 2003
Prx3$^{-/-}$	Yes	NM	NM	C57BL/6	Li et al., 2007
WT				C57BL/6	Ran et al., 2007
Gpx4^{+-}	Yes	No	0.32	C57BL/6	Ran et al., 2007
WT				C57BL/6 × 129	Perez et al., 2009a
Trx2$^{+/-}$	Yes	No	0.23	C57BL/6 × 129	Perez et al., 2008, 2009a
Sod2$^{+/-}$ × *Gpx1*$^{+/-}$	NM	No	0.61	C57BL/6	Zhang et al., 2009
Sod2$^{+/-}$ × *Gpx1*$^{-/-}$	NM	No	0.76	C57BL/6	Zhang et al., 2009
Gpx1$^{-/-}$ × *Sod1*$^{-/-}$	NM	No	<0.001	C57BL/6	Perez et al., 2009a
Gpx4$^{+/-}$ × *Sod1*$^{-/-}$	NM	No	0.004	C57BL/6	Perez et al., 2009a
Sod1$^{-/-}$ × *Sod2*$^{+/-}$	NM	No	<0.001	C57BL/6	Perez et al., 2009a
Gpx4$^{+/-}$ × *Gpx1*$^{-/-}$	NM	No	0.85	C57BL/6	Perez et al., 2009a
Gpx4$^{+/-}$ × *Sod2*$^{+/-}$	NM	No	0.85	C57BL/6	Perez et al., 2009a
WT				C57BL/6 × 129	Moskovitz et al., 2001
MsrA$^{-/-}$	Yes	Yes	<0.001	C57BL/6 × 129	Moskovitz et al., 2001
WT				C57BL/6 × 129	Salmon et al., 2009
MsrA$^{-/-}$	NM	No	0.87	C57BL/6 × 129	Salmon et al., 2009
WT				C57BL/6 × 129	Osterod et al., 2001
Ogg1$^{-/-}$	Yes	NM	NM	C57BL/6 × 129	Osterod et al., 2001

alterations in a wide variety of genes involved in antioxidant protection. With regard to the pathogenesis and biological toxicity of oxygen free radicals, knockout mice have strengthened established views considerably, but they also have provided a string of surprises. In the following section, and in Table 8.3, we review the results of several studies that have examined the role of antioxidant enzyme ablation on life span.

Cu,Zn-SOD

A null mutation for *Sod1* (*Sod1*$^{-/-}$) has been shown to result in viable mice that live to at least 20 months of age (Ho et al., 1997; McFadden et al., 1999; Reaume et al., 1996) and were initially thought to have no deleterious phenotypes (Huang et al., 1997). However, significant reductions in median, mean, and maximal life span have been identified for *Sod1*$^{-/-}$ mice (Elchuri et al., 2005). Although *Sod1*$^{-/-}$ mice are viable, these animals are exquisitely sensitive to exogenous oxidative stress and have some defects (see below), indicating that cytoplasmic $O_2^{\bullet -}$ has pathogenic potential. Virtually all markers of protein, lipid, and DNA oxidative damage are increased in *Sod1*$^{-/-}$ mice (Muller et al., 2006). The increase in all forms of macromolecular oxidative damage in *Sod1*$^{-/-}$ mice is associated with an increased incidence of hepatocellular carcinoma. Approximately 70% of *Sod1*$^{-/-}$ mice develop this condition, compared with a 7% incidence in wild-type mice. To date, evidence from *Sod1*$^{-/-}$ mice provides the strongest evidence in support of the free radical theory of aging.

Mn-SOD

The importance of superoxide removal in the mitochondrion is illustrated by the finding that a null mutation for *Sod2* is neonatally lethal (Lebovitz et al., 1996; Li et al., 1995). The cause of death in *Sod2*$^{-/-}$ mice has been shown to strain specific, with cardiomyopathy, metabolic acidosis, and neurodegeneration present in C57BL/6, DBA, and (C57DBA)F1 mice, respectively (Huang et al., 2001). Similarly, mice null for *Sod2* in both heart and muscle have elevated mitochondrial oxidative stress and mitochondrial dysfunction in both heart and skeletal muscle and have a median life span of 4 months and a maximal life span of 6 months (Nojiri et al., 2006), with the cause of death determined to be heart failure.

Knockout mice heterozygous for *Sod2* (*Sod2*$^{+/-}$) are viable and show reduced (30–80%) Mn-SOD activity in all tissues studied without any compensatory upregulation of other antioxidant enzymes (Van Remmen et al., 1999). The median and maximal life span of *Sod2*$^{+/-}$ mice has been found not to be different from that of controls (Mansouri et al., 2006). *Sod2*$^{+/-}$ mice do show increases in oxidative stress and oxidative damage, as evidenced by a decrease in aconitase activity and increased DNA oxidative damage (Williams et al., 1998; Van Remmen et al., 2001). In contrast to *Sod1*$^{-/-}$ mice, the evidence found in *Sod2*$^{+/-}$ mice argues against the free radical theory of aging.

Catalase, Glutathione Peroxidase 1, and Peroxiredoxins

CAT (*CAT*$^{-/-}$; Ho et al., 2004) knockout mice are viable and have been shown to develop normally. Unfortunately, oxidative damage and life span have yet to be measured in *CAT*$^{-/-}$ mice.

A null mutation for *Gpx1* (*Gpx1*$^{-/-}$), which encodes the major H_2O_2-degrading protein in the cytoplasm, results in mice that are viable to at least 22 months of age (Reddy et al., 2001) and that have been shown to have a normal median, mean, and maximal life span, relative to controls (Zhang et al., 2009). Oxidative damage was not measured by Reddy and colleagues (2001) or by Zhang and colleagues (2009). Although *Gpx1*$^{-/-}$ mice develop cataracts at an earlier age than wild-type animals, old *Gpx1*$^{-/-}$ mice did not show differences in age-related pathology and tumor burden, relative to controls (Reddy et al., 2001).

Peroxiredoxins (Rhee et al., 2001) are primarily responsible for the degradation of lipid peroxides and H_2O_2. Peroxiredoxins 1 and 2 are both localized to the cytosol. Peroxiredoxin 3 is located in the mitochondrion. *Prdx1* (*Prdx1*$^{-/-}$; Neumann et al., 2003) knockout mice exhibit an increase in oxidative DNA damage, hemolytic anemia, and neoplasms, beginning at 9 months of age. A significant 14% decrease in mean life span was found by Neumann and colleagues (2003), but maximal life span was not reported. *Prdx2* (*Prdx2*$^{-/-}$; Lee et al., 2003) knockout mice develop normally, but have an increase in protein oxidative damage, as measured by oxidized protein cysteines. *Prdx2*$^{-/-}$ mice have also been shown to exhibit hemolytic anemia. *Prdx3*$^{-/-}$ knockout mice have elevated levels of protein and DNA damage in lung, but otherwise do not demonstrate any dramatic phenotypes (Li et al., 2007). Life-span studies on *Prdx2*$^{-/-}$ and *Prdx3*$^{-/-}$ mice have yet to be conducted.

Phospholipid Glutathione Peroxidase 4

In contrast to the absence of a dramatic phenotype in *Gpx1*$^{-/-}$ mice, null mutations in phospholipid glutathione hydroperoxidase (PHGPx; the product of the *Gpx4* gene) result in embryonic lethality (Yant et al., 2003). Furthermore, Seiler and colleagues (2008) used an inducible Cre–Lox system to knock out PHGPx specifically in brain and found that this resulted in lethality within 1 week of birth. PHGPx is a unique glutathione peroxidase in that it reduces lipid hydroperoxides to their respective alcohols; in the absence of this catalytic activity, lipid hydroperoxides could decompose to $^{\bullet}OH$ and propagate lipid peroxidation or damage nearby macromolecules in cellular membranes (Imai et al., 2003; Yant et al., 2003). *Gpx4* is expressed globally in tissues at low levels, except in testis, where it is found in relatively high levels. PHGPx is found in various cellular compartments, including the mitochondria (Imai et al., 2003). It is impossible to say whether the lethality of *Gpx4*$^{-/-}$ is due to lipid peroxidation or some unrelated function of *Gpx4*. Oxidative damage was not measured in *Gpx4* null embryos (Yant et al., 2003). It is unknown whether it is the lack of antioxidant function that is responsible for the death of the *Gpx4*$^{-/-}$ mice; failing that, it is regretful that the strongest possible conclusion, namely that lipid hydroperoxides are incompatible with life, cannot be drawn from this work.

Despite the embryonic lethality of *Gpx4*$^{-/-}$ mice, *Gpx4*$^{+/-}$ mice are viable and have been reported to have a significant 7% increase in median life span (Ran et al., 2007). However, mean and maximal life span was not different comparing the life span of *Gpx4*$^{+/-}$ mice and controls (Ran et al., 2007). The increase in median life span was correlated with an increase in F_4-neuroprostanes, a marker of brain lipid peroxidation, data that are in opposition to the oxidative stress theory of aging. It was concluded that the increase in median life span in *Gpx4*$^{+/-}$ mice was most likely because of altered pathology, namely the delayed occurrence of fatal lymphoma and a reduced severity of glomerulonephritis (Ran et al., 2007). No significant difference in lipid oxidative damage (measured as F_2-isoprostanes) in plasma or liver was identified in old *Gpx4*$^{+/-}$ mice, compared with controls.

Thioredoxin and Thioredoxin Reductase

The homozygous knockout for each *Trx1* and *Trx2* in mice is embryonic lethal (*Txn1*$^{-/-}$, Matsui et al., 1996; *Txn2*$^{-/-}$, Nonn et al., 2003), again illustrating the importance of antioxidant enzymes for existence. Mice heterozygous for *Trx2* (*Txn2*$^{+/-}$) are viable but do not show changes in either mean or maximal life span, relative to controls (Perez et al., 2009a). However, *Txn2*$^{+/-}$ mice have been shown to have elevated oxidative stress, as indicated by an increased rate of mitochondrial H_2O_2 production. Lipid, DNA, and protein oxidative damage are each elevated in *Trx2*$^{+/-}$ mice. An increase in disulfide content (indicative of elevated oxidative stress) was found in both the cytosol and the mitochondria in *Trx2*$^{+/-}$ mice. In a manner similar to that of *Sod2*$^{+/-}$ mice, evidence from *Trx2*$^{+/-}$ mice provides evidence that strongly argues against the free radical theory of aging.

Thioredoxins are maintained in the reduced state via the combination of NADPH and thioredoxin reductase (Txnrd). Thioredoxin reductase 1 (Txnrd1) is localized to the cytosol (Gladyshev et al., 1996). Thioredoxin reductase 2 (Txnrd2) is found within the mitochondria (Gasdaska et al., 1999). Mice homozygous null for Txnrd1 (*Txnrd1*$^{-/-}$) and Txnrd2 (*Txnrd2*$^{-/-}$) are embryonic lethal (*Txnrd1*$^{-/-}$, Jakupoglu et al., 2005; *Txnrd2*$^{-/-}$, Conrad et al., 2004). These data create another point with respect to the oxidative stress theory of aging, that certain antioxidant enzymes are necessary for development, making study of their role on life span impossible. Future studies aimed at the postdevelopment ablation of antioxidant enzymes will better address this issue.

Combined Antioxidant Enzyme Knockouts

The mean and maximal life span of mice homozygous null for both *Sod1* and *Gpx1* (*Sod1*$^{-/-}$*Gpx1*$^{-/-}$ mice) is not significantly different from that of *Sod1*$^{-/-}$ mice (Perez et al., 2009a; Zhang et al., 2009). The life spans of mice heterozygous for both *Sod2* and *Gpx1* (*Sod2*$^{+/-}$*Gpx1*$^{+/-}$) or null for *Gpx1* (*Sod2*$^{+/-}$*Gpx1*$^{-/-}$) are not significantly different from those of controls (Perez et al., 2009a; Zhang et al., 2009). The life span of mice null for *Gpx1* and heterozygous for *Gpx4* (*Gpx1*$^{-/-}$*Gpx4*$^{+/-}$) is not different from the life span of wild-type, *Gpx1*$^{-/-}$, or *Gpx4*$^{+/-}$ mice (Perez et al., 2009a). Finally, mice null for both *Gpx1* and *Gpx2* (*Gpx1*$^{-/-}$*Gpx2*$^{-/-}$) have been shown to have intestinal inflammation (Esworthy et al., 2001) and neoplasia (Chu et al., 2004), but life span has yet to be determined. Unfortunately, oxidative damage was not measured in any of these combined antioxidant knockout mice.

KNOCKOUTS OF OXIDATIVE DAMAGE REPAIR ENZYMES

While the biochemistry and biological significance of antioxidant enzymes have received considerable experimental attention, far fewer studies exist as to the biochemistry and physiological significance of oxidative damage repair pathways. Two such systems, namely the repair of oxidized methionine (methionine sulfoxide) and the repair of oxidized guanine (8-oxo-dG) have been probed using gene knockout techniques. Table 8.3 provides a summary of the roles of oxidative damage repair enzyme knockouts on life span.

Methionine Sulfoxide Reductase

Methionine sulfoxide reductase is a protein responsible for the reduction of oxidized methionine. The null mutation for methionine sulfoxide reductase A (*MsrA*$^{-/-}$) has been shown to shorten life span considerably compared with controls (Moskovitz et al., 2001). Levels of oxidized proteins (protein carbonyls) were increased after hyperoxia (100% O_2) in various tissues isolated from *MsrA*$^{-/-}$ mice, compared with controls. In addition, exposure to chronic hyperoxia shortened the life span of *MsrA*$^{-/-}$ mice by 10% and provides evidence that *MsrA*$^{-/-}$ mice are sensitive to oxidative stress. Because the substrate required to form superoxide is oxygen, an increase in the oxygen concentration would be expected to increase the superoxide content. That *MsrA*$^{-/-}$ mice have an increase in oxidative damage and a reduction of life span provides support for the free radical theory of aging. However, a problem with the study performed by Moskovitz and colleagues (2001) is that the life span of the wild-type mice used (mean of 22–23 months) was significantly shorter than the mean life span of at least 27 to 29 months commonly reported for most mouse strains and could be indicative of suboptimal animal husbandry conditions. By maximizing the life span of the mice, the effect of genotype/environment interactions on life span is minimized, i.e., one has a more accurate measure of the effect of the genetic manipulation on aging. Conversely, Salmon and colleagues (2009) showed no significant difference in mean or maximum life span comparing the life span of *MsrA*$^{-/-}$ mice and controls. The average and maximal life span for *MsrA*$^{-/-}$ and wild-type controls was 32 and 40 months, respectively, data that indicate good animal husbandry conditions. *MsrA*$^{-/-}$ mice were more sensitive to paraquat toxicity (paraquat exposure has been shown to lead to the production of superoxide radicals and to initiate lipid peroxidation; Bus et al., 1976), and skin-derived fibroblasts from *MsrA*$^{-/-}$ mice are more sensitive to cell death caused by paraquat, hydrogen peroxide, *t*-butyl hydrogen peroxide, and sodium hypochlorite. These data indicate that *MsrA*$^{-/-}$ mice are indeed sensitive to oxidative stress, but this was insufficient to affect life span in Salmon and colleagues (2009).

Separately, mice lacking MsrB1, an isoform of MsrB, showed no difference in life span through 20 months of age (Fomenko et al., 2008), suggesting again that methionine sulfoxide reductases may not be critical determinants of longevity under careful animal husbandry.

Ogg1

Genetic ablation of *Ogg1*, the major glycosylase responsible for the removal of the mutagenic base 8-oxo-dG, results in mice with no overt pathologies (Klungland et al., 1999; Minowa et al., 2000) and that are viable to at least 27 months (Osterod et al., 2001). Steady-state levels of 8-oxo-dG are increased two- to threefold in *Ogg1*$^{-/-}$ mice and the rate of accumulation of this lesion with age is also accelerated (Osterod et al., 2001; Nishimura, 2002). Spontaneous mutagenesis has been found to be increased in these mice, albeit only in the liver (approximately twofold). Quite surprisingly, spontaneous carcinogenesis was not increased in *Ogg1*$^{-/-}$ mice (Epe, 2002). On the other hand, a null mutation in the *Mth1* gene, which codes for a protein that hydrolyzes 8-oxo-dGTP and prevents 8-oxo-dG incorporation into DNA by rapidly dividing cells, results in an increased tumor burden by 18 months of age (Tsuzuki et al., 2001). Suppression of MTH1 expression has been shown to increase total cellular 8-oxoguanine levels and cause early passage primary and telomerase-immortalized human skin fibroblasts to rapidly undergo cell senescence, doing so without altering cellular reactive oxygen species levels (Rai et al., 2009). Whether this actually leads to a decrease in life span has not yet been reported.

Overall, these studies indicate that many antioxidant (and repair) enzymes are dispensable for a normal life span (under optimized laboratory conditions) despite an apparent increase in oxidative damage markers, which at face value is not consistent with the free radical theory. However, upon close inspection of the data, whether in vivo oxidative damage is truly increased is ambiguous and depends on the particular marker of oxidative damage being investigated.

FACTS AND ARTIFACTS OF OXIDATIVE DAMAGE MARKERS AND THEIR RELATION TO THE FREE RADICAL THEORY OF AGING

The lack of a life span decrease (or accelerated aging pathologies) in *Sod2*$^{+/-}$ mice despite increased mitochondrial oxidative stress (Mansouri et al., 2006) and increased oxidative mitochondrial and nuclear DNA damage (Osterod et al., 2001) would seem to pose a formidable challenge to the free radical theory of aging. However, other markers of oxidative damage in *Sod2*$^{+/-}$ mice are not changed, compared with controls. While 8-oxo-dG content found in mitochondrial DNA (mtDNA) is elevated in *Sod2*$^{+/-}$ mice, mtDNA deletions are not increased (Lynn et al., 2001). While nuclear 8-oxo-dG content is elevated in *Sod2*$^{+/-}$ mice, in vivo mutation rates, as measured by the LacZ technique, are not increased (J. Vijg, personal communication). Furthermore, the expression pattern of oxidative stress-inducible genes is not upregulated in *Sod2*$^{+/-}$ mice (Edwards et al., 2007). Finally, plasma F_2-isoprostanes are not elevated in *Sod2*$^{+/-}$ mice (Muller et al., 2007). In short, whether *Sod2*$^{+/-}$ mice are in fact oxidatively stressed, and whether the lack of life-span shortening poses a challenge to the free radical theory, very much depends on which marker of oxidative damage is being considered. To support this interpretation, it is necessary to discuss the methodological procedures involved in measuring oxidative stress.

Measurement of markers of oxidative damage invariably requires the homogenization or breaking of tissues under an environmental oxygen concentration of ~21%. However, the mean tissue concentration of oxygen has been reported to be 3% (Guyton &, Hall, 1996) or less (Silver & Erecinska, 1988). As mentioned earlier, the substrate for the formation of superoxide is oxygen. An increase in the oxygen concentration will increase superoxide content, based on mass action. It could be argued that the increase in the oxygen concentration that arises when tissues are homogenized also increases superoxide production, thereby leading to an artifactual rise in oxidative damage, one that was not present in vivo. In support of this notion, most markers of oxidative damage suffer some degree of artifactual signal that arises during the extraction procedure (i.e., the NaI method, as reported by Hamilton et al., 2001). An extensive literature documents the problems associated with measuring lipid hydroperoxides using the TBARS assay (Hamberg et al., 1975; Gutteridge, 1986; Kosugi et al., 1987; Yeo et al., 1994) or, more recently, the 8-oxo-dG HPLC assay (Helbock et al., 1998). Although much work has gone into eliminating some of these artifacts, the ultimate problem, breaking tissues in aerated environments, remains. For instance, there is an ~100-fold discrepancy when 8-oxo-dG is measured by the Fapy–glycosylase comet assay and HPLC–EC (Trapp et al., 2007) in control tissue. This problem is even more acute in knockout mice: according to the HPLC assay, *Ogg1*$^{-/-}$ mice have an ~6-fold increase in nuclear and a 22-fold increase in mitochondrial 8-oxo-dG, but according to the Fapy–glycosylase assay, no increase in 8-oxo-dG is found (Osterod et al., 2002; Stuart et al., 2005; Trapp et al., 2007).

Which result is more biologically plausible? It is important to note that *Ogg1*$^{-/-}$ mice do not show an increase in spontaneous mutagenesis (Trapp et al., 2007) or cancer or a shortened life span (Osterod et al., 2002; Klungland & Bjelland, 2007). We suggest the following explanation for these discrepancies: a substantial amount of oxidative damage that is measured by the 8-oxo-dG assay is generated during the tissue homogenization procedure and the observed difference between knockout and control tissue arises because these enzymes protect against the oxidative modifications that occur during tissue homogenization and sample preparation. In other words, the output from the 8-oxo-dG assay would suggest that levels of oxidative damage are elevated in vivo, but in fact, this difference was not endogenously present but arose during the isolation procedure. This effect is probably compounded in assays that require extensive subpurification, such as the measurement of 8-oxo-dG in mitochondria or the measurement of mitochondrial function in vitro. In fact, most mitochondrial function assays are likely to suffer from this problem. Regarding in vitro mitochondrial defects (decreased aconitase activity, respiratory function, etc., in *Sod2*$^{+/-}$ mice (Williams et al., 1998), it is well known that mitochondria deteriorate (e.g., the respiratory control ratio drops, aconitase activity decreases) rapidly once the tissue is broken and this decline continues as mitochondria are stored on ice (Chan & Higgins, 1978). It is likely that autoxidation (continuous exposure to 21% vs the in vivo 3% O_2 tension)-driven oxidative stress is critical in the functional decline of isolated mitochondria (Chan & Higgins, 1978; Brewer et al., 2004). For this reason, it is conceivable that the differences in oxidative stress observed between mitochondria isolated from *Sod2*$^{+/-}$ and from wild-type mice may in fact arise as a consequence of the oxidative stress generated during isolation and in vitro handling, rather than being endogenously present.

What is clear is that *Sod2*$^{+/-}$ mice are sensitized to pathophysiological conditions thought to arise because of increased mitochondrial superoxide production. For example, *Sod2*$^{+/-}$ mice are more susceptible to ischemia–reperfusion injury (Murakami et al., 1998; Lewén et al., 2000; Asimakis et al., 2002) and when crossed to the transgenic *Sod1* G93A ALS mouse model, this results in an accelerated disease course and a shortened life span (Andreassen et al., 2000). On the other hand, when *Sod2*$^{+/-}$ mice are crossed to the *Sod1* ALS mouse model (which does not exhibit obvious mitochondrial dysfunction), heterozygosity for *Sod2* does not worsen pathology or shorten life span (Muller et al., 2008). Our interpretation of these data is that a 50% reduction in Mn-SOD content is sufficient to control the levels of superoxide produced during "normal" metabolism (i.e., there is no elevation in oxidative stress under basal conditions), but not during pathophysiological situations that result in elevated mitochondrial superoxide production, or in vitro when homogenized tissues or organelles are exposed to the high environmental oxygen concentration.

Although these arguments are made with respect to *Sod2*$^{+/-}$ mice, they also apply toward other antioxidant-deficient mice such as *Gpx4*$^{+/-}$ and *Txn2*$^{+/-}$. For these reasons, we favor oxidative damage assays (that do not require homogenization or extensive purification) that are performed in a near-native state, such as measurement of plasma F_2-isoprostanes. No tissue homogenization is required: a simple hydrophobic extraction is necessary. F_2-isoprostanes have been reported to be a sensitive marker of endogenously induced oxidative damage (Roberts & Milne, 2009).

An additional technique that bypasses the artifact issues raised above consists of measuring gene expression changes that are responsive to oxidative stress. We have previously conducted a microarray study leading to the conclusion that metallothioneins and *Nrf2* and *p53* target genes are reliably induced by oxidative stress in vivo as well as in multiple in vitro systems (Han et al., 2008). Therefore, one can investigate the oxidative stress status of various animal models by measuring the expression of oxidative stress-response genes. Using these criteria, none of the antioxidant knockout mice except *Sod1*$^{-/-}$ show evidence of elevated oxidative stress (Han et al., 2008). *Sod1*$^{-/-}$ knockout mice show a potent induction of *p53* and *Nrf2* target genes and the metallothioneins. In fact, the induction of *p53* is well known to mediate the "accelerated aging" phenotypes of nucleotide excision repair enzyme-deficient mice (Garinis et al., 2008). It is therefore intriguing to consider whether *Sod1*$^{-/-}$ mice also exhibit features of accelerated aging.

A comprehensive review of the data on oxidative damage in various knockout mice leaves little doubt that only *Sod1*$^{-/-}$ mice exhibit a clear, unambiguous increase in oxidative damage. Plasma F_2-isoprostanes are elevated two- to threefold in *Sod1*$^{-/-}$ mice (Muller et al., 2006). This is the highest level of plasma isoprostanes that we have observed in any antioxidant knockout animal in our laboratory. Furthermore, oxidative damage to DNA is consistently high and there is an increase in DNA mutation frequency as measured by the LacZ method in *Sod1*$^{-/-}$ mice. The LacZ method has been shown to be free from oxidative isolation artifacts (Busuttil et al., 2005). In addition, protein oxidative damage (protein carbonyls) has also been shown to be elevated in *Sod1*$^{-/-}$ mice (Elchuri et al., 2005).

The Richardson/Van Remmen group and several other investigators have reported that the life span of *Sod1*$^{-/-}$ mice is reduced from a maximum of 41 months and a mean of 31 months to 30 and 22 months, respectively, relative to controls. In colonies in which the wild-type strain is long-lived, the life span of *Sod1*$^{-/-}$ mice is reduced by 30% (Elchuri et al., 2005; Sentman et al., 2006). However, in colonies in which the same wild-type mice have an average life span of less than 24 months, the life span reduction brought about by *Sod1* deletion is considerably less (Erker et al., 2006). Although 70% of males and 35% of females develop hepatocellular carcinoma (Elchuri et al., 2005), the survival curves diverge before the age of onset of these tumors (22 months), indicating that the shortened life span of *Sod1*$^{-/-}$ mice is not simply due to liver cancer. In addition, *Sod1*$^{-/-}$ mice develop a range of pathologies reminiscent of natural aging, including an accelerated rate of sarcopenia (Muller et al., 2006), an earlier onset of cataracts (Reddy et al., 2004), macular degeneration (Imamura et al., 2006), and an earlier onset of hearing loss (McFadden et al., 1999). It is interesting to note that *Sod1*$^{-/-}$ mice further resemble nucleotide excision repair (NER)-deficient mice in their gene expression signature, e.g., downregulation of the somatotropic axis (Han et al., 2008). NER-deficient mice are thought to exhibit accelerated aging because the DNA damage-sensing pathway causes activation of p53, thereby leading to cell senescence and apoptosis. We have observed that p53 targets are continuously upregulated in *Sod1*$^{-/-}$ mice, evidence that suggests an accelerated rate of cell senescence and apoptosis may also occur in *Sod1*$^{-/-}$ mice. If the criteria set by NER-deficient mice are taken as a standard (Hasty et al., 2003; Hasty & Vijg, 2004), then an even stronger case could be made that *Sod1*$^{-/-}$ mice exhibit accelerated aging.

SYNOPSIS, CONCLUSIONS, AND PERSPECTIVES

In this chapter, we have presented a review of the literature that suggests that increasing the antioxidant concentration, whether exogenously through the diet or through use of transgenic mouse models, is not sufficient to extend the life span of mice bred under optimal animal husbandry conditions. However, in some cases the presence of an increase in antioxidants under less than ideal animal husbandry conditions or in animal models that are genetically short-lived has been shown to extend life span. We suggest that the first premise of the free radical theory of aging, i.e., that an increase in antioxidants should extend life span, appears to be true only when short-lived animal models are used (Bezlepkin et al., 1996; Lemon et al., 2005; Navarro et al., 2005), whether it is because they are genetically short-lived or because of suboptimal husbandry conditions. Conversely, in a few of these studies there is fairly clear evidence that steady-state levels of oxidative damage were in fact decreased, yet no life-span extension was found (Blackett, 1981b; Jang et al., 2009). Unfortunately, in most of these studies steady-state oxidative damage levels (or aging-related increases in oxidative damage) were not measured.

With regard to the second premise of the free radical theory of aging, that an increase in oxidants should shorten life span, the data accumulated so far leave little doubt that uncontrolled levels of reactive oxygen species are incompatible with mammalian life. The deleterious phenotypes of *Sod2*$^{-/-}$ and *Gpx4*$^{-/-}$ mice make this point very clearly. In addition, the increased levels of oxidative damage brought about by the absence of *Sod1* are clearly sufficient to result in an increase in oxidative damage (Muller et al., 2006) and a significant reduction in life span (Elchuri et al., 2005) as well as to accelerate the course of several age-related pathologies. However, the effect of high levels of oxidative damage on aging is difficult to interpret because *Sod1*$^{-/-}$ mice also have a significant increase in cancer, which may contribute to the reduction in life span (Elchuri et al., 2005). The question now remains, how high does oxidative stress need to be to be life-span limiting? Is there a dose–response curve between life span and elevated oxidative damage? The evidence regarding oxidative stress parameters and life span in *Sod2*$^{+/-}$ mice could be taken to indicate that moderate elevations in oxidative damage have no negative effects on life span. This conclusion also holds when considering other antioxidant knockout mouse models such as those that potentially affect redox status (e.g., *Trx2*$^{+/-}$). Furthermore, reduction of other antioxidant enzymes results in an increase in oxidative damage without changes in life span (Perez et al., 2009a), and in one case, reduction of phospholipid hydroperoxide glutathione peroxidase (*Gpx4*$^{+/-}$ mice) actually increases median life span. Finally, it is also interesting to note that the naked mole rat has been shown to have very high levels of several types of oxidative damage throughout its life span (Andziak et al., 2006), but has a life span ≈10 times greater than that of *Mus musculus*. The most stringent interpretation of the sum of these data is that the free radical theory is simply incorrect and that oxidative damage (unless very high) has no bearing on life span.

Overall, our integrated assessment is that research in the 50 years after the free radical theory was first proposed resulted in a significant amount of correlative data consistent with the theory; however, definite proof is still lacking. A number of studies have yielded results inconsistent with the theory as originally proposed and suggest a need for a reevaluation or modification of the theory. On the other hand, with the exception of *Sod1*$^{-/-}$ and *Prx1*$^{-/-}$ mice, no instance of an unambiguous elevation of oxidative stress, without also observing a decrease in life span, has been reported. Thus, the hypothesis that an increase in oxidative stress is limiting to life span has not been disproved. We suggest that a modest version of the free radical theory, that oxidative stress is one of the several life-span-limiting factors, may well prove to hold true.

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Chapter | 9 |

TOR: A Conserved Nutrient-Sensing Pathway that Determines Life-Span Across Species

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CHAPTER CONTENTS

Introduction 203

Target of Rapamycin (TOR) Couples Nutrients to Growth 203

TOR: A Hub Protein that Relays Signals from Nutrients, Growth Factors, and Various Stresses 204

Links between Nutrients, TOR, and Life-Span in *D. melanogaster* 205

Links between Nutrients, TOR, and Life-Span in *S. cerevisiae* 206

Links between Nutrients, TOR, and Life-Span in *C. elegans* 207

Links between TOR and Life-Span in *Mus Musculus* 209

Conclusions and Outlook 209

Acknowledgments 210

References 210

INTRODUCTION

Dietary restriction (DR) is the most robust environmental method of life-span extension in species as diverse as yeast, worms, fruit flies, and rodents (Masoro, 2003; Rogers & Kapahi, 2006). DR also slows the progression of most age-related diseases, including cancer, neurodegeneration, and cardiovascular diseases (Masoro, 2003). DR is defined as the reduction of particular or total nutrient intake without causing malnutrition. In practice, DR is applied as an overall reduction of the caloric intake (calorie restriction), the restriction of a particular class of nutrients, or temporal variations in food intake. Although DR has been known to extend life-span in rodents since the 1930s, the mechanisms of this protective response have remained elusive. However, in the past few years a number of researchers have exploited the strengths of simple model organisms to understand how DR exerts its protective effects. The short life-span and powerful genetic tools that are available in *Saccharomyces cerevisiae, Caenorhabditis elegans,* and *Drosophila melanogaster* have made them attractive models for investigating the mechanisms of life-span extension.

TARGET OF RAPAMYCIN (TOR) COUPLES NUTRIENTS TO GROWTH

One of the proposed evolutionary mechanisms of DR is that under conditions of nutrient limitation there is a shift in metabolic investment from reproduction and growth toward somatic maintenance to ensure extended survival under DR (Holliday, 1989). The evidence for TOR as a conserved nutrient sensor makes it an attractive candidate to mediate this switch between growth and somatic maintenance to extend life-span by DR across species. Remarkably, TOR acts as a nutrient sensor in species as diverse as plants, fungi, and mammals, which are in different kingdoms (Whittaker, 1969).

Rapamycin was discovered as a product of the bacterium *Streptomyces hygroscopicus* in a soil sample from the island of Rapa Nui, hence the name (Vezina et al., 1975). Originally discovered for its potent antifungal

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properties, rapamycin was later shown to inhibit the growth of cells and also act as an immunosuppressant. Insight into the mechanism of its action came upon identification of mutants that suppressed the cell-cycle-arrest properties of rapamycin using *S. cerevisae* (Heitman et al., 1991). Mutations that conferred rapamycin resistance altered conserved residues in FPR1, a peptidyl–prolyl *cis–trans* isomerase, that are critical for drug binding (Heitman et al., 1991). The growth-related targets of FPR1 were identified to be TOR1 and TOR2. TOR belongs to a group of kinases from the phosphatidylinositol kinase-related kinase family and exists in two complexes with different functions in yeast (Martin & Hall, 2005). TOR complex I (TORC1), which includes the TOR1 and TOR2 subunits, is rapamycin sensitive and controls cell growth in mass, as opposed to proliferation, whereas TORC2, which excludes the TOR1 subunit, is rapamycin insensitive and controls spatial aspects of growth within the cell (Jacinto et al., 2004; Loewith et al., 2002). Since its discovery in *S. cerevisae* TOR has been observed in all eukaryotes examined (Wullschleger et al., 2006).

In *D. melanogaster*, larvae lacking TOR show similarities to amino acid-deprived animals, such as reduced nucleolar size, developmental arrest, and lipid vesicle aggregation in the larval fat body (Oldham et al., 2000; Zhang et al., 2000). The role of orthologs of TOR has also been examined in anautogenous mosquitoes (Hansen et al., 2004). These mosquitoes use the reproductive strategy of anautogeny, requiring a blood meal to initiate egg maturation. Production of egg yolk protein precursors requires both the steroid hormone 20-hydroxyecdysone and activation of the TOR pathway (Hansen et al., 2004), indicating that TOR couples nutrients in the form of blood ingestion to egg production in mosquitoes. In *C. elegans*, deletion of TOR leads to developmental arrest at the L3 larval stage and intestinal atrophy (Long et al., 2002). A similar phenotype was observed for homozygous *daf-15* mutants (Jia et al., 2004). DAF-15 is the worm ortholog of the mammalian protein raptor (regulatory-associated protein of mammalian TOR) and forms a stoichiometric complex with TOR. Similar to invertebrates, loss of function of mammalian TOR in mice leads to embryonic lethality (Hentges et al., 2001; Murakami et al., 2004). Thus, mutations in TOR show developmental or growth-arrest phenotypes in different species, similar to those observed upon nutrient deprivation, supporting the notion that it is a conserved nutrient sensor across species. TOR couples environmental nutrient signals to growth and coordinates anabolic activities during times when nutrients are plentiful. However, when nutrients are limited, inhibition of TOR reduces growth signaling and enhances catabolic activities as the cell hunkers down for survival until conditions become once again conducive for growth and replication.

TOR: A HUB PROTEIN THAT RELAYS SIGNALS FROM NUTRIENTS, GROWTH FACTORS, AND VARIOUS STRESSES

In addition to playing a conserved role as a common nutrient sensor, TOR is also a versatile protein that acts as a major hub that integrates signals emanating from a variety of inputs. TOR integrates signals from growth factors, nutrient availability, energy status, and various stressors. Each signaling input is specialized to sense a particular relevant signal(s) and conveys it to the TOR signaling core. In turn, the signal is relayed toward the regulation of a number of outputs to respond appropriately to the environmental change by influencing mRNA translation, autophagy, transcription, metabolism, cell survival, proliferation, and growth, among a number of other cellular processes, some of which influence organismal life-span (Figure 9.1).

The cell has solved the problem of integrating information from multiple nutrient signals by utilizing molecules such as TOR as important checkpoints to balance various nutrient inputs appropriately for growth. Withdrawal of amino acids leads to inhibition of TORC1 activity (Hara et al., 1998). Two small heterodimeric GTPases, RagA/RagC and RagB/RagD, have been shown to relay the signal of amino acid sufficiency to TORC1 (Kim et al., 2008; Sancak et al., 2008). TOR is also an energy sensor. A drop in the cell's energy content triggers accumulation of AMP and results in the activation of the AMP-dependent kinase (AMPK). AMP binds directly to the β-subunit of AMPK and its activation leads to the inhibition of TORC1 activity (Gwinn et al., 2008; Inoki & Guan, 2006; Inoki et al., 2003). In addition to nitrogen starvation, carbon and phosphate starvation also leads to inhibition of TOR1 in *S. cerevisae* (Kuruvilla et al., 2001; Shamji et al., 2000; Urban et al., 2007).

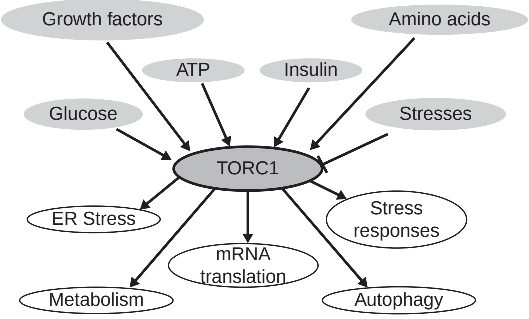

Figure 9.1 TOR signaling.

In contrast to unicellular organisms, in multicellular organisms the requirement for coordination of growth in various tissues creates a need for intercellular communication, which is achieved by diffusible growth factors. One of the major upstream regulators TORC1 is AKT, which is downstream of the insulin and IGF signaling pathways. The autonomous TOR pathway may have evolved in multicellular organisms to interact with other growth pathways such as insulin-like signaling (ILS) to coordinate growth in multicellular organisms. Evidence from a number of studies suggests that signaling through TOR is both parallel to and interactive with signaling in the ILS pathway and has been reviewed elsewhere (Kapahi & Zid, 2004; Marygold & Leevers, 2002; Shamji et al., 2003). In addition, further descriptions of insulin signaling can be found in Chapters 1, 2, and 17 of this book.

In addition to nutrients and growth factors, TOR also integrates information on environmental stresses. TOR1 is rendered less active by high temperature, hydrogen peroxide, and high-salt stress in *S. cerevisiae* (Urban et al., 2007). TORC1 also responds to hypoxia (Brugarolas et al., 2004; Reiling & Hafen, 2004). Hence, sensing of inputs from nutrients, growth factors, and cellular stress signals positions TORC1 uniquely to synchronize growth in tune with its environment. It also explains how TOR mediates changes in survival and life-span in response to various forms of nutrient manipulation in multiple species (Table 9.1). The evidence for TOR as a mediator of life-span extension by DR in various species is discussed below.

LINKS BETWEEN NUTRIENTS, TOR, AND LIFE-SPAN IN *D. MELANOGASTER*

In *D. melanogaster*, restriction of total food yields extended life-span (Clancy et al., 2002; Mair et al., 2003; Rogina et al., 2002). A number of groups have also shown that reducing yeast (the major source of protein in fly diet) alone is also sufficient to extend life-span in *D. melanogaster* (Chippindale et al., 1993; Kapahi et al., 2004; Mair et al., 2005). Support for the idea that restricting protein alone is sufficient for life-span extension comes from rodent studies. In rodents, restricting the intake of single amino acids, such as methionine or tryptophan, yields an extended life-span comparable to restricting ad libitum feeding (Miller et al., 2005; Orentreich et al., 1993; Richie et al., 1994; Zimmerman et al., 2003). Reduction of protein fails to extend life-span in rats (Masoro et al., 1989) and whether reduction of protein would extend life-span in other species remains to be seen. Overexpression of either of the tuberous sclerosis complex (TSC) genes, TSC1 or TSC2, or dominant negative forms of TOR extends life-span in *D. melanogaster* (Kapahi et al., 2004). Under conditions of DR imposed by restricting dietary yeast, inhibition of the TOR pathway resulted in no further extension of life-span. These findings support the idea that TOR, which responds to amino acid concentrations, mediates life-span extension by DR in flies (Kapahi et al., 2004).

Increase in protein synthesis is one of the key downstream effects of enhanced TOR activity (Ma & Blenis, 2009; Shamji et al., 2003). The regulation of protein synthesis also plays a key role in keeping organismal growth and development in tune with environmental conditions (Sonenberg et al., 2000). Regulation of protein synthesis by TORC1 takes place at multiple levels, which include modulation of S6 kinase (S6K), eukaryotic initiation factor eIF4E binding protein (4E-BP), and ribosomal biogenesis. Modulation of mRNA translation by these outputs is likely to be a major mechanism by which TORC1 modulates life-span and is discussed below.

TORC1 enhances translation initiation of mRNA by inhibitory phosphorylation of the 4E-BP family of proteins (Shamji et al., 2003). Hypophosphorylated 4E-BPs act as translational repressors by binding to the translation initiation factor eIF4E. This inhibits the activity of the eIF4F translation initiation complex. eIF4E plays a key role in initiation by recruiting mRNAs via their 5′UTRs. This is accomplished by regulating the association of the mRNA cap binding protein eIF4E to the scaffold protein eIF4G, both components of the eIF4F complex. eIF4G helps assemble the eIF4F complex by bridging the poly(A) binding proteins (PABPs) with eIF4E (Sonenberg et al., 2000). This leads to the circularization of mRNAs, which has a synergistic effect on enhancing the rate of translation (Sonenberg et al., 2000). TOR also activates S6K, which also modulates mRNA translation initiation by influencing the eIF3F complex (Ma & Blenis, 2009).

Activation of ribosomal p70 S6K by phosphorylation is one of the most well established downstream events of the TOR pathway. Loss of S6K shows slowed growth and reduced body size in multiple species, including flies (Montagne et al., 1999), worms (Hansen et al., 2007; Pan et al., 2007), and mice (Um et al., 2004). The role of S6K as a mediator of the life-span extension by TOR is supported by findings that inhibition of S6K extends life-span in *D. melanogaster* (Kapahi et al., 2004). Another target of TOR, 4E-BP, was recently shown to be important for the protective effects of DR in *D. melanogaster*. DR enforced by restriction of yeast in the diet requires intact 4E-BP to cause fully extended life-span (Zid et al., 2009). DR also induces 4E-BP protein levels independent of FoxO, suggesting that nutrients regulate 4E-BP by changes in both gene expression and TORC1-dependent phosphorylation. Overexpression of a gain-of-function form of 4E-BP, which has increased affinity for eIF4E (Miron et al., 2001), is sufficient to

Table 9.1 Components of the TOR pathway that modulate nutrient-modulated life-span changes in various species

MODEL SYSTEM	DIETARY MANIPULATION/ASSAY	PROTEINS IMPLICATED	REFERENCE
S. cerevisiae	Glucose restriction (replicative life-span)	TOR, SCH9	Kaeberlein et al., 2005
S. cerevisiae	Glucose restriction (replicative life-span)	TOR, MSN2, MSN4, PNC1	Medvedik et al., 2007
S. cerevisiae	Glucose restriction (replicative life-span)	GCN4, 60S ribosomal subunit, TOR	Steffen et al., 2008
C. elegans	Bacterial restriction	AMPK, DAF-16	Greer et al., 2007
C. elegans	*eat-2*	TOR	Hansen et al., 2007
C. elegans	*eat-2*	Autophagy	Hansen et al., 2008
C. elegans	Intermittent fasting	TOR, RHEB-1, DAF-16	Honjoh et al., 2009
C. elegans	Bacterial restriction	S6K, HIF-1, EGL-9, IRE-1, raptor	D. Chen et al., 2009
D. melanogaster	Yeast restriction	TOR, TSC1, TSC2, S6K	Kapahi et al., 2004
D. melanogaster	Yeast restriction	4E-BP, mitochondrial electron transport chain components	Zid et al., 2009

A list of studies that have shown a role for genes in the TOR pathway to mediate life-span extension by nutrient manipulation in various model systems.

extend life-span under rich nutrient conditions, which is not further extended upon DR (Zid et al., 2009). Together these experiments support the hypothesis that 4E-BP acts as a mediator of life-span extension by DR and that a gain-of-function 4E-BP displays the hallmarks of a DR mimetic in *D. melanogaster.*

How does a reduction in protein synthesis extend life-span? Methods to assess genome-wide changes in translation have been established, which are likely to provide useful insights into how changes in mRNA translation modulate life-span. These methods assess the mRNA translation state based on the separation of mRNAs bound to varying numbers of polysomes via density gradient centrifugation. Given that mRNA translation initiation is the major rate-limiting step in mRNA translation, this indicates the efficiency with which each mRNA is translated (Serikawa et al., 2003; Zong et al., 1999). Genome-wide translational changes revealed an increase in the translation of certain mRNAs, though there was an overall decrease in mRNA translation under DR in *D. melanogaster* (Zid et al., 2009). Using this method, a novel observation was made that under DR some mRNAs exhibit a significant increase in ribosome loading compared to the average change in mRNA ribosome loading across the entire mRNA pool. Surprisingly, a number of nuclear-encoded mitochondrial genes, including those encoding the electron transport chain (ETC) complexes I and IV and mitochondrial ribosomal proteins, displayed increased ribosomal binding that was dependent on their simple 5′UTR structures (Zid et al., 2009). Consistent with enhanced ribosomal loading increasing mRNA translation, DR was found to increase mitochondrial density and function (Zid et al., 2009). These results suggest a novel mode of regulation of nuclear-encoded mitochondrial genes by enhancing the mRNA translation under DR. Furthermore, the study also showed that in *D. melanogaster,* inhibition of the mitochondrial ETC abrogated the benefits of TOR deletion on chronological life-span. These observations support the proposed idea that a deficiency of ETC function with age, observed in multiple species, may be responsible for organismal aging (McCarroll et al., 2004). The enhanced translation of mitochondrial ETC genes may serve as a protective mechanism not only by increasing mitochondrial efficiency but also by maintaining the function of the ETC and, hence, ATP production, which is known to decline with age (McCarroll et al., 2004).

LINKS BETWEEN NUTRIENTS, TOR, AND LIFE-SPAN IN *S. CEREVISIAE*

In *S. cerevisiae,* DR by limitation of glucose has been shown to extend life-span robustly (Jiang et al., 2000; Lin et al., 2000). Replicative life-span, measured by the number of replication events from a single mother yeast cell, is increased when TOR activity is diminished (Kaeberlein et al., 2005). Furthermore, lowering glucose levels did not further extend life-span of a *tor1* deletion mutant (Kaeberlein et al., 2005). Similar results are obtained in a background deficient in the yeast ortholog of S6K, known as sch9 (Fabrizio et al., 2001; Kaeberlein et al., 2005; Urban et al., 2007). Hence, the TOR pathway mediates life-span extension by DR imposed by limiting glucose concentrations in *S. cerevisiae.*

The role of protein synthesis downstream of the TOR pathway in mediating the life-span extension by DR has also been demonstrated in *S. cerevisiae.* Ribosomal proteins play a conserved and established role in protein synthesis and growth in various species. Ribosome production is an energetically expensive process and it has been proposed that TORC1 couples nutrient availability to ribosome production (Wullschleger et al., 2006). TORC1 signaling regulates ribosome biogenesis by multiple mechanisms (Guertin & Sabatini, 2007; Wullschleger et al., 2006). TORC1 is known to affect the transcription of mRNAs encoding ribosomal proteins by RNA polymerase II, as well as transcription of ribosomal RNAs and transfer RNAs by RNA polymerase I and RNA polymerase III, respectively (Wullschleger et al., 2006). In *S. cerevisiae,* it has been proposed that reduced ribosomal biogenesis downstream of the TOR signaling pathway mediates the effects of DR. This has been supported by the observation that the loss of genes encoding the 60S ribosomal subunit extends life-span (Steffen et al., 2008). Furthermore, the life-span extension by DR, inhibition of TORC1 signaling, and inhibition of 60S ribosomal subunits utilize overlapping mechanisms. Inhibition of various ribosomal subunits has also been shown to extend life-span in *C. elegans* (Bell et al., 2009; Chen et al., 2007; Curran & Ruvkun, 2007; Hansen et al., 2007). To identify the downstream effectors that mediate life-span extension upon ribosomal biogenesis the authors examined the transcription factor GCN4, which is upregulated upon starvation. Upon starvation, while the global rate of protein synthesis decreases, production of GCN4 protein increases by an enhanced ribosomal loading of GCN4 mRNA. Mechanistically, this is driven by short upstream open reading frames in the 5′UTR of this gene (Hinnebusch, 2005; Tzamarias et al., 1989). GCN4 levels were found to increase under DR and contribute to the increased life-span caused by RNAi against 60S ribosomal subunits (Steffen et al., 2008).

DR enhances the resistance to various environmental stresses (Martin et al., 1996; Masoro, 2003). The modulation of stress pathways has been well established as contributing to life-span extension in multiple species (Martin et al., 1996). Inhibition of the TOR signaling network and also a number of translation factor genes enhances resistance to various environmental stresses (Hansen et al., 2007; Kaeberlein et al., 2005; Pan et al., 2007; Powers et al., 2006), which is best understood in yeast. In yeast, TORC1 inhibition leads to enhanced resistance to stress and nuclear translocation of Msn2 and Msn4, a stress-induced transcription factor (Beck & Hall, 1999; Crespo et al., 2002). Msn2/4 contribute to life-span extension upon inhibition of TOR by enhancing the levels of the nicotinamidase gene, PNC1 (Medvedik et al., 2007). The serine/threonine kinase Rim15 positively regulates the stress-response transcription factors Gis1 and Msn2/4 and is required for yeast chronological life-span extension caused by deficiencies in *Tor1* and *Sch9* and by DR. A recent study in *S. cerevisiae* also found that DR and inhibition of the TOR pathway enhance stress resistance by switching metabolism to enhance glycerol synthesis (Wei et al., 2009). Glycerol biosynthesis genes were upregulated in long-lived TOR pathway mutants, and their inhibition was sufficient to reverse the chronological life-span extension and enhanced stress-resistance phenotypes upon loss of TOR. Together, these experiments support the idea that inhibition of TORC1 plays an important role in mediating the switch of cellular resources from growth and reproduction toward somatic maintenance as revealed by the increase in resistance to various stresses that leads to life-span extension.

LINKS BETWEEN NUTRIENTS, TOR, AND LIFE-SPAN IN *C. ELEGANS*

Inhibition of the TOR signaling network has also been shown to mediate life-span extension by DR imposed by various methods in *C. elegans.* Downregulation of TOR expression by RNAi in *C. elegans* during adulthood results in life-span extension (Vellai et al., 2003). This effect is independent of the FoxO ortholog DAF-16, a transcription factor that is required to mediate the life-span extension caused by reduced ILS pathway activity (Vellai et al., 2003). Mutants heterozygous for *daf-15,* the worm ortholog of the mammalian protein raptor, display an extended life-span, again supporting the hypothesis that partial inhibition of TORC1 activity suffices to extend life-span in model organisms (Jia et al., 2004). Genes in the TOR pathway have been implicated in mediating the life-span extension imposed by reducing bacterial food concentration in the diet of *C. elegans* (D. Chen et al., 2009). In a model distinct from reducing available food, mutation in *eat-2,* which cause pharyngeal pumping defects in *C. elegans,* have been proposed to extend life-span by eliciting a DR-like response (Lakowski & Hekimi, 1998). Additional inhibition of the TOR pathway and its downstream gene in an *eat-2* mutant background does not further extend life-span, suggesting that DR and lowering of TORC1 signaling act by overlapping mechanisms (D. Chen et al., 2009; Hansen et al., 2008). Furthermore, the life-span extension by *eat-2*, similar to DR, is independent of DAF-16 (Houthoofd et al., 2003; Lakowski & Hekimi, 1998). In *C. elegans*, DR can also be imposed by intermittent fasting. A regimen of a single day of fasting followed by 2 days of feeding significantly extends life-span (Honjoh et al., 2009). Both the TORC1-activating GTPase Rheb and TOR inhibition have been shown to be involved in life-span extension due to intermittent fasting (Honjoh et al., 2009).

The role of protein synthesis in life-span extension has also been observed in *C. elegans*. Studies from a number of groups have shown that inhibition of several components of the eIF4F complex and PABPs extends life-span (Curran & Ruvkun, 2007; Hansen et al., 2007; Henderson et al., 2006; Long et al., 2002; Pan et al., 2007; Syntichaki et al., 2007). Inhibition of *ifg-1*, which encodes the *C. elegans* ortholog of eIF4G, during development causes larval arrest similar to that observed upon inhibition of TOR, while its reduced expression during adulthood increases life-span and stress resistance (Hansen et al., 2007; Pan et al., 2007). These results support the notion that reduction of mRNA translation initiation by inhibition of the eIF4F complex might be a conserved mechanism to extend life-span in multiple species.

Inhibition of S6K has previously been shown to extend life-span in *C. elegans* (Hansen et al., 2007; Pan et al., 2007). Loss of S6K shows slowed growth and reduced body size in worms (Hansen et al., 2007; Pan et al., 2007). Inhibition of S6K reduces protein synthesis and fecundity but leads to increased resistance to starvation (Pan et al., 2007). Recently, it has been shown that the AMPK acts downstream of S6K and mediates the S6K-dependent effects on body size and life-span (Selman et al., 2009) in *C. elegans*. Both, in mice and in worms, loss of S6K causes increased AMPK activity (Aguilar et al., 2007; Selman et al., 2009). In *C. elegans*, loss of AMPK in an S6K mutant background rescued the body size and fecundity defects of the S6K mutant. These data suggest that AMPK is an important mediator of the effects of S6K on growth and life-span. A previous study also showed that AMPK mediates life-span extension by DR in *C. elegans* (Greer et al., 2007), though not under all regimens of DR (Mair et al., 2009) (see Chapter 1 for further discussion). As described earlier, a number of studies have demonstrated that active AMPK inhibits TORC1. Hence, DR under which AMPK becomes active would lead to TORC1 inhibition, while DR that leads to TORC1 inhibition by one or more of its upstream inputs may also lead to AMPK activation. This model suggests that under DR the cell achieves a state in which both AMPK is active and TORC1 is inhibited because of a positive feedback signaling loop that involves AMPK, TORC1, and S6K.

Autophagy is a highly regulated cellular starvation response that leads to degradation of cellular components to maintain essential nutrient levels and viability (Cecconi & Levine, 2008; Diaz-Troya et al., 2008) (see Chapter 13 for more discussion on this topic). Inhibition of TORC1 enhances autophagy and provides an attractive mechanism to explain the effects of reduced TORC1 activity on life-span. Autophagy is required for the life-span extension mediated by DR or inhibition of TORC1 (Hansen et al., 2008; Jia & Levine, 2007; Toth et al., 2008). In addition, life-span extension by the *eat-2* mutation can be suppressed by inhibition of autophagy genes using RNAi (Hansen et al., 2008; Jia & Levine, 2007). Increased autophagy under DR requires the FoxA transcription factor PHA-4 (Hansen et al., 2008), which is discussed below. Together these experiments implicate an important role for autophagy in life-span extension by DR. Given the key role of mRNA translation in mediating the effects of life-span extension in multiple species, future work examining the regulation of autophagy in response to changes in the protein translation apparatus would be of great interest.

The transcription factor PHA-4 plays an essential role in the embryonic development of the foregut in *C. elegans*. Its mammalian orthologs, the FoxA transcription factors, FoxA1, FoxA2, and FoxA3, also play important roles during development and act later in life to regulate glucagon production and glucose homeostasis in response to fasting. The inactivation of *let-363/tor or rsks-1/s6k* can suppress the lethality associated with *pha-4* mutants in *C. elegans* (Sheaffer et al., 2008), suggesting a critical interaction between PHA-4 and TOR signaling. This is further demonstrated by the finding that the life-span extension by inhibition of S6K was dependent on *pha-4* (Sheaffer et al., 2008). PHA-4 has previously been described as a critical regulator of life-span extension mediated by DR (Panowski et al., 2007). Together these findings argue for the role of S6K in mediating life-span extension by DR through PHA-4-dependent mechanisms.

Another target of TOR is HIF-1α, which plays a key role in responding to hypoxia (Kaelin & Ratcliffe, 2008). HIF-1α helps cells adapt to low-oxygen stress by regulating angiogenesis, glycolysis, and cell survival (Semenza, 2000). The regulation of HIF-1α is under the control of TORC1 activity at both the transcriptional and the translational levels (Bernardi et al., 2006; Hui et al., 2006). This is also supported by findings in cell culture studies showing that rapamycin-treated cells fail to adapt to hypoxia (Thomas et al., 2006). A 2009 study suggests that HIF-1 participates in a nutrient-responsive pathway that mediates the effects of DR on life-span extension in *C. elegans* (D. Chen et al., 2009; Kaeberlein & Kapahi, 2009). Genetic epistasis analysis places HIF-1 downstream of the S6K to mediate life-span extension. A mutation in *hif-1* extends life-span under rich nutrient conditions but does not cause further life-span extension under DR, whereas the *egl-9* mutant, with elevated HIF-1, failed to show maximal life-span extension by DR. Downstream of HIF-1, endoplasmic reticulum (ER) signaling was observed to be important for its life-span extension effects. An increase in ER stress and activation of the unfolded protein response in mammalian cells has been observed in responses to excess nutrients (Ozcan et al., 2004) or enhanced TORC1 signaling due to loss of the tuberous sclerosis complex gene TSC1 or TSC2 (Ozcan et al., 2008). Consistent with a role for ER signaling in aging of *C. elegans, ire-1* was found to be required for life-span determination by DR and loss of *hif-1* function (D. Chen et al., 2009). IRE-1 encodes an ER transmembrane protein that senses misfolded proteins in the ER lumen and responds by splicing the *xbp-1* mRNA. This allows the translation of functional XBP-1 protein, which regulates target genes required for increased ER stress resistance (Ron & Walter, 2007). These studies open up a rich area of investigation to examine the mechanisms by which ER stress signaling and hypoxia signaling, which have been linked with cancer, obesity, and diabetes, mediate DR-dependent life-span extension.

LINKS BETWEEN TOR AND LIFE-SPAN IN *MUS MUSCULUS*

The role of the TOR signaling network in mediating mammalian aging phenotypes is also beginning to emerge. Research in mammalian models allows better examination of age-related pathologies, providing a model for dissecting the genetic pathways that modulate mammalian age-related diseases. As described earlier, a number of studies from invertebrates have demonstrated that genes in the TOR signaling pathway may play important roles in mediating the effects of DR in yeast, worms, and flies. Though it remains to be seen whether the TOR pathway also mediates the effects of DR in mammalian species, some reports suggest that it may indeed be involved in slowing aging and age-related pathology in mammals.

Long-lived Ames dwarf mice harbor mutations in a gene called *Prop-1* that disrupts pituitary gland development; these mice display reduced levels of circulating growth hormone and insulin-like growth factor 1 (Bartke & Brown-Borg, 2004; Brown-Borg, 2009). It has been proposed that these mice also show reduced protein synthesis possibly via inhibition of TOR signaling (Sharp & Bartke, 2005). The dwarf mice show reduced phosphorylation of the p70 S6K1 and increased levels of the translation repressor 4E-BP1 compared to control animals. These data suggest that reduced signaling downstream of TOR is associated with extended longevity in mice.

The role of S6K1 in causally influencing aging has been tested. Mice lacking S6K1 show a 20% extension in the median life-span of females, although no significant extension was seen in males (Selman et al., 2009). One of the expected outcomes of extended life-span is that it will also slow down age-related pathologies. Consistent with this, the authors find mice lacking S6K1 are protected against age-related declines in motor, bone, and immune dysfunction. Furthermore, the older S6K1 mutant mice also show better insulin sensitivity compared to control animals of the same age. It had previously been shown that mice lacking S6K1 are resistant to high-fat diet and show improved insulin sensitivity (Um et al., 2004). These findings support the idea that the life-span increase of inhibiting S6K is conserved in yeast, worms, flies, and mice, supporting its role as a key player relaying the life-span effect of reduced mammalian TORC1 signaling (Rogers & Kapahi, 2006).

The possibility of translating the increase in health span observed in S6K1-defective mice to humans relies on identifying drugs that target the TOR pathway. Rapamycin, an inhibitor of TOR, reduces the activity of S6 kinase from yeast to human cells and is used clinically as an immunosuppressant and as an anticancer therapeutic (Sonenberg & Hinnebusch, 2009). In 2003, the National Institute on Aging initiated the Interventions Testing Program (ITP) to evaluate drugs that putatively delay aging or prevent multiple forms of late-life disease in laboratory mice (Miller et al., 2007). To avoid effects specific to inbred genetic backgrounds, the ITP uses mice produced by a standardized four-way cross (Miller et al., 2007). The mice are exposed to selected drugs by a standardized feeding regimen, and the effects on life-span variations are tested at three sites in parallel (Miller et al., 2007). The ITP found that in mice, the administration of rapamycin late in life (starting at 600 days) was sufficient to cause increased life-span (Harrison et al., 2009). The effect of rapamycin on life-span was significant in both sexes at all three sites ($P < 0.05$). Another study also demonstrated that rapamycin treatment initiated past middle age (22–24 weeks) showed a significant increase in life-span in mice (C. Chen et al., 2009). This study also showed that under these conditions, rapamycin treatment can boost immune function and rejuvenate hematopoietic stem cells. Though the effects of rapamycin in reversing age-related pathologies remain to be seen, these data give hope that the use of TOR inhibitors such as rapamycin may help guide the development of DR-mimetic drugs to slow aging and age-related diseases in humans.

CONCLUSIONS AND OUTLOOK

TOR is emerging as one of the strongest conserved candidates for mediating the effects of life-span extension by dietary restriction. These data emphasize the importance of TORC1 signaling in mediating life-span extension in multiple species and upon various methods of nutrient manipulations, which are consistent with TORC1 being a conserved nutrient sensor. However, future work in this area examining whether limiting various types of nutrients (glucose, protein, or fat) in various species extends life-span by similar mechanisms is needed to assess better the role of TOR in life-span extension. As noted above, strong inhibition of TORC1 early in life (embryogenesis or larval stages) drastically slows or even stops development in multiple species. In contrast, inhibition of this pathway during adulthood (late life) extends life-span. This is consistent with the predictions of the theory of antagonistic pleiotropy, which suggests that genes that are important for fitness early in life limit life-span later in life (Williams, 1957). Hence, TOR is likely to be an important mediator of antagonistic pleiotropic mechanisms of aging. The discovery that the TOR signaling network mediates life-span extension caused by nutrient modulation in invertebrate and vertebrate species provides a framework for examining the genetic and molecular basis of how DR extends life-span. Various groups have now been able to link various processes downstream of TORC1 mechanistically to aging, which provides hope for yielding a better understanding of this phenomenon. One of the challenges in the field is to understand better the cross talk of TOR with other aging pathways and the mechanisms by which TOR mediates life-span changes through some of the effectors mentioned in this chapter and those that remain undiscovered. Another challenge is to understand the relative contribution of each of the downstream effectors and the various inputs that influence them in determining life-span in different species. These studies may provide drug targets to slow a number of age-related diseases and even postpone aging. Further analyses of this highly conserved pathway will also shed light on the link between diet and various age-related diseases such as cancer, neurodegeneration, and diabetes in humans.

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Chapter 10

Comparative Genetics of Aging

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CHAPTER CONTENTS

Introduction 215
Common Nonmammalian Models of Aging 216
S. cerevisiae 216
Yeast Replicative Aging 216
Yeast Chronological Aging 220
C. elegans 220
Drosophila 221
Conserved Longevity Interventions 221
Dietary Restriction 222
Genetic Manipulation of Insulin/IGF-1-like Signaling 222
Overexpression of Sirtuins 224
Reduced TOR Signaling 226
Quantitative Evidence for Conserved Mechanisms of Longevity Control 226
Demonstration of Conservation between Yeast and Worms 226
TOR Signaling Accounts for Many Conserved Longevity Factors 227
Interaction Between Dietary Restriction and Conserved Longevity Pathways 228
IIS: A Partial Interaction with DR with Respect to Secondary Aging Phenotypes 228
Sirtuins: A Complex and Unresolved Connection to DR 229
TOR Signaling: A Conserved Mediator of Life-Span Extension by DR 230
Conclusions 231
References 231

INTRODUCTION

Aging is a degenerative process affecting virtually all known organisms that is characterized by progressive deterioration of cellular components and deregulation of cellular processes, resulting in mortality. Investigation toward understanding the molecular processes and environmental factors that influence the rate of aging is a primary focus of research related to the basic biology of aging. The identification and characterization of genetic pathways that interact with these processes to modulate longevity is paramount to understanding why we age and will probably facilitate the development of therapeutic strategies for combating aging and age-related disease.

Life span is the primary endpoint to consider when studying aging; however, directly measuring life span in mammals is both difficult and expensive because of the relatively high longevity of most mammalian species. For this reason, research into mammalian aging has often been limited to looking at secondary endpoints that tend to correlate with longevity, such as stress-resistance and metabolic parameters. The cost and labor associated with maintaining relatively large cohorts of long-lived animals in a laboratory environment is particularly prohibitive to large-scale approaches looking for longevity phenotypes in mammals, such as high-throughput genetic or chemical screens. An alternative approach has been to develop model systems with characteristics that lend themselves to the types of studies common in aging research. These characteristics include short life span, rapid reproduction resulting in a large number of offspring, ease of maintenance in the laboratory environment, well-characterized biology including fully sequenced genomes, and availability of powerful tools for genetic manipulation. Three

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nonmammalian organisms have emerged as particularly prominent models of aging: the budding yeast *Saccharomyces cerevisiae*, the nematode *Caenorhabditis elegans*, and the fruit fly *Drosophila melanogaster*.

We are ultimately interested in interventions that can be applied to reduce mortality and fight age-related disease in humans. The use of nonmammalian models in aging-related research raises an important question: are findings in relatively simple eukaryotes applicable to human aging? From a genetic standpoint the relevant question is whether genetic pathways that play a role in controlling aging and longevity in one species are common among evolutionarily divergent species or unique to that particular lineage. If genetic mechanisms can be identified that modulate life span across evolutionarily divergent simple eukaryotes, it is reasonable to expect that at least a subset of these mechanisms will be conserved in humans (Kaeberlein, 2004).

The best-studied example of a conserved longevity intervention is dietary restriction (DR), which has been defined as a reduction in food consumption in the absence of malnutrition (Kennedy et al., 2007; Masoro, 2005; Spindler, 2009; Weindruch & Walford, 1988). DR has long been known to increase life span in many different species, including yeast, worms, flies, and rodents. DR has also been reported to increase life span and health span in a nonhuman primate, the rhesus macaque, with the caveat that significance was achieved only when more than two-thirds of the deaths were censored as non-age-related (Colman et al., 2009). Several factors have been proposed to contribute to the health and longevity benefits of DR. To date, while DR is effective at extending life span in a variety of species, it remains unknown whether DR acts via similar mechanisms in different species (discussed further below), let alone whether DR can significantly improve longevity or health span in humans.

The rationale that conserved longevity interventions are more likely to be relevant for human health has spurred the hunt for "conserved aging genes," which for the purposes of this chapter will be defined as genes that function to modulate aging in multiple evolutionarily divergent species (Kaeberlein, 2004). Work from several groups has led to the identification of more than two dozen conserved aging genes, and comparative genetic analyses are beginning to place these genes into known aging pathways (Table 10.1). In this chapter we describe the current state of knowledge in this area. We first discuss how aging is studied in each of the common invertebrate model systems and then describe major classes of conserved aging genes. We also describe how genome-scale longevity studies in yeast and nematodes have accelerated progress in the comparative genetics of aging and are leading to mechanistic insights for how these genes may be influencing longevity across multiple species. Finally, we present an overview of the complex relationships between known conserved pathways that influence aging and how they interact with the response to environmental nutrients.

COMMON NONMAMMALIAN MODELS OF AGING

Aging has been studied in a wide variety of model organisms, both mammalian and nonmammalian. Among nonmammalian model systems, three species are widely used in aging-related studies: *S. cerevisiae*, *C. elegans*, and *D. melanogaster.* Several other nonmammalian organisms are also actively being used to study aging on a smaller scale, including bacteria (Ackermann et al., 2003; Nystrom, 2007; Stewart et al., 2005), fission yeast (Barker & Walmsley, 1999; Roux et al., 2006), other nematode and fly species (Carey et al., 2002; Davies et al., 2005; Sutphin & Kaeberlein, 2008), and fish (Terzibasi et al., 2007; Valenzano et al., 2006). For the purposes of this chapter, we focus primarily on comparative genetics of aging in the three most common models.

S. cerevisiae

The budding yeast *S. cerevisiae* has been used as a model organism for aging research for more than 50 years (Mortimer & Johnston, 1959). Two distinct paradigms have been defined for yeast aging: chronological and replicative. Chronological life span refers to the length of time that a yeast cell can retain viability in a nondividing state, while replicative life span refers to the number of viable daughter cells produced by a mother cell during vegetative growth (Fabrizio et al., 2001; Kaeberlein, 2006; Mortimer & Johnston, 1959).

Yeast Replicative Aging

Replicative life span is the older of the two yeast aging models and has been studied in greater detail. To date, nearly 100 genes are reported to modulate yeast replicative aging (Bitterman et al., 2003; Jazwinski, 2000; Steinkraus et al., 2008). Replicative life span is measured by microdissection of daughter cells away from mother cells while tallying the number of daughters produced at each age point. Replicative longevity varies widely among different laboratory strains, with the most strains having an average replicative life span between 18 and 26 generations (Kaeberlein, 2006). The most extensively studied yeast strains are the parental strains of the yeast open reading frame (ORF) deletion collection, which are closely related to the *S. cerevisiae* wild-type strain S288C (Kaeberlein et al., 2005a; Mortimer & Johnston, 1986). This collection has been used for genome-wide screens for single-gene

Table 10.1 Conserved aging genes

LONGEVITY PATHWAY	KNOWN OR PREDICTED PROTEIN FUNCTION	ENCODING GENE				REFERENCES
		YEAST	WORMS	FLIES	MICE	
Insulin/IGF-1-like signaling	AKT/protein kinase B	*SCH9*[a]	*akt-1, akt-2*[b]			Fabrizio et al., 2001; Hamilton et al., 2005; Hertweck et al., 2004
	FoxO family transcription factor	n/a	*daf-16*	*dFOXO*		Henderson & Johnson, 2001; Giannakou et al., 2004; Hwangbo et al., 2004
	Insulin receptor substrate (IRS)	n/a		*Chico*	*Irs1, Irs2*	Clancy et al., 2001; Selman et al., 2008; Taguchi et al., 2007
	Insulin/IGF-1-like receptor	n/a	*daf-2*	*InR*	*Insr, Igf1r*	Kenyon et al., 1993; Tatar et ssal., 2001; Bluher et al., 2003; Holzenberger et al., 2003
	Phosphoinositide 3-kinase (PI3K)	n/a	*age-1, aap-1*		*PI3Kγ*	Klass, 1983; Dorman et al., 1995; Wolkow et al., 2002; Barber et al., 2006
Sirtuins	Histone Deacetylase	*SIR2*	*sir-2.1*	*dSir2*		Kaeberlein et al., 1999; Tissenbaum & Guarente, 2001; Rogina & Helfand, 2004
	Histone Deacetylase	*RPD3*		*Rpd3*		Kim et al., 1999; Rogina et al., 2002
mRNA translation/TOR signaling	Large subunit ribosomal protein	*RPL19A*	*rpl-19*			Smith et al., 2008a,b; Hansen et al., 2007
	Large subunit ribosomal protein	*RPL6B*	*rpl-6*			Smith et al., 2008a,b; Hansen et al., 2007
	Large subunit ribosomal protein	*RPL9A*	*rpl-9*			Smith et al., 2008a,b; Hansen et al., 2007
	S6 kinase	*SCH9*[a]	*rsks-1*	*dS6K*	*S6K1*	Fabrizio et al., 2001, 2004; Urban et al., 2007; Hansen et al., 2007; Pan et al., 2007; Kapahi et al., 2004; Selman et al., 2009
	Small subunit ribosomal protein	*RPS6B*	*rps-6*			Kaeberlein et al., 2005a,b,c; Hansen et al., 2007

(*Continued*)

Table 10.1 (Continued)

LONGEVITY PATHWAY	KNOWN OR PREDICTED PROTEIN FUNCTION	ENCODING GENE				REFERENCES
		YEAST	WORMS	FLIES	MICE	
	Target of rapamycin kinase	*TOR1*	*let-363*	*dTOR*	*mTOR*[c]	Kaeberlein et al., 2005a,b,c; Powers et al., 2006; Vellai et al., 2003; Kapahi et al., 2004; Harrison et al., 2009
	Translation initiation factor	*TIF1*, *TIF2*	*inf-1*			Smith et al., 2008a,b; Curran & Ruvkun, 2007
	Translation initiation factor	*TIF4631*	*ifg-1*			Smith et al., 2008a,b; Henderson et al., 2006; Curran & Ruvkun, 2007; Pan et al., 2007
Stress resistance	Catalase			*Cat*	*Cat*[d]	Orr & Sohal, 1994; Schriner et al., 2005
	Heat shock protein		*hsp-6*	*Hsp70*		Yokoyama et al., 2002; Tatar et al., 1997
	Superoxide dismutase	*SOD1*		*Sod1*		Fabrizio et al., 2003; Orr & Sohal, 1994
Unknown	3-Phosphoinositide-dependent kinase	*PKH2*	*pdk-1*			Smith et al., 2008a,b; Paradis et al., 1999
	α-Mannosyltransferase	*ALG12*	*T27F7.3*			Smith et al., 2008a,b; Curran & Ruvkun, 2007
	Ammonium transporter	*MEP1*, *MEP2*	*amt-2*			Powers et al., 2006; Kim & Sun, 2007
	CCCH-type Zn-finger protein	*TIS11*	*pos-1*			Smith et al., 2008a,b; Curran & Ruvkun, 2007
	Ceramide synthase component	*LAG1*	*hyl-1*			D'Mello et al., 1994; Tedesco et al., 2008; Menuz et al., 2009
	Coenzyme Q7 homolog		*clk-1*		*Coq7*	Lakowski & Hekimi, 1998; Liu et al., 2005
	Cytoskeletal linker protein	*YGR130C*	*erm-1*			Smith et al., 2008a,b; Curran & Ruvkun, 2007
	DEAD-box helicase	*DBP3*	*B0511.6*			Smith et al., 2008a,b; Curran & Ruvkun, 2007

Table 10.1 (Continued)

LONGEVITY PATHWAY	KNOWN OR PREDICTED PROTEIN FUNCTION	ENCODING GENE				REFERENCES
		YEAST	WORMS	FLIES	MICE	
	Dehydrogenase	*ADH1*	*W09H1.5*			Smith et al., 2008a,b; Hamilton et al., 2005
	Endosomal complex adaptor protein	*HSE1*	*sem-5*			Smith et al., 2008a,b; Curran & Ruvkun, 2007
	G protein, α subunit	*GPA2*	*gpa-1, gpa-5, odr-3*			Lin et al., 2000; Lans & Jansen, 2007
	Golgi membrane ATPase ion transporter	*PMR1*	*eat-6*			Smith et al., 2008a,b; Lakowski & Hekimi, 1998
	Isocitrate dehydrogenase	*IDH1, IDH2*	*F43G9.1*			Smith et al., 2008a,b; Hamilton et al., 2005
	Metalloprotease	*AFG3*	*spg-7*			Smith et al., 2008a,b; Curran & Ruvkun, 2007
	Methionine sulfoxide reductase A	*MXR1*		*Eip71CD*		Koc et al., 2004; Ruan et al., 2002
	Polyphosphoinositide phosphatase	*INP51, INP53*	*unc-26*			Smith et al., 2008a,b; Lakowski & Hekimi, 1998
	Protein phosphatase regulatory subunit	*SIS2*	*Y46H3C.6*			Smith et al., 2008a,b; Hamilton et al., 2005
	RAB-family GTPase	*YPT6*	*rab-10*			Smith et al., 2008a,b; Hansen et al., 2005
	S-adenosylmethionine synthetase	*SAM1*	*sams-3*			Smith et al., 2008a,b; Curran & Ruvkun, 2007
	Surfeit gene 1			*Surf1*	*Surf1*	Zordan et al., 2006; Dell'Agnello et al., 2007
	Thioredoxin	*TrxT*			*Txn1*[d]	Umeda-Kameyama et al., 2007; Mitsui et al., 2002
	Transcription elongation factor	*SPT4*	*spt-4*			Smith et al., 2008a,b; Hamilton et al., 2005

Homologous genes for which altered expression or activity is reported to extend life span in two or more evolutionarily divergent organisms are shown.

[a] *SCH9* has been suggested as a yeast homolog to both mammalian Akt/PKB and mammalian S6K and shows S6K activity.

[b] *akt-1(RNAi) akt-2(ok393)* is longer lived than wild type, *akt-1(ok525)*, and *akt-2(ok393)*.

[c] Predicted based on life-span extension from treatment with the TOR inhibitor rapamycin.

[d] Mouse life-span extension shown by overexpressing the human version of the gene.

deletions that increase either chronological life span or replicative life span (Kaeberlein et al., 2005c; Powers et al., 2006) (described in more detail below). Environmental parameters such as temperature and medium composition are also known to influence replicative life span. One primary molecular cause of replicative aging in yeast is thought to be the mother-cell-specific accumulation of extrachromosomal rDNA circles (ERCs) (Defossez et al., 1999; Sinclair & Guarente, 1997), although additional uncharacterized factors are also known to contribute to replicative aging. Evidence suggests that these factors may include age-associated genomic instability, mitochondrial retrograde signaling, accumulation of oxidatively damaged proteins in the mother cell, and altered histone acetylation near telomeres (Aguilaniu et al., 2003; Dang et al., 2009; Kaeberlein et al., 1999; Kirchman et al., 1999; McMurray & Gottschling, 2003).

DR has been studied in the context of replicative life span by reducing either the amino acid or, more commonly, the glucose content of the growth medium (Jiang et al., 2000; Lin et al., 2000). Replicative life span extension has been reported at multiple glucose concentrations ranging from 0.5 to 0.005% glucose (M. Kaeberlein et al., 2006; Lin et al., 2002) compared to the standard concentration in yeast medium of 2%. The glucose concentration at which replicative life span is maximally extended is dependent on the genetic background of the strain, and there has been substantial debate regarding whether the mechanism by which DR extends life span is similar at differing glucose concentrations (Kaeberlein & Powers, 2007).

Yeast Chronological Aging

Chronological aging has typically been measured by culturing cells into a postdiauxic quiescent-like state in synthetically defined growth medium and monitoring the viability of cells over time, where viability is defined by the ability of cells to reenter the cell cycle and resume vegetative growth in the presence of a nutrient-rich medium (Fabrizio & Longo, 2003). Alternative growth conditions for monitoring chronological aging have been described but not widely used, including maintaining cells in water at a high temperature after 2–3 days of standard culture and aging cells in rich growth medium rather than synthetic defined medium (Harris et al., 2001; Piper et al., 2006). Like replicative life span, chronological life span varies among different laboratory strains and is robustly influenced by the composition of the growth medium (Fabrizio et al., 2005; Murakami et al., 2008; Smith et al., 2007). Chronological senescence is correlated with an accumulation of oxidatively damaged proteins, mitochondrial dysfunction, and induction of the yeast apoptotic-like response (Aerts et al., 2009; Fabrizio et al., 2003; Herker et al., 2004). Recently, acetic acid toxicity associated with acidification of the growth medium has been identified as a primary molecular cause of chronological senescence under standard conditions (Burtner et al., 2009). Although the molecular causes of chronological senescence appear to be distinct from replicative senescence, chronologically aged cells show a reduced replicative life span, suggesting that underlying similarities may exist (Ashrafi et al., 1999).

Similar to the case for replicative aging, life-span extension from DR in the yeast chronological aging paradigm can be accomplished by reducing the glucose concentration of the growth medium from 2 to 0.5% or lower (Murakami et al., 2008). A form of extreme DR has also been described in which cells are transferred from expired growth medium to water after 2–4 days of aging (Fabrizio et al., 2005).

C. elegans

C. elegans has arguably become the most informative model organism for genetic studies of basic mechanisms of aging. When measured under standard conditions (20 °C on solid nematode growth medium), the life span of the common lab strain (N2) is about 3 weeks. *C. elegans* are typically fed a diet of *Escherichia coli* OP50 bacteria grown as a lawn on the surface of the agar plate and viability is determined by the ability of adult animals to move in response to touch (Sutphin & Kaeberlein, 2009). The *C. elegans* life cycle takes around 3 days and consists of externally laid eggs, four larval stages, and a reproductively active adult stage. The majority of adult animals are hermaphrodites and self-fertilize to produce several hundred offspring per individual. Rare male worms arise spontaneously and mate with hermaphrodites to produce broods that are half male and half hermaphrodite. Cells in adult animals are postmitotic with the exception of the germ line.

Studies in *C. elegans* have identified more than 300 genes that are associated with increased life span when their function is diminished (Braeckman & Vanfleteren, 2007; Smith et al., 2008b). Most of these genes were identified from large-scale RNA interference (RNAi) screens carried out using libraries that cover roughly 90% of the known ORFs in the nematode genome (Arum & Johnson, 2007; Chen et al., 2007; Curran & Ruvkun, 2007; Dillin et al., 2002; Hamilton et al., 2005; Hansen et al., 2005; Lee et al., 2003). RNAi is particularly powerful in *C. elegans*, as efficient gene knockdown can be achieved by simply feeding animals bacteria expressing double-stranded RNA with sequence corresponding to the gene of interest. Many of the currently known *C. elegans* aging genes can be broadly classified based on epistasis grouping and known or predicted function into one or more of the following classes: (1) insulin/IGF-1-like signaling, (2) mitochondrial function, (3) protein synthesis/mRNA translation, (4) chemosensory function, (5) dietary restriction, or (6) hypoxic response.

The molecular mechanisms that cause *C. elegans* to age are not known, but analysis of tissue-specific aging has led to the conclusion that neuronal cells largely retain function in old animals, while muscle cells in many animals show a gradual decline in function beginning near the transition to the postreproductive stage of adulthood (Herndon et al., 2002). Associated with this general decline in muscle function is a decrease in pharyngeal pumping, resulting in reduced food consumption (Huang et al., 2004; Kenyon et al., 1993; Smith et al., 2008a), and an accumulation of autofluorescent age pigment throughout the body (Gerstbrein et al., 2005; Klass, 1977). If a live food source is used, bacterial colonization of the gut can also contribute to senescence; however, the relevance of this to normal aging is unclear, as animals fed a killed bacterial food source show a similar progression of age-associated phenotypes with life span extended by only a few days (Garigan et al., 2002; Garsin et al., 2003).

DR in *C. elegans* has been studied using a variety of methods and there is currently little consensus regarding which methods are most appropriate (Greer & Brunet, 2009; Mair et al., 2009). Most methods of DR in *C. elegans* involve reducing the amount of bacterial food provided to the worms, but differ in whether the food is alive or killed, whether the growth environment is solid agar-based or liquid, and whether the amount of food is constant or varied (akin to feeding/fasting cycles in rodents) over the course of the experiment (Greer & Brunet, 2009). Under at least some conditions on agar-based medium, complete removal of the bacterial food during adulthood has been observed to increase life span maximally, a DR regimen referred to as bacterial deprivation (T. L. Kaeberlein et al., 2006; Lee et al., 2006). Age at onset of DR also varies from study to study and may influence the resulting life span; however, at least in the case of bacterial deprivation, similar median and maximal life-span extension has been demonstrated for DR initiated between day 4 and day 14, with similar maximal life-span extension achieved for DR initiated as late as day 24 (Smith et al., 2008a).

Drosophila

The fruit fly *D. melanogaster* is the earliest invertebrate player in aging research, with studies of life span dating back to 1916 (Loeb & Northrop, 1916). The fly life cycle lasts 1 to 2 weeks and consists of three easily distinguishable growth stages (embryo, larva, and pupae) followed by the reproductively active adult stage. Similar to *C. elegans*, the majority of the cells in the adult fruit fly are postmitotic, with exceptions in the germ line and a subset of gut cells. Flies are typically maintained in vials with a cornmeal–sugar–yeast or sugar–yeast agar-based food source. Unlike yeast and worms, flies cannot be frozen and must be actively maintained. Wild-type *D. melanogaster* has a median life span between 1 and 2 months when maintained at 25°C.

The fruit fly has been used extensively to explore nongenetic environmental manipulations that extend life span. DR can be accomplished by diluting yeast or other components in the food source (Bass et al., 2007a; Chapman & Partridge, 1996; Good & Tatar, 2001). Fruit flies also experience a strong inverse relationship between environmental temperature and life span (Helfand & Rogina, 2003; Miquel et al., 1976), and brief exposure to mild stressors such as high temperature or low-level radiation can result in increased life span (Hercus et al., 2003; Le Bourg et al., 2004; Vaiserman et al., 2003). Flies generally have a strong inverse correlation between reproduction and longevity. Strains bred for longevity by selecting offspring from late life reproduction show reduced egg laying early in life relative to ancestral strains (Luckinbill et al., 1984; Rose, 1984). In *Drosophila subobscura*, life-span extension observed in response to DR is accompanied by a reduction in egg production (Marden et al., 2003). Preventing mating can also double female life span (Smith, 1958), though, in *D. melanogaster*, seminal factors have been implicated in shortening female life span as opposed to some intrinsic cost associated with reproduction (Ueyama & Fuyama, 2003).

As a model system, *Drosophila* offers a variety of powerful genetic techniques for studying aging at a genetic level. While high-throughput methodology for studying life span has yet to be employed, gene- and pathway-specific approaches as well as smaller-scale candidate gene and random mutation studies have been useful in testing a variety of aging theories and in identifying new players in fly aging. *Drosophila* genes that play a role in modifying aging have been identified in a variety of pathways, including insulin/IGF-1-like signaling, mitochondrial function, oxidative stress resistance, sirtuins, and TOR signaling.

CONSERVED LONGEVITY INTERVENTIONS

The growing body of aging research using multiple divergent species has led to the discovery and characterization of several aspects of longevity control that have been evolutionarily conserved, of which DR is the most studied. Three (at least partially) distinct genetic pathways have been found to modulate aging in evolutionarily divergent organisms: insulin/IGF-1-like signaling (IIS), sirtuins, and the nutrient-responsive target of rapamycin (TOR) kinase. Each of these genetic pathways has also been proposed to play a role in life-span extension from DR. This section describes the current state of knowledge surrounding DR and each of these conserved longevity pathways with respect to aging. The relationship between each pathway and DR is discussed in a later section, and additional discussion of DR is provided in Chapters 1, 9, and 21. Additional details regarding TOR signaling are provided in Chapter 9, and sirtuins in Chapter 11.

Dietary Restriction

The effects of DR on longevity are clearly shared among diverse organisms, but it remains an open question as to whether the underlying molecular mechanisms are also shared. Several hypotheses for how DR might mediate a reduced rate of aging have been proposed, including reduced inflammation, reduced damage from reactive oxygen species, improved glucose homeostasis, and enhanced resistance to a variety of stresses (Spindler, 2009). To date none of these hypotheses has been definitively shown to play a primary role in mediating the effects of DR. In addition to enhanced longevity and reduced age-associated disease, two DR-associated phenotypes that seem to be shared between different organisms are a reduction in reproductive rate and an increase in broad-spectrum stress resistance. This observation has led to the hypothesis that life-span extension in response to DR is an evolutionarily conserved mechanism for maintaining reproductive potential in response to transient environmental fluctuations in food availability (Harrison & Archer, 1988; Holliday, 1989).

One unresolved question regarding DR is whether the longevity and health benefits are solely due to reduced caloric consumption, as was initially assumed, or whether other dietary factors may also be involved. In support of a more general view of DR, simply restricting the dietary abundance of methionine in both mice and rats is sufficient to increase life span (Miller, et al., 2005; Orentreich et al., 1993). Similar observations have been made with respect to tryptophan in rats (Ooka et al., 1988; Segall & Timiras, 1976; Timiras et al., 1984). An alternative way to interpret these results is that the standard laboratory mouse diet does not contain an ideal balance of amino acids and that a subset, including methionine and tryptophan, is overly abundant. Indeed, Masoro et al. (1989) found that methionine restriction did not contribute to the life-span extension resulting from a 40% decrease in food intake in rats. The most convincing evidence for a model of DR that is not limited to restriction of caloric intake is the finding that food sensing can modulate longevity independent of food consumption in both nematodes and flies (Libert & Pletcher, 2007; Smith et al., 2008a). Whether food sensing modulates longevity in mammals is unknown.

Genetic Manipulation of Insulin/IGF-1-like Signaling

Among multicellular eukaryotes, IIS pathways mediate growth, stress resistance, and longevity in response to environmental conditions. The longevity-related IIS pathways share a core set of similar features in divergent organisms, including insulin-like molecules, one or more insulin/IGF-1-like receptors, a phosphatidylinositol 3-kinase (PI3K), an Akt kinase, and a FoxO-family transcription factor (Figure 10.1). Downstream genetic targets of IIS are regulated by controlling nuclear localization of the FoxO-family transcription factor, with increased IIS resulting in decreased transcription factor activity.

Worms and flies each possess a single IIS receptor that mediates signals from multiple insulin-like ligands (at least 30 in worms and 8 in flies; Bartke, 2008; Toivonen & Partridge, 2008). The *C. elegans* insulin/IGF-1-like receptor, PI3K, Akt kinase, and FoxO-family transcription factor are encoded by *daf-2*, *age-1*, *akt-1/2*, and *daf-16*, respectively (Kimura et al., 1997; Lin et al., 1997; Morris et al., 1996; Ogg et al., 1997). Worms with reduced IIS caused by mutations that decrease activity of either *daf-2* or *age-1* have a life span increased in a nonadditive, *daf-16*-dependent manner (Dorman et al., 1995; Kenyon et al., 1993). Life-span extension by reduced IIS therefore requires DAF-16, which regulates a diverse set of processes including fat storage, metabolism, development, fertility, and resistance to heat and oxidative stress (Finch & Ruvkun, 2001; Gems et al., 1998; Larsen, 1993). Extension of life span via mutation of *daf-2* also requires AAK-2, the catalytic subunit of the adenosine monophosphate-activated protein (AMP) kinase, and overexpression of *aak-2* is sufficient to increase life span (Apfeld et al., 2004).

Reduced IIS is thought to increase life span in worms, at least in part, by upregulating stress-response proteins. Long-lived worms with reduced IIS are resistant to multiple forms of environmental stress including reactive oxygen species, exposure to UV, and increased temperature (Martin et al., 1996; Murakami & Johnson, 1996). Transient heat shock is sufficient to extend life span (Butov et al., 2001; Lithgow et al., 1995; Michalski et al., 2001; Yashin et al., 2002) and causes nuclear localization of DAF-16 (Henderson & Johnson, 2001; Lin et al., 2001). Overexpression of the *C. elegans* heat-shock factor-1 (HSF-1) is sufficient to increase life span, and deletion of the *hsf*-1 blocks life-span extension from *daf-2* knockdown (Hsu et al., 2003; Morley & Morimoto, 2004). HSF-1 also activates multiple longevity genes including several that encode small heat-shock proteins (Hsu et al., 2003). Reducing IIS probably does not optimally activate the heat-shock response for life-span extension, however, as heat shock produces a further increase in life span and upregulation of small heat-shock protein genes in long-lived *age-1* mutants (Walker et al., 2001).

In addition to their role in aging, *daf-2* and *daf-16* regulate entry into the dauer larval stage, a long-lived alternate development pathway. Dauer larvae are sexually immature and characterized by a thick cuticle,

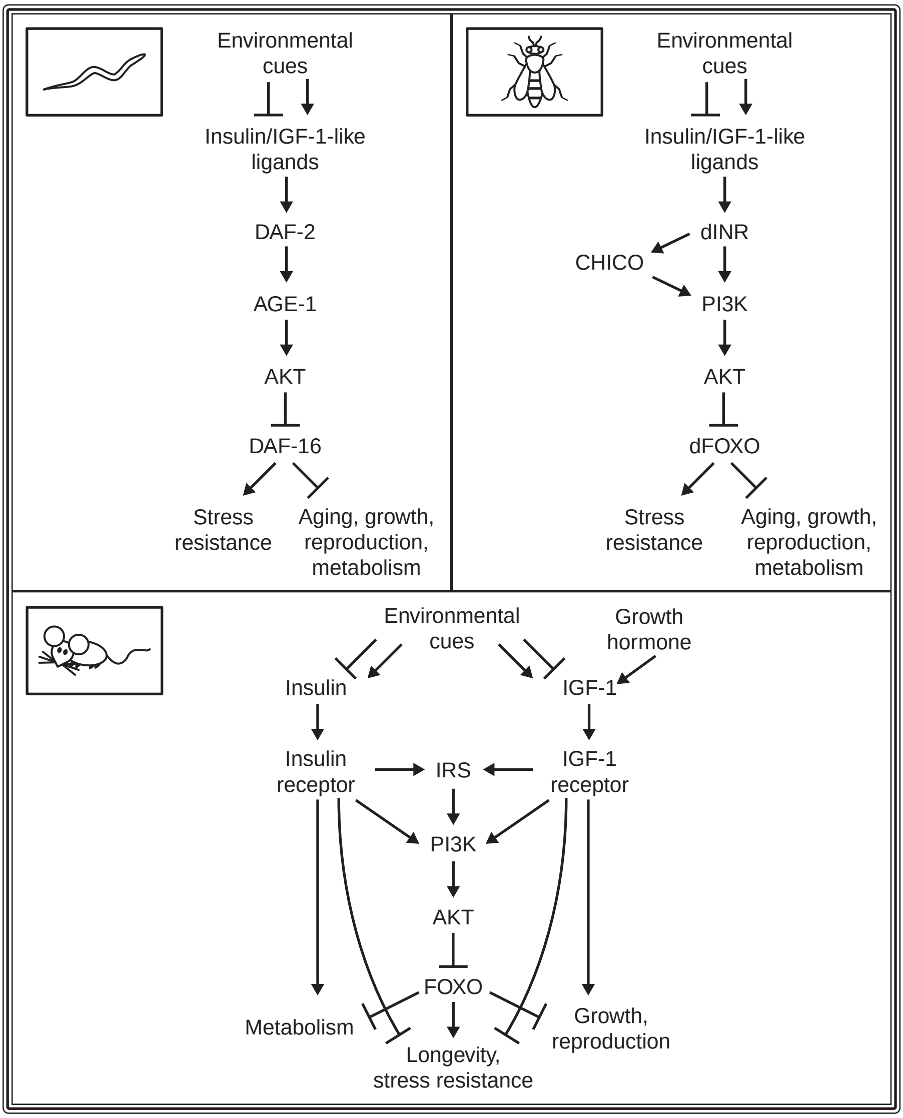
Environmental cues
Insulin/IGF-1-like ligands
DAF-2
AGE-1
AKT
DAF-16
Stress resistance
Aging, growth, reproduction, metabolism
Environmental cues
Insulin/IGF-1-like ligands
dINR
CHICO
PI3K
AKT
dFOXO
Stress resistance
Aging, growth, reproduction, metabolism
Environmental cues
Growth hormone
Insulin
IGF-1
Insulin receptor
IRS
IGF-1 receptor
PI3K
AKT
FOXO
Metabolism
Longevity, stress resistance
Growth, reproduction

Figure 10.1 Insulin and IGF-1-like signaling pathways play a conserved role in aging in nematodes, flies, and mice.

constricted pharynx, and sealed buccal and anal cavities, resulting in an inability to eat or defecate and an increased resistance to environmental stresses such as harsh chemical treatment and desiccation (Cassada & Russell, 1975; Riddle, 1988). At least three environmental factors contribute to the decision to enter the dauer larval stage: population density, temperature, and food availability (Golden & Riddle, 1982, 1984). Dauer larvae exposed to favorable environmental conditions (i.e., low population density, reduced temperature, and abundant food) resume development and proceed to become reproductively active adults. Complete inhibition of *daf-2* results in constitutive entry into the dauer larva stage regardless of environmental signals, and worms with mutations in *daf-16* fail to enter the dauer larva stage or do so inefficiently (Gottlieb & Ruvkun, 1994). As might be expected, dauer larvae share many similarities with worms that have reduced (but not abolished) IIS, including enhanced longevity and stress resistance, suggesting that the benefits of reduced IIS may represent an adult dauer-like state and may potentially be subject to the same trade-offs, such as reduced reproduction. Notably, the dauer response can be decoupled from the prolongevity effects of reduced IIS, as RNAi knockdown of *daf-2* starting well into adulthood—even postreproductively—dramatically increases life span without altering development or influencing reproductive potential (Dillin et al., 2002; Smith et al., 2008a).

Similar to worms, reduction of IIS signaling in flies via mutations in *InR*, the gene encoding the insulin/IGF-1-like receptor, or *Chico*, the gene encoding the insulin receptor substrate (IRS), increases stress resistance and longevity (Clancy et al., 2001; Tatar et al., 2001; Tu et al., 2002). The influence of IIS on life span appears to be partially gender specific in flies, as mutation of *InR* extends only the female life span (Tatar et al., 2001). In the *Chico* mutants, both heterozygous and homozygous female flies displayed increased life span, whereas only heterozygous males displayed increased life span, relative to wild type (Clancy et al., 2001; Tu et al., 2002). This is in contrast to worms, in which mutation of *daf-2* increases hermaphrodite and male life spans to similar degrees (Gems & Riddle, 2000). Transient heat shock also extends fly life span (Hercus et al., 2003). Furthermore, partial genetic ablation of the median neurosecretory cells (MNCs) both reduces expression of MNC-specific *dilp* (*Drosophila* insulin-like peptide) genes and increases life span (Broughton et al., 2005). This also suggests that the role of IIS in aging is cell nonautonomous. While it is not currently known whether these phenotypes are dependent on dFOXO, the *D. melanogaster* FoxO-family transcription factor (Junger et al., 2003; Kramer et al., 2003; Puig et al., 2003), there is some evidence pointing in that direction. Reduced cell division caused by mutations that decrease IIS require dFOXO (Junger et al., 2003), and adult fat-body-specific overexpression of dFOXO is sufficient to extend life span (Giannakou et al., 2004, 2007; Hwangbo et al., 2004).

Unlike invertebrates, mammalian IIS involves only three insulin-like peptides (insulin, IGF-1, and IGF-2), but three receptor peptides (one insulin receptor and two IGF-1 receptors) dimerize to form five types of dimeric receptors. These include separate receptors for insulin and IGF-1 ligands (Taguchi & White, 2008), both of which appear to play a role in determining life span. Female mice with a heterozygous IGF-1 receptor knockout live ~30% longer than wild-type mice (Holzenberger et al., 2003), while both male and female fat-specific insulin receptor knockout mice with an adipose-specific insulin receptor knockout live ~18% longer than wild type (Bluher et al., 2003). Growth hormone, which is not present in invertebrates, also appears to interact with IIS in modulating life span in mice. IGF-1 production is promoted by increased growth hormone activity and mice with mutations in the growth hormone receptor or defects in the pituitary gland (Ames and Snell dwarf mice) show reduced growth hormone and IGF-1 levels and increased life span relative to controls (Brown-Borg et al., 1996; Coschigano et al., 2003; Flurkey et al., 2002). As with flies, the role of FoxO proteins in mouse life-span extension from reduced IIS is unknown. However, FoxO proteins are known to function in IIS pathways that affect metabolism (Burgering & Kops, 2002) and have been implicated in the increased stress resistance of certain long-lived mouse strains (Nemoto & Finkel, 2002).

Interestingly, while insulin sensitivity is typically associated with longevity in mice, there are several examples of mutations that both increase insulin resistance and extend life span. These include *KLOTHO* overexpression (Kurosu et al., 2005), IRS1$^{-/-}$ knockout (Selman et al., 2008), and brain-specific IRS2 knockout (Taguchi et al., 2007). These findings are difficult to interpret in light of the potential for pleiotropic effects, as resistance to both insulin and IGF-1 was observed in all cases.

The evolution of multiple IIS pathways in mammals has several implications for the role of IIS in aging. Functions performed by the single IIS pathway in invertebrates that are related to life-span extension may be divided between the insulin and the IGF-1 branches of IIS in mammals. Indeed, while there is evidence for overlapping function, insulin signaling is primarily involved in regulating metabolism, while IGF-1 modulates growth and development (Kim & Accili, 2002; Rincon et al., 2005). Multiple pathways would also have eased pleiotropic evolutionary restrictions and allowed the insulin and IGF-1 branches to specialize further and/or acquire new functions.

Overexpression of Sirtuins

Sir2 orthologs (sirtuins) are present in organisms from yeast to humans and function as NAD-dependent protein deacetylases (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000; Tanner et al., 2000). Sir2 is a histone deacetylase that promotes transcriptional silencing at three specific loci in the yeast genome: the ribosomal DNA (rDNA), the silent mating (HM) loci, and regions near the telomeres (Aparicio et al., 1991; Bryk et al., 1997; Gottschling et al., 1990; Ivy et al., 1986; Rine & Herskowitz, 1987; Smith & Boeke, 1997). Unlike yeast, the reported substrates of Sir2 orthologs in multicellular eukaryotes appear to be primarily nonhistone and include endoplasmic reticulum-stress response factors (Viswanathan et al., 2005), FoxO-family transcription factors (Brunet et al., 2004; Motta et al., 2004; van der Horst et al., 2004), peroxisome proliferator-activated receptor γ coactivator 1α (Gerhart-Hines et al., 2007; Rodgers et al., 2005), p53 (Luo et al., 2001; Vaziri et al., 2001), and several others (Dali-Youcef et al., 2007; Finkel et al., 2009).

A role for Sir2 orthologs in aging was first demonstrated by the observation that overexpression of Sir2 is sufficient to increase yeast replicative life span (Kaeberlein et al., 1999). Subsequent studies demonstrated a similar longevity-enhancing effect associated with overexpression of *sir-2.1* in worms and dSir2 in flies (Rogina & Helfand, 2004; Tissenbaum & Guarente, 2001). To date, there has been no report showing that increased expression of SIRT1 in

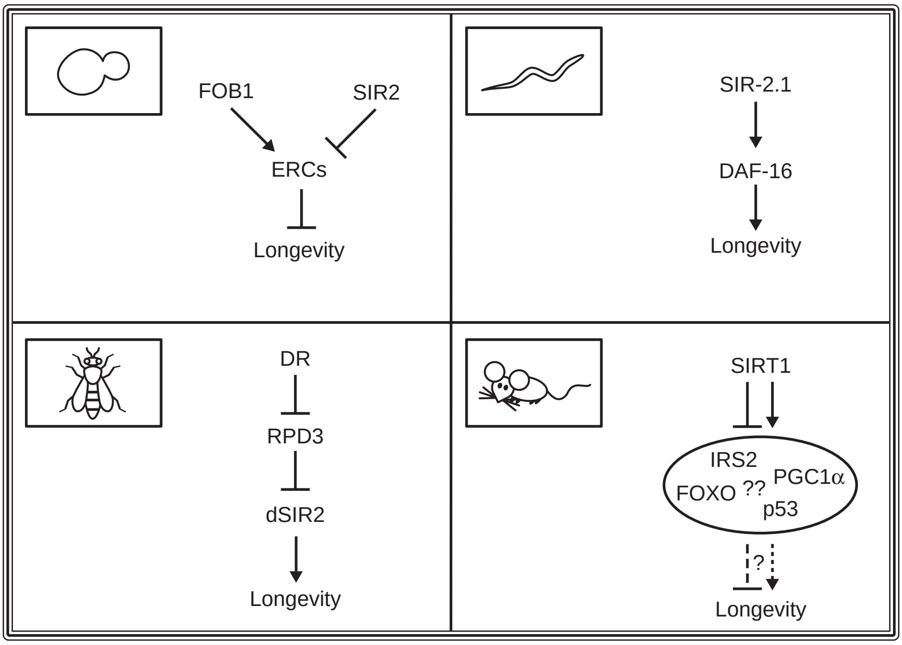

Figure 10.2 Sir2 orthologs promote longevity in yeast, nematodes, and flies by distinct mechanisms. The ability of SIRT1 overexpression to increase mouse life span has yet to be established, but SIRT1 influences a variety of age-associated phenotypes in mice, possibly via multiple substrate targets.

mammals is sufficient to increase life span, although SIRT1 transgenic mice are reported to have improved metabolic profiles (Banks et al., 2008; Bordone et al., 2007) and show resistance to colon cancer (Firestein et al., 2008).

One surprising feature of the longevity-promoting functions of sirtuins is the apparently distinct mechanisms by which they act in different organisms (Figure 10.2). In yeast, Sir2 is thought to slow replicative aging by promoting genomic stability in the rDNA and repressing the formation of extrachromosomal rDNA circles (Kaeberlein et al., 1999), one cause of replicative senescence in yeast cells (Sinclair & Guarente, 1997). Unlike yeast, there is no evidence that Sir2 orthologs modulate the formation of extrachromosomal rDNA circles in multicellular eukaryotes, nor are their data suggesting that rDNA circles cause aging in higher organisms. Instead, sirtuins appear to have evolved different prolongevity functions in these organisms. For example, in *C. elegans,* evidence suggests that *sir-2.1* modulates the downstream targets of IIS by interacting with *daf-16* in a 14-3-3-dependent manner (Berdichevsky et al., 2006; Wang et al., 2006). In flies, the relevant downstream targets of dSir2 have yet to be described, but it has been proposed that dSir2 acts in a longevity-promoting pathway with the Rpd3 histone deacetylase (Rogina & Helfand, 2004). Whether Sir2 orthologs really function to slow aging by different mechanisms in different organisms, or whether there exist as yet uncharacterized conserved sirtuin functions, is a question of continuing interest.

In contrast to the prolongevity effects associated with sirtuins, recent studies have suggested that sirtuin proteins may also promote aging in some systems or tissues. For example, yeast chronological life span is limited by Sir2 activity (Fabrizio et al., 2005; Kennedy et al., 2005). SIRT1-deficient mouse embryonic fibroblasts are highly resistant to replicative senescence and have increased replicative potential under chronic oxidative stress (Chua et al., 2005), in stark contrast to the observed decrease in replicative life span of yeast lacking Sir2 (Kennedy et al., 1995). A recent study found reduced IIS and Ras/ERK signaling in mice lacking SIRT1 (Li et al., 2008). Li et al. (2008) also found that SIRT1 knockdown enhanced oxidative stress resistance in mouse neuronal cell culture and that SIRT1 knockout mice had reduced oxidation of proteins and lipids in the brain, in contrast to the finding in flies that neuron-specific overexpression of dSir2 is sufficient for life-span extension (Rogina & Helfand, 2004), suggesting that both increasing and decreasing sirtuin activity may have neuroprotective consequences. These studies reinforce the idea that sirtuins perform different functions in different organisms and imply that the biology of sirtuins is more complex than initially suspected. Further effort will be required to unravel the intricacies of the action of sirtuins on longevity and to determine what similarities and differences exist between evolutionarily divergent species.

Reduced TOR Signaling

The TOR kinase is a highly conserved nutrient- and growth factor-responsive protein that is essential for viability in eukaryotic species (Stanfel et al., 2009). TOR was first identified as the molecular target of an antifungal compound (rapamycin) produced by the bacterium *Streptomyces hygroscopicus* (Vezina et al., 1975). Rapamycin was subsequently shown to inhibit the activity of protein products of two partially redundant yeast genes: TOR1 and TOR2 (Heitman et al., 1991). TOR proteins have since been identified in a variety of species, including humans, and have been shown to act in two distinct complexes: TOR complex 1 (TORC1) and TOR complex 2 (TORC2) (De Virgilio & Loewith, 2006; Martin & Hall, 2005). Although both TOR complexes are essential for viability (Guertin et al., 2006; Helliwell et al., 1998), only TORC1 is sensitive to rapamycin. It is currently thought that TOR-mediated longevity control occurs exclusively via altered TORC1 activity. TORC1 serves as a key regulatory nexus important for mounting an appropriate response to nutrients, growth cues, and cellular energy status (Wullschleger et al., 2006). TORC1 is activated by environmental nutrient availability in the form of both amino acids and glucose and is also responsive to IIS (through Akt) as well as the energy-sensing AMP-activated protein kinase (AMPK) (Arsham & Neufeld, 2006; Bhaskar & Hay, 2007).

The link between TOR and aging has been definitively demonstrated in four different organisms (Stanfel et al., 2009). Reduced TOR signaling is sufficient to increase life span in mice (Harrison et al., 2009), worms (Jia et al., 2004; Vellai et al., 2003), flies (Kapahi et al., 2004), and both yeast aging paradigms (Kaeberlein et al., 2005c; Powers et al., 2006). Aside from DR, TOR inhibition is the only intervention known to slow aging in each of these model systems (Kaeberlein & Kennedy, 2009). The importance of TOR signaling in yeast replicative and chronological life-span determination was uncovered from independent, unbiased longevity screens of the yeast ORF deletion collection. Deletion of *TOR1* was found to increase both replicative and chronological life span, as did pharmacological inhibition of TOR using rapamycin (Kaeberlein et al., 2005c; Powers et al., 2006). RNAi knockdown of the gene coding for TOR (*let-363*) or the TORC1 component raptor (*daf-15*) is sufficient to increase life span in worms (Jia et al., 2004; Vellai et al., 2003) and transgenic expression of a dominant-negative allele of TOR increases life span in flies (Kapahi et al., 2004). The effect of reduced TOR signaling on life span in a mammalian system was recently demonstrated by a study in which mice were fed a diet supplemented with rapamycin. Supplementation with rapamycin beginning at 600 days of age resulted in a significant increase in life span (Harrison et al., 2009).

The precise molecular mechanisms by which TOR signaling modulates aging in evolutionarily divergent organisms have yet to be completely characterized. Unlike the sirtuin pathway, components of TOR signaling are highly conserved both upstream and downstream of TORC1, including several TOR-regulated processes that have been suggested to play a role in longevity determination such as regulation of mRNA translation, autophagy, stress response, and mitochondrial metabolism. For example, autophagy is induced in a TOR-dependent manner by both DR and reduced IIS in *C. elegans* and is required for life-span extension in both cases (Hansen et al., 2008; Jia & Levine, 2007; Melendez et al., 2003). Altered TOR signaling is thought to be partially responsible for the beneficial effects of DR, which is discussed further below.

QUANTITATIVE EVIDENCE FOR CONSERVED MECHANISMS OF LONGEVITY CONTROL

A handful of conserved longevity factors have been known to exist for some time, but the degree to which mechanisms that control longevity are generally conserved between evolutionarily disparate organisms has only recently begun to be addressed. Unbiased genome-wide analyses of longevity in yeast and worms have afforded the first opportunities to assess the overlap between genes that modulate longevity in these two evolutionarily divergent organisms on a genomic level.

Demonstration of Conservation between Yeast and Worms

In a study published in 2008 in *Genome Research,* Smith et al. (2008b) took advantage of the large number of known longevity-associated genes in *C. elegans* to address the question of whether genetic control of aging has been conserved between yeast and worms. Underlying this analysis was the rationale that if genetic control of aging has been conserved, then yeast homologs of worm longevity-associated genes should have a greater likelihood of influencing longevity than randomly selected yeast genes. Since a majority of the known longevity-associated genes in *C. elegans* were derived from RNAi screens, Smith et al. (2008b) restricted their study to a set of 276 *C. elegans* genes reported to increase life span when expression or function is decreased. Yeast homologs of these genes could then be examined as deletion alleles and the corresponding effect on life span determined.

To identify orthologous gene pairs between worms and yeast, a two-tiered approach was taken. A high-stringency set of ortholog pairs was defined based on a modified reciprocal BLASTp best-match criterion.

Mapping of two yeast orthologs to one worm gene was allowed in cases in which BLASTp scores for yeast paralogs were within 10% of each other. A low-stringency set of homologs included all cases in which one or more yeast proteins could be identified with at least 20% sequence identity and 10% amino acid alignment to the worm aging protein, with a maximum of 6 yeast homologs allowed per worm gene. From 276 worm aging genes, 264 nonessential yeast genes (viable as single-gene deletions) were identified in the low-stringency homolog set, of which 78 also met the high-stringency ortholog criterion (Smith et al., 2008b).

Replicative life-span analysis was performed on each of the 264 single-gene deletion strains contained in the low-stringency homolog set. Using a rigorous iterative procedure for large-scale life-span analysis in yeast (Kaeberlein & Kennedy, 2005; Kaeberlein et al., 2005c), 25 single-gene deletions (9.5%) from this set were determined to be significantly long lived, of which 11 (14.1%) were also in the high-stringency ortholog set. In both sets, the frequency of long replicative life span significantly exceeded the frequency of long replicative life span observed in a study of 564 randomly chosen deletion strains (2.3%) (Kaeberlein et al., 2005c).

The results of Smith et al. allow for the conclusion that genetic control of longevity has been evolutionarily conserved between yeast and worms (Smith et al., 2008b). This study provides the first quantitative evidence for conservation of genetic determinants of aging. The nature of the aging models used in the comparison makes this finding particularly striking. Yeast replicative life span is a measure of mitotic aging. In contrast, cells in the adult *C. elegans* are completely postmitotic with the exception of the germ line. Such genetic conservation between mitotic and nonmitotic aging is not intuitively obvious. Demonstration of aging conservation also has important implications for human aging. On an evolutionary time scale, yeast and worms are separated by approximately 1.5 billion years, while worms and humans are separated by only approximately 1.0 billion years (Wang et al., 1999). We can thus speculate that a subset of genes that play a conserved role in aging in yeast and worms is likely to play a similar role in humans.

TOR Signaling Accounts for Many Conserved Longevity Factors

The most notable feature of the conserved longevity factors identified by Smith et al. (2008b) is the substantial enrichment for genes that code for proteins involved in regulating mRNA translation. Among the 25 ortholog pairs, only 2 were previously known to modulate aging in both yeast and worms: *TOR1/let-363* and *SCH9/rsks-1*. *SCH9* and *rsks-1* are homologs of mammalian ribosomal S6 kinase, which functions downstream of TOR signaling to modulate mRNA translation initiation (Pan et al., 2007; Urban et al., 2007). Excluding *TOR1/let-363* itself, 6 of the 10 remaining ortholog pairs in the high-stringency set can be definitively assigned functions related to mRNA translation: three ribosomal proteins of the large subunit (*RPL19A/rpl-19*, *RPL6B/rpl-6*, *RPL9A/rpl-9*) and three translation initiation factors (*TIF1/inf-1*, *TIF2/inf-1*, *TIF4631/ifg-1*). Given that TOR and S6 kinase are known to regulate positively both ribosome biogenesis and translation initiation factor activity, it is reasonable to speculate that all of these factors act in a single conserved longevity pathway (Figure 10.3).

The best evidence supporting the hypothesis that TOR signaling modulates longevity via regulation of mRNA translation comes from yeast replicative aging studies. Epistasis analysis clearly places *TOR1*, *SCH9*, and genes encoding ribosomal proteins of the large subunit (RPLs) into a single pathway (Stanfel et al., 2009). This is evidenced by nonadditivity of life-span extension when deletion of *TOR1* or *SCH9* is combined with a life-span-extending deletion of an RPL and Sir2-independent life-span extension from deletion of *TOR1*, *SCH9*, or RPLs (Kaeberlein et al., 2005c; Steffen et al., 2008).

Further support for this hypothesis has recently been provided by the identification of the nutritionally regulated Gcn4 transcription factor as a potential downstream mediator of life-span extension in response to reduced TOR signaling and altered mRNA translation (Kaeberlein et al., 2005c; Steffen et al., 2008). Cellular levels of Gcn4 are primarily controlled by translation and protein degradation (as opposed to transcription; Hinnebusch, 2005) and both RPL mutations and reduced TOR signaling have been shown to induce Gcn4 activity (Cherkasova & Hinnebusch, 2003; Foiani et al., 1991; Kubota et al., 2003; Martin-Marcos et al., 2007; Valenzuela et al., 2001). Steffen et al. (2008) showed that deletion of *GCN4* partially blocks replicative life-span extension in yeast lacking either an RPL or *TOR1*. Furthermore, Gcn4 activity was specifically upregulated in two long-lived RPL mutants (*rpl20bΔ* and *rpl31aΔ*) but not in mutants lacking paralogs of these genes that are not long lived (*rpl20aΔ* and *rpl31bΔ*), demonstrating that the increase in Gcn4 activity is linked to the increase in life span (Steffen et al., 2008).

A model placing TOR, S6 kinase, protein synthesis, and a Gcn4-like transcription factor in a linear pathway controlling life span may be overly simplistic on the broader stage of public mechanisms of longevity control. While both TOR signaling and TOR-regulated protein synthesis factors modulate life span in both yeast and worms (Smith et al., 2008b), longevity epistasis studies in *C. elegans* map *let-363* (TOR), but not *rsks-1* (S6 kinase) and translation initiation factors, to the same pathway as DR (Hansen et al., 2007). The results of Hansen et al. (2007) suggest that DR extends life span by reduced protein

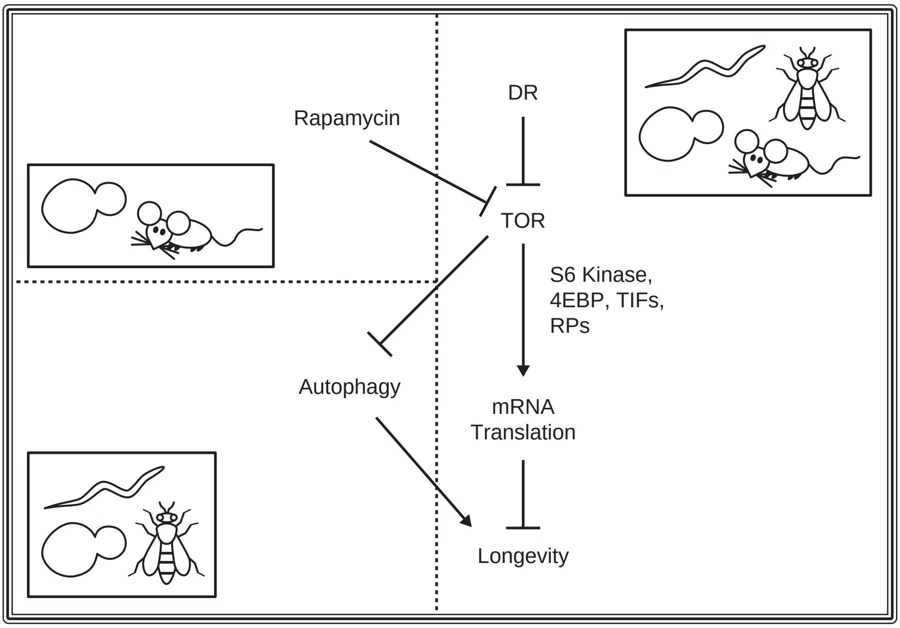

Figure 10.3 A reduction in TOR signaling extends life span in evolutionarily divergent organisms. Mutation of S6 kinase increases life span in yeast, nematodes, flies, and mice; the TOR inhibitor rapamycin increases life span in yeast and mice; and autophagy has been implicated in life-span extension from reduced TOR signaling in yeast, nematodes, and flies.

translation via TOR signaling, while knockdown of S6 kinase and other protein synthesis factors may act through a different mechanism.

Notably absent from the set of 25 conserved longevity ortholog pairs, not to mention any the genome-wide longevity screens in worms, are genes known to function in the same pathway as *SIR2*/*sir-2.1* (Curran & Ruvkun, 2007; Dillin et al., 2002; Hamilton et al., 2005; Hansen et al., 2005; Lee et al., 2003; Smith et al., 2008b). This lack of evidence for Sir2-related conservation of longevity control may reflect the limited understanding of upstream and downstream factors involved in *SIR2*/*sir-2.1*-mediated longevity control. It may also be due to the apparently dissimilar mechanisms by which Sir2 orthologs modulate longevity in different organisms, as discussed above.

INTERACTION BETWEEN DIETARY RESTRICTION AND CONSERVED LONGEVITY PATHWAYS

Life extension in response to DR was first observed in rats in 1934 (McCay & Crowell, 1934) and has since been demonstrated in a wide range of model systems. DR alters a multitude of physiological processes, and each of the conserved aging pathways discussed under Conserved Longevity Interventions—IIS, TOR signaling, and sirtuins—has been independently proposed to mediate the response to DR. In this section we discuss the relationship between DR and each of these pathways.

IIS: A Partial Interaction with DR with Respect to Secondary Aging Phenotypes

DR and mutations that reduce IIS have many phenotypic similarities, including enhanced longevity, stress resistance, reduced TOR signaling, and increased autophagic protein degradation. This is not surprising, since one of the major environmental factors to modulate IIS is nutrient availability. Thus, reduced IIS is a natural candidate for mediating the beneficial effects of DR. Interestingly, while DR and IIS clearly overlap, genetic studies have indicated that they also act through at least partially distinct mechanisms to control longevity.

The relationship between IIS and DR has been studied most extensively in *C. elegans*. Life-span extension from multiple approaches to DR, including bacterial dilution in liquid culture, axenic growth in liquid culture, bacterial deprivation, and mutation of *eat-2* (a genetic model resulting in reduced food intake due to decreased pharyngeal pumping), has been repeatedly shown to extend life span by a mechanism different from mutations that reduce IIS (Houthoofd et al., 2003; T. L. Kaeberlein et al., 2006; Lakowski & Hekimi, 1998; Lee et al., 2006). Specifically, all of these DR methods increase life span in animals lacking DAF-16. In contrast, one study found both DAF-16 and AAK-2 to be required for a specific method of DR, termed sDR, involving maintenance of worms on solid agar plates in the presence of diluted bacterial food (Greer et al., 2007). Another study found that mutations in *daf-2* produced increased growth and stress resistance in *eat-2* mutants (Iser & Wolkow, 2007). Similarly, growth impairment normally observed in response to DR was suppressed in *daf-2* mutants (Iser & Wolkow, 2007). Thus, IIS and most forms of DR are thought to act in parallel pathways to mediate longevity in worms, but have potential to interact downstream by influencing AAK-2 and DAF-16 activity under some circumstances.

In flies, the life span on a range of food concentrations of long-lived *Chico* mutants, which have reduced IIS, is right-shifted relative to wild type, meaning that *Chico* mutants are shorter lived than controls on low food concentrations and longer lived on normal to high food concentrations (Clancy et al., 2002). This was initially taken as an indication that DR requires IIS to extend life span. However, a 2008 study found that deletion of dFOXO did not block the ability of DR to extend life span (Giannakou et al., 2008). Overexpression of dFOXO in the adult fat body partially mimicked the long-lived *Chico* mutants in that the mutant flies were longer lived at normal to high food concentrations (Giannakou et al., 2008). Thus IIS and DR interact when IIS is active, but IIS is not required for DR to extend life span. One possible explanation is that *Drosophila* IIS does not act entirely through dFOXO, but influences life span through a different mediator.

IIS shares a complex relationship with DR in mice as well. Growth hormone receptor knockout (GHRKO) mice are longer lived than wild-type controls and have reduced levels of both insulin and IGF-1 (Coschigano et al., 2000; Liu et al., 2004; Zhou et al., 1997). GHRKO mice are not longer lived than wild-type mice subject to DR, nor do GHRKO mice show increased longevity or improved insulin sensitivity when subjected to DR, with the exception of an increase in maximum life span in females (Al-Regaiey et al., 2007; Bonkowski et al., 2006). In contrast, DR extends the lives of mice with pituitary mutations, which are defective for production of several hormones, including growth hormone (Bartke et al., 2001). This suggests that DR and IIS may act via partially distinct pathways, although it is also possible that they act via a similar mechanism, but that neither intervention optimally activates that mechanism with respect to longevity.

Sirtuins: A Complex and Unresolved Connection to DR

The connection between Sir2 and DR in yeast has been a source of controversy (Guarente, 2005; Kaeberlein & Powers, 2007; M. Kaeberlein et al., 2006; Kennedy et al., 2005; Lamming et al., 2005). Sirtuins were first proposed as mediators of DR based on the known role of Sir2 in yeast replicative aging and the discovery that Sir2 is activated in yeast in a NAD-dependent manner (Guarente, 2000). This hypothesis was supported by early evidence that reducing glucose in the medium did not extend the replicative life span of short-lived yeast lacking *SIR2* (Lin et al., 2000). An alternative interpretation of this result is that accumulation of ERCs in the *sir2Δ* strain causes enough damage that cells die before they can respond to DR, masking the life-span extension normally observed. Indeed, subsequent studies from independent labs found that Sir2 is not required for life-span extension by DR under conditions where ERC accumulation is reduced (Jiang et al., 2000; Kaeberlein et al., 2004; Kaeberlein & Powers, 2007; M. Kaeberlein et al., 2006; Lamming et al., 2005). More specifically, while DR does not extend life span of *sir2Δ* strains (Kaeberlein et al., 2004; Lin et al., 2000), suppression of the short-lived *sir2Δ* phenotype by deletion of *FOB1* allows robust life-span extension by DR (Kaeberlein et al., 2004). Combining DR with overexpression of Sir2 or deletion of *FOB1* also results in an additive life-span extension (Kaeberlein et al., 2005a). DR has therefore been shown to control longevity via at least one Sir2-independent mechanism in yeast, and two studies have reinforced this model that DR does not act through Sir2 by showing that Sir2 activity is not enhanced in vivo by DR (Riesen & Morgan, 2009; Smith et al., 2009).

In multicellular eukaryotes the interaction between DR and sirtuins is unresolved. Reports concerning *sir-2.1* and DR in worms are conflicting, but, with the exception of one study (Wang & Tissenbaum, 2006), support the idea that DR by a variety of methods does not require *sir-2.1* for life-span extension (Greer & Brunet, 2009; Hansen et al., 2007; T. L. Kaeberlein et al., 2006; Lee et al., 2006; Mair et al., 2009). Consistent with a model in which *sir-2.1* and DR act via distinct mechanisms, life-span extension by *sir-2.1* overexpression requires *daf-16* (Tissenbaum & Guarente, 2001), while life-span extension by DR does not (Lakowski & Hekimi, 1998). Thus, while the majority of evidence supports a model in which DR and *sir-2.1* act in parallel, further work will be required to determine definitively how *sir-2.1* interacts with DR in worms, if at all.

The situation in the published literature is less complicated in flies. Epistasis maps dSir2 to the same pathway as both DR and the histone deacetylase Rpd3 with respect to longevity (Rogina & Helfand, 2004), and both DR and reduced Rdp3 activity increase transcription of *dSir2* (Rogina et al., 2002). Unlike the case in other organisms, the role of dSir2 in the response to DR in flies has not been studied extensively, however, and would benefit from additional characterization.

The relationship between SIRT1 and DR in mice is complex. SIRT1 has been linked to both stress resistance and the regulation of metabolic processes, including hormone levels and fat storage, providing a potential connection to DR via diet and nutrient sensing (Guarente & Picard, 2005). While the effect of DR on longevity in mice with elevated SIRT1 levels is not known, knocking out SIRT1 in mice represses the increase in physical activity normally observed in response to DR (Chen et al., 2005) and prevents life-span extension from DR (Li et al., 2008). SirT1 mRNA and protein levels are reported to be increased in some tissues in response to DR, but there is evidence that DR also downregulates SIRT1 in some tissues. One study in mice looking specifically at the liver found that SIRT1 activity is decreased in response to DR and increased in response to high-fat diet (Chen et al., 2008). Liver-specific SIRT1 knockout mice are also partially protected from fat accumulation and have improved metabolic characteristics on a high-fat diet relative to wild-type animals with similar food intake (Chen et al., 2008).

A common approach used to study the interaction between sirtuins and diet is to look at the response to pharmacological activators of sirtuins. The most common is resveratrol, a potent small-molecule activator of Sir2 found in the skin of grapes and other plants (Howitz et al., 2003). Resveratrol has been reported to increase life span in yeast (Howitz et al., 2003), worms (Viswanathan et al., 2005; Wood et al., 2004), flies (Bauer et al., 2004; Wood et al., 2004), and one short-lived species of fish (Valenzano et al., 2006), though the findings in yeast, worms, and flies have proven difficult to replicate (Bass et al., 2007b; Kaeberlein et al., 2005b). In mice, resveratrol was protective against the health consequences of a high-fat diet (Baur et al., 2006; Lagouge et al., 2006). A potential confounding factor in studies using resveratrol is specificity. Resveratrol activates AMPK in addition to SIRT1, raising the question as to which effects are caused by increased SIRT1 activity and which are caused by increased AMPK activity (Baur et al., 2006). SIRT1720, a small-molecule activator of SIRT1 that does not activate AMPK and has improved potency relative to resveratrol, was identified in a small-molecule screen (Milne et al., 2007). Like resveratrol, SIRT1720 was found to protect mice fed a high-fat diet from developing obesity and insulin resistance by promoting oxidative metabolism in metabolic tissues (Feige et al., 2008). While feeding mice a high-fat diet cannot exactly be considered the opposite of DR, these studies do provide a clear link between diet and sirtuins, and high-fat diet may indeed be a more appropriate model for modern human societies. Notably, Feige et al. (2008) also observed transcriptional changes typically associated with low energy levels in response to treatment with SIRT1720, suggesting a potential link between sirtuins and DR.

TOR Signaling: A Conserved Mediator of Life-Span Extension by DR

Among genetic pathways that regulate life span, the evidence is most consistent for TOR signaling as a mediator of the response to DR (Stanfel et al., 2009). As noted above, reduced TOR signaling is the only intervention aside from DR that extends life span in mice, flies, worms, and both yeast paradigms. The TOR signaling pathway is also a conserved nutrient-responsive pathway (Kapahi & Zid, 2004) that has been observed to be inhibited, as measured through a reduction in autophagy and S6 kinase (S6K) activity, in response to DR in a variety of model organisms (Arsham & Neufeld, 2006; Bhaskar & Hay, 2007; De Virgilio & Loewith, 2006).

Findings in invertebrate models point strongly toward TOR signaling as a mediator of dietary restriction. In yeast, replicative life-span extension by DR and *TOR1* deletion is nonadditive (Kaeberlein et al., 2005c). Replicative life-span extension by DR, deletion of *TOR1*, and deletion of *SCH9* (yeast S6K) is additive with deletion of *FOB1* and independent of *SIR2* (Kaeberlein et al., 2004; Kaeberlein et al., 2005c; M. Kaeberlein et al., 2006; Tsuchiya et al., 2006), placing DR and TOR in a common pathway that is distinct from Sir2 and Fob1. Life-span extension from reduced TOR signaling and DR is similarly nonadditive in worms (Hansen et al., 2007). Studies have also found that autophagy induced by reduced TOR signaling is required for DR life-span extension in both worms (Hansen et al., 2008; Jia & Levine, 2007; Toth et al., 2008) and flies (Juhasz et al., 2007). A connection between DR and TOR signaling has not been tested directly in the yeast chronological paradigm, though one group has linked chronological life-span extension by deletion of *TOR1* to mitochondrial respiration (Bonawitz et al., 2007). Bonawitz et al. (2007) proposed a model in which DR derepresses respiration by inhibiting TOR signaling, leading to increased mitochondrial oxygen consumption and resulting in decreased damage from reactive oxygen species and extension of chronological life span.

As with IIS and sirtuins, a role for TOR signaling in mammalian response to DR has yet to be definitively demonstrated, though indirect evidence from several studies examining phenotypes in mice treated with rapamycin provides some cause for optimism about a connection between aging, DR, and TOR signaling. For example, treatment with rapamycin prevents weight gain in both humans and rats (Rovira et al., 2008) and improves resistance to cancer, neurodegeneration, and cardiac disease in mice (Gao et al., 2006; Wullschleger et al., 2006). DR has long been known to reduce the occurrence of cancer in rodents (Ross & Bras, 1965; Tannenbaum, 1942; Weindruch & Walford, 1982; Yu et al., 1982), is currently in clinical trials for treatment of cancer in humans (Weil, 2008), and has been found to suppress proteotoxicity in models of neurodegenerative diseases in nematodes (Steinkraus et al., 2008b).

Several studies have also examined the role of components of the TOR signaling pathway in the context of high-fat diet. A 2008 study found that mice with an adipose-specific knockout of raptor, an essential gene and specific component of the mammalian TORC1 (mTORC1) complex, were lean, had less adipose tissue, exhibited improved insulin sensitivity, and were resistant to diet-induced obesity relative to control mice (Polak et al., 2008). Polak et al. (2008) also found increased expression of genes encoding mitochondrial uncoupling proteins and heightened energy expenditure caused by an increase in uncoupled respiration, suggesting that mTORC1 regulates energy homeostasis by controlling adipose metabolism. Whole-body knockout of S6K, which is positively regulated by mTORC1, results in mice that are lean and have improved insulin sensitivity and resistance to age- and diet-induced obesity because of increased energy expenditure (Pende et al., 2000; Um et al., 2004). Consistent with these findings, whole-body knockout of 4E-BP1 and 4E-BP2, which are negatively regulated by mTORC1, results in obese mice that are hypersensitive to diet-induced obesity (Le Bacquer et al., 2007). These studies indicate that reduced TOR signaling is protective against the damaging effects of eating a high-fat diet, which is consistent with a model in which DR action on longevity is mediated by reduced TOR signaling. Chapter 2 provides further discussion of the relationship between TOR and DR.

CONCLUSIONS

The past few decades have seen the emergence of IIS, TOR signaling, sirtuins, and DR as important and evolutionarily conserved regulators of aging and longevity. Pharmacological agents that target components of these pathways, such as resveratrol and rapamycin, are being developed and tested for aging-related activities in model organisms. Clinical trials for some of these agents are already under way for treatment of cancer and diabetes and will probably be expanded to other age-related disorders. These trials mark the first clinical benefits derived from comparative genetics of aging in model organisms.

As key players in these conserved aging pathways continue to be uncovered and characterized using model systems, we will also gain a better understanding of how they function and interact to integrate environmental signals into cellular responses that modulate aging. It has become apparent that, although longevity interventions can be mapped to genetically distinct pathways through epistasis and other types of studies, in reality most (or all) of these conserved longevity modifiers interact within cells as part of a complex network. For example, TOR activity both modulates and is modulated by insulin-like signaling, while DR alters signaling through both pathways. In future studies, it will be important to consider not only which proteins play a conserved longevity role, but which interactions between longevity factors have also been conserved. Such an approach should make it possible to develop a more comprehensive picture of the overarching longevity network and may resolve lingering questions and controversies in the field while providing more effective routes toward therapies for improving human health span and longevity.

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Chapter | 11 |

Sirtuins in Aging and Age-Related Diseases

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CHAPTER CONTENTS

Introduction 243
Discovery of Sirtuins 244
Sirtuin Enzymology 245
Sirtuins and Aging 246
Sirtuins and Calorie Restriction 246
  Lower Organisms 247
  Mammals 247
Endogenous Modulators of Sirtuins 248
  Protein Abundance 248
  NAD^+, NADH, and Nicotinamide 248
  Protein–Protein Interactions 250
Chemical Inhibitors and Activators of Sirtuins 250
Sirtuins and Energy Metabolism 251
  SIRT1 and Energy Metabolism 251
  SIRT3 and Energy Metabolism 253
  SIRT4 and Energy Metabolism 254
  SIRT5 255
  SIRT6 255
Sirtuins in Cell Survival and Apoptosis 255
  SIRT1 as a Prosurvival Gene 255
  SIRT1 as a Tumor Suppressor 255
  Other Sirtuins in Cancer 258
Sirtuins and the Aging Cardiovascular System 258
Sirtuins in Inflammation and Immunity 260
Sirtuins in the Aging Brain 261
Perspective 262
Acknowledgments 263
References 263

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INTRODUCTION

For most of the 20th century, aging was thought to be too complex for any single gene or drug to have a significant impact on overall health and life span. This all changed in the early 1990s, when single-gene alterations were shown to increase dramatically the life span of simple laboratory organisms and even mice (Guarente & Kenyon, 2000). These were the first insights into the fact that eukaryotes possess regulatory pathways that influence an organism's life span in response to diet and biological stress. Such genes have been given the name "longevity assurance genes," or simply "longevity genes," and include components of the insulin/IGF-1 pathway, TOR (target of rapamycin) signaling, AMP-activated kinase (AMPK), and the sirtuins (Figure 11.1). These four main pathways, and presumably others yet to be discovered, are part of an interconnected network that senses the environment and adjusts how much energy an organism stores away for harsher times or utilizes for survival (Kirkwood & Shanley, 2005).

This chapter is about the sirtuins, a set of closely related longevity genes that are found in most living things, including bacteria, yeast, plants, and animals. Evidence strongly indicates that sirtuins have evolved to sense the environment and modulate cell-protective mechanisms, which include DNA break repair and base excision repair, energy utilization, autophagy, oxidative stress defenses, and protein folding, to maximize survival during times of adversity.

In yeast, nematode worms, and fruit flies, overexpression of the *SIR2* gene extends life span. This chapter focuses on diseases of aging and how sirtuins have the ability prevent them. The reason for this focus is that it remains unclear whether they by themselves

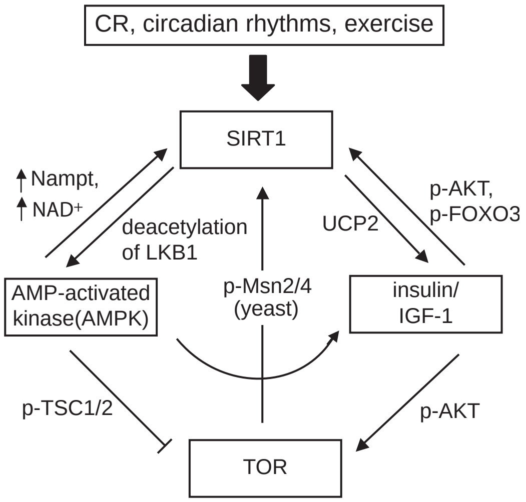

Figure 11.1 SIRT1 is part of an energy-sensing longevity control network. Four main pathways have emerged in recent years as key regulators governing health and life span in mammals: sirtuins, AMPK, TOR, and the insulin signaling pathways. The sirtuin SIRT1 forms a key component in this complex regulatory network. These interconnected pathways impact processes that control health and life span, including cell defenses, apoptosis, DNA repair, energy metabolism, and circadian rhythms. AMPK, AMP-activated kinase; TOR, target of rapamycin; IGF-1, insulin-like growth factor; LKB1, AMPK regulatory kinase; Msn2/4, yeast stress response transcription factors; SIRT1, NAD^+-dependent deacetylase, sirtuin 1.

can extend life span in mammals. What sirtuins do have is the unprecedented ability to prevent a wide variety of diseases that occur with aging, including type 2 diabetes, cancer, atherosclerosis, Alzheimer disease, and osteoporosis (Haigis & Sinclair, 2010; Lavu et al., 2008). Human genetic studies indicate that certain type of SIRT1 alleles influence susceptibility to diseases of aging such as metabolic diseases (Peeters et al., 2008; Weyrich et al., 2008; Zillikens et al., 2009). If sirtuins are able to slow these major diseases in humans, it seems inconceivable that average life span would not increase. But even if sirtuins merely extend health span, allowing us to live better, more productive lives, this would still be a major advance in medicine and one that is becoming increasingly appreciated as a primary goal of aging research.

DISCOVERY OF SIRTUINS

The sirtuins are some of the better-known longevity genes, but this was not always the case. The first sirtuin, *SIR2* from *Saccharomyces cerevisiae,* was originally known as *MAR1,* for mating-type regulator. Amar Klar and colleagues discovered *MAR1* by virtue of a spontaneous mutation that caused sterility by relieving silencing at the mating-type loci *HMR* and *HML* (Klar, 1979). Around the same time, Jasper Rine's lab was isolating a variety of mutations with a sterile phenotype and named the genes *SIR1* through *SIR4*, for silent information regulators (Ivy et al., 1985; Rine & Herskowitz, 1987; Shore et al., 1984). The *SIR* nomenclature ended up in common use, replacing MAR.

In 1991, Daniel Gottschling and colleagues showed that *SIR2*, *SIR3*, and *SIR4* are also required for silencing at telomeres and that this silencing tended to alternate back and forth, from a silent to an active state, in what is known as heritable epigenetic switching. Two years later, Braunstein and coworkers showed that silent regions at telomeres and mating-type loci are associated with histones that are relatively hypoacetylated at the ε-amino of N-terminal lysines (Aparicio et al., 1991; Braunstein et al., 1993). *SIR2* overexpression caused substantial histone deacetylation, an additional characteristic that distinguished *SIR2* from the other *SIR* genes. Around the same time, Gottlieb & Esposito (1989) demonstrated that *SIR2* is the only *SIR* gene required to suppress recombination between the 100–200 copies of tandemly repeated ribosomal RNA genes (rDNA). In 1995, four additional *S. cerevisiae* genes with high homology to *SIR2* were reported: *HST1–4* (homologs of *SIR2*;Brachmann et al., 1995; Derbyshire et al., 1996). In 1997, it was found that the rDNA was in a heterochromatic state that silenced marker genes inserted there and that *SIR2*, but not the other *SIR* genes, was essential for rDNA silencing. Thus, by 1997, the yeast field had shown that *SIR2* was required for the silencing and stability of three kinds of loci: *HM*, telomeres, and rDNA.

In the late 1990s, the sequencing of mammalian genomes identified *SIR2* homologs in mice and humans, demonstrating that *SIR2* is a member of a large, ancient family of genes we now refer to as "sirtuins." Despite their key role in yeast, *SIR3* and *SIR4* homologs have not been discovered in mammals. Mammals contain seven sirtuins, SIRT1–7 (Table 11.1), that were identified by their highly conserved central NAD^+-binding and catalytic domain (Frye, 2000). Mammalian sirtuins are found in a variety of subcellular compartments. SIRT1, SIRT6, and SIRT7 are found predominantly in the nucleus (Michan & Sinclair, 2007), whereas SIRT3, SIRT4, and SIRT5 reside in mitochondria (Haigis et al., 2006; Michishita et al., 2008; Nakagawa et al., 2009; Schwer et al., 2002, 2006), and SIRT2 is primarily cytoplasmic (Perrod et al., 2001). The subcellular localization of these proteins depends upon cell type, stress status, and molecular interactions.

Table 11.1 The mammalian sirtuins

SIRTUIN	LOCATION	INTERACTIONS	MAJOR FUNCTIONS	NULL PHENOTYPE
SIRT1	Nucleus/cytoplasm	p53, NF-κB, Ku70, FoxO, PGC-1α, etc.	Metabolism, stress resistance	Developmental defects, lethal
SIRT2	Cytoplasm	Tubulin, H4, FoxO	Cell cycle	Developmentally normal
SIRT3	Mitochondria	ACS2, GDH, complex I, LCAD	Thermogenesis, ATP	Developmentally normal, cardiac hypertrophy, long-chain fatty acid build-up
SIRT4[a]	Mitochondria	GDH, IDE, ANT	Insulin secretion	Developmentally normal
SIRT5	Mitochondria	CPS1	Urea cycle	Developmentally normal
SIRT6	Nucleus	Histone H3, NF-κB	Base excision repair, glucose metabolism	Premature aging-like, hypoglycemia
SIRT7	Nucleolus	RNA Pol I	rDNA transcription	Short life span, heart defects

[a]SIRT4 is the only sirtuin not known to have deacetylase activity. It may be exclusively a mono-ADP-ribosyltransferase.

SIRTUIN ENZYMOLOGY

The first reaction characterized for a sirtuin was a ribosyltransfer reaction catalyzed by a Sir2 homolog, cobB from bacteria, that converted 5,6-dimethylbenzimidazole to α-ribose-5,6-benzimidazole (Tsang & Escalante-Semerena, 1998). This finding led to the surprising discovery that sirtuins are NAD$^+$-dependent deacetylases and mono-ADP-ribosyltransferases. Both enzymatic activities require NAD$^+$ cleavage during each reaction cycle (Imai et al., 2000; Moazed, 2001; Sauve et al., 2001; Tanner et al., 2000; Tanny et al., 1999). Thus, sirtuin activity is intimately linked to the metabolic state of the cell. Thus far, the majority of studies involve the deacetylase activity of sirtuins. Most mammalian sirtuins catalyze NAD$^+$-dependent deacetylation (SIRT1, SIRT2, SIRT3, SIRT5, SIRT6, and SIRT7; Imai et al., 2000; Landry et al., 2000; North et al., 2003; Tanner et al., 2000), while SIRT4 catalyzes NAD$^+$-dependent mono-ADP-ribosyltransfer (Haigis et al., 2006; Liszt et al., 2005) (Figure 11.2). Both SIRT1 and SIRT6 have been shown to catalyze auto-ADP-ribosylation and substrate-specific deacetylation (Michishita et al., 2008), but the latter is by far the more active based on in vitro studies.

The mechanistic basis for deacetylation by sirtuins is both elegant and unprecedented. The reaction begins with an amide cleavage of NAD$^+$ and the formation of nicotinamide (NAM) and a covalent ADP-ribose (ADPR) peptide–imidate intermediate. The intermediate is resolved to form *O*-acetyl-ADP-ribose (AADPR or AAR) and the deacetylated substrate is released (Borra et al., 2004; Sauve et al., 2001; Schmidt et al., 2004). The amide-to-AADPR acyltransfer is energetically unfavorable but hydrolysis of NAD$^+$ can provide a favorable driving force for the overall sirtuin reaction (Sauve, 2009; Sauve et al., 2006; Smith et al., 2008). Although some details remain to be resolved, it is thought that peptide binding facilitates an allosteric change in enzyme structure that enables reaction of NAD$^+$ with a nucleophile from the enzyme to generate the enzyme-stabilized ADPR intermediate (Sauve et al., 2006).

For the mono-ADP-ribosylation reaction of sirtuins (Tanny et al., 1999), arginine is the major ADP-ribose acceptor and is not merely a side reaction of deacetylation (Fahie et al., 2009; Haigis et al., 2006; Hawse & Wolberger, 2009). The AADPR product is a novel metabolite that is poorly studied, but may have major physiological significance. For example, in yeast, AADPR promotes the assembly of the SIR complex, specifically Sir3 with the Sir2/Sir4 dimer, and catalyzes the spreading of the SIR complex across chromatin (Liou et al., 2005). In mammals, AADPR binds and activates the transient receptor potential melastatin-related channel 2 (Grubisha et al., 2006). AADPR can also be metabolized by nudix hydrolases in vitro and may be metabolized in vivo to acetate by an unidentified enzyme (Rafty et al., 2002). Thus, the metabolites of sirtuin-mediated deacetylation may hold functions that are biologically relevant to aging and are an important area of future discovery.

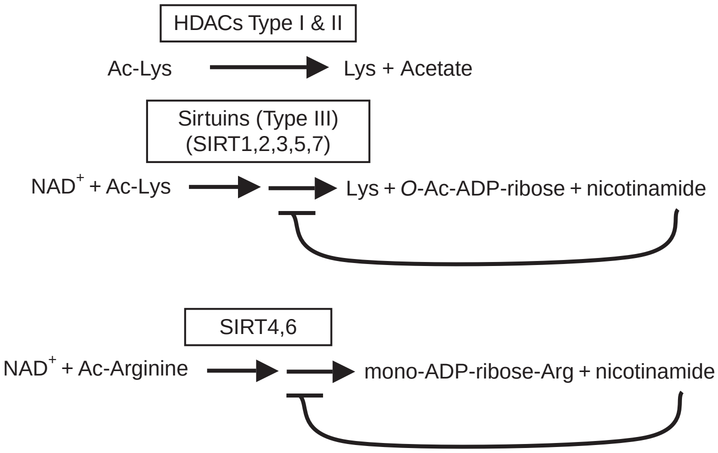

Figure 11.2 Sirtuins are class III deacetylases and mono-ADP-ribosyltransferases. Unlike the type I and II histone deacetylases, sirtuins require NAD^+ as a cosubstrate to remove an acetyl group from a lysine of a target protein (SIRT1, 2, 3, 5, 6, 7) or add an ADP-ribose subunit to an arginine (SIRT4). For amino acids already carrying an acetyl group, whereas arginine does not appear to be the major ADP-ribose acceptor in reactions using acetylated histone H1.1. Amide-to-ester acyltransfer is unfavorable but hydrolysis of NAD^+ can provide a favorable driving force for the overall sirtuin reaction. Evidence favors a mechanism in which electrophilic capture of the acetyl oxygen in an ADP-ribosyltransfer reaction forms an ADPR–peptidyl–imidate complex. This intermediate may last a few seconds, enough time for nocotinamide (NAM) to enter the "C-pocket" and catalyze the reverse reaction.

SIRTUINS AND AGING

The role of sirtuins in aging was discovered in the Guarente lab, which in the 1990s was studying the replicative life span of *S. cerevisiae* (Guarente, 2000). Replicative life span measures the number of times a yeast mother cell produces a daughter cell. The other measure is "chronological life span," which is the length of time cells remain viable under starvation conditions (Sinclair, 2002). In 1997, the cause of yeast replicative aging was identified: recombination at the rDNA locus results in toxic extrachromosomal rDNA circles (ERCs) that accumulate preferentially in aged mother cells. Based on the fact that *sir2* mutants have hyper-rDNA recombination, it was hypothesized that adding an extra copy of the *SIR2* gene should suppress ERC formation. In 1999, Guarente and colleagues published that an additional copy extends replicative life span by ≈30% by suppressing rDNA recombination and decreasing ERC accumulation. As predicted, deletion of *SIR2* increased ERC formation and shortened life span by about 50% (Kaeberlein et al., 1999).

Thomas Nystrom's group discovered a novel role for *SIR2* in the segregation of oxidized proteins to the mother cell (Aguilaniu et al., 2003; Liu et al., 2010). Sir2 controls the CCT chaperonin and how actin folds, promoting the clearance of damaged and aggregated proteins from daughter cells (Liu et al., 2010).

Since then, sirtuins have been discovered in the genomes of most species, ranging from bacteria to plants to mammals, and seem to have a conserved role in modulating health and life span (North & Verdin, 2004). The role of *SIR2* in aging is evident from studies involving more complex model organisms, such as *Caenorhabditis elegans* and *Drosophila*. In *C. elegans*, lifespan extension by *sir-2.1* requires the worm forkhead protein DAF-16 but may not require an intact insulin signaling pathway (Tissenbaum & Guarente, 2001; Wang & Tissenbaum, 2006). In response to stress, sir-2.1 binds to DAF-16 and activates it directly (Wang & Tissenbaum, 2006), a step that is controlled by two 14-3-3 proteins (Berdichevsky & Guarente, 2006; Y, Wang et al., 2006). Increasing the copy number of the *SIR2* ortholog in *Drosophila* also extends life span, an effect that can be achieved by overexpressing *dSir2* in neurons (Rogina & Helfand, 2004). The regulation of *dSir2* expression in response to diet is mediated in part by the fly p53 protein and its downstream target, the Rpd3 deacetylase, a *dSir2* repressor (Bauer et al., 2009; Rogina et al., 2002).

SIRTUINS AND CALORIE RESTRICTION

Calorie restriction (CR) increases life span in numerous species, from yeast to worms, flies, and mice (Sinclair, 2005). It is the most reproducible way to slow aging and delay the appearance of age-related diseases such as type 2 diabetes, cancer, and cardiovascular disease. The specific physiology of CR is comprehensively reviewed in Chapter 21 of this book and elsewhere (Sinclair, 2005). It is important to note that CR does not work in all species: exceptions include wild mice, some mouse genetic backgrounds, and house flies (Cooper et al., 2004; Harper et al., 2006). Even so, researchers believe there is validity in trying to understand how CR works so that some of the protective mechanisms might be utilized in medicine.

Lower Organisms

While most researchers agree that sirtuins are important for CR, there is some debate about their exact role. Life-span extension by CR does require Sir2 using the standard regimen of 0.5% glucose (Anderson et al., 2003; Lin et al., 2000). A more severe "CR" regimen (0.05% glucose) also extends yeast replicative life span, but does not require Sir2 or mitochondrial respiration (Kaeberlein et al., 2004, 2005a), potentially because of the *SIR2* homolog *HST2*, which can cover for a loss of *SIR2* under severe CR conditions. For yeast chronological life span, *SIR2* appears instead to reduce survival of certain exceptionally long-lived mutant strains such as *sch9* (Fabrizio et al., 2005).

In worms the role of sirtuins in CR is debated. Most worm diets that induce a state of calorie restriction have no requirement for *Sir-2.1* to extend life span. The one exception is a genetic mimic of CR called *eat-2*, which does require *Sir-2.1* for its long life span (Bishop & Guarente, 2007). For an excellent summary of pathways that mediate CR in the worm and how their functions overlap see Greer & Brunet (2009). *Sir2* in *Drosophila* (*dSir2*) is required for CR to extend life span: fly mutants lacking *dSir2* do not live longer when placed on CR, and there is no additive effect between CR and *Sir2* overexpression (Rogina & Helfand, 2004).

The Helfand and Rogina laboratories have been instrumental in understanding the links between *dSir2* and CR. Using complex genetics experiments, they have assembled a genetic pathway by which CR decreases the expression of the deacetylase Rpd3, a dSir2 repressor. The increase in dSir2 levels inactivates p53, which they show is sufficient to extend life span (Rogina & Helfand, 2004; Rogina et al., 2002). No additive effects of *dSir2* overexpression, dominant-negative p53, and caloric restriction are observed, arguing that they work via the same pathway (Rogina & Helfand, 2004). More recent work showed that *dSir2* is required to mediate increases not only in life span but also in the physical activity induced by calorie restriction (Parashar & Rogina, 2009), reminiscent of similar results in mammals using the SIRT1 knockout mouse (D. Chen et al., 2005).

The involvement of yeast *SIR2* in CR-mediated life-span extension led researchers to ask: how are sirtuins regulated by diet? Because the amount of yeast Sir2 protein does not increase during CR (Anderson et al., 2003), other explanations have been proposed for the apparent increase in Sir2 activity in response to this diet. The Guarente laboratory has proposed that during CR, a reduction in NADH, an inhibitor of Sir2, results in increased Sir2 activity (S. J. Lin et al., 2004). The Sinclair and Smith laboratories proposed that the increase in Sir2 activity is due to upregulation of *PNC1* during CR, which depletes NAM and increases flux through the NAD^+ salvage pathway (Anderson et al., 2002, 2003; Gallo et al., 2004; Medvedik et al., 2007). Interestingly, *PNC1* is also upregulated by mild stresses that extend life span such as increased temperature (37°C) and nitrogen restriction. These data are seen as evidence that CR is a form of hormesis, or a mild stress that induces a beneficial defense response (Masoro, 2000; Rattan, 2004; Sinclair & Howitz, 2006). The NADH and *PNC1* mechanisms are fundamentally different: the former is a passive mechanism, whereas the latter is an active genetic pathway that responds specifically to stress.

Mammals

The findings in lower organisms have generated considerable interest in the potential role of the mammalian sirtuins in CR-mediated physiology. In rodents and humans, fasting and CR result in an increase in SIRT1 expression in a variety of tissues, including brain, kidney, liver, white adipose tissue, and skeletal muscle (Civitarese et al., 2007; Cohen et al., 2004b; Nemoto et al., 2004; Nisoli et al., 2005; Rodgers et al., 2005). SIRT1 increases are not observed in all CR studies and may be induced in a tissue-specific manner or even decreased (Chen et al., 2008). It is interesting to note that NAD^+ levels are also increased in some tissues during CR. Thus, it is likely that in many tissues, a combination of boosting SIRT1 protein levels and NAD^+ concentration (or flux though the NAD salvage pathway) contributes to increased activity of this sirtuin during CR.

Genetic manipulations of SIRT1 have further validated the link between this gene and CR. The Guarente lab was the first to show that Sirt1 is required for the induction of a phenotype by calorie restriction: the increase in physical activity (D. Chen et al., 2005). Transgenic mice that overexpress SIRT1 also display several metabolic benefits that overlap with CR phenotypes. For example, the first study using a knock-in mouse model with SIRT1 expression driven by the β-actin promoter demonstrated that SIRT1-overexpressing mice are leaner, are more glucose tolerant, and display reduced levels of blood cholesterol, adipokines, and insulin compared to wild-type controls (Bordone et al., 2007).

In another series of elegant studies, wild-type or mutant SIRT1 was overexpressed in mice using its own promoter from a bacterial artificial chromosome. These animals were used to demonstrate that elevated SIRT1 did not improve basal glucose tolerance, but attenuated obesity-induced glucose intolerance (Banks et al., 2008). Another study showed that transgenic SIRT1 mice are resistant to liver steatosis (fatty liver) and insulin resistance (Pfluger et al., 2008). These SIRT1 phenotypes are similar to, and were predicted by, the effects of treating mice with SIRT1 activators such as resveratrol and SRT1720 (Baur et al., 2006; Lagouge et al., 2006; Milne et al., 2007). As many of these studies were performed using whole-body transgenic animals it will be useful to identify tissues that drive these protective phenotypes. Thus far, there is no study indicating that overexpressing SIRT1 can extend mean or maximum life span, only health span. Treatment of mice with resveratrol does extend life span on normal chow when the diet is provided every other day (Pearson et al., 2008). The reason feeding every other day rather than ad libitum enhances the effects of resveratrol are not yet known.

SIRT1 null mice also have provided evidence that SIRT1 mediates aspects of CR. The first study investigated the behavior of mice fed a CR diet. CR causes an increase in activity in wild-type mice, whereas whole-body SIRT1 null mice do not show this increase (Boily et al., 2008; D. Chen et al., 2005). Recently, life spans of SIRT1 null mice have been measured. SIRT1 null mice have a shorter life span than their wild-type littermates, and CR does not increase the life span of these animals (Li et al., 2008). These data provide important evidence that SIRT1 may be required for life-span extension by CR in mammals. However, firm conclusions are complicated by the fact that SIRT1 null inbred mice that survive to adulthood die prematurely and have developmental defects (McBurney et al., 2003). Future life-span studies using SIRT1 tissue-specific and whole-body knockout mice will be important to ascertain the role of SIRT1 in mediating aspects of CR physiology.

ENDOGENOUS MODULATORS OF SIRTUINS

The discovery that Sir2 has enzymatic activity has led to a great deal of effort to identify small molecules and macromolecules that modulate the activity sirtuins. Some of these are endogenous regulators, whereas others come from other species. The most potent activators have been identified in large-scale chemical library screens and then modified by medicinal chemistry efforts. Modulators of sirtuin activity fall into one of five categories: transcriptional regulators, cosubstrates and enzymatic products, posttranslational modifications, and protein–protein interactions (Figure 11.3). Small-molecule inhibitors and activators are discussed under Chemical Inhibitors and Activators of Sirtuins.

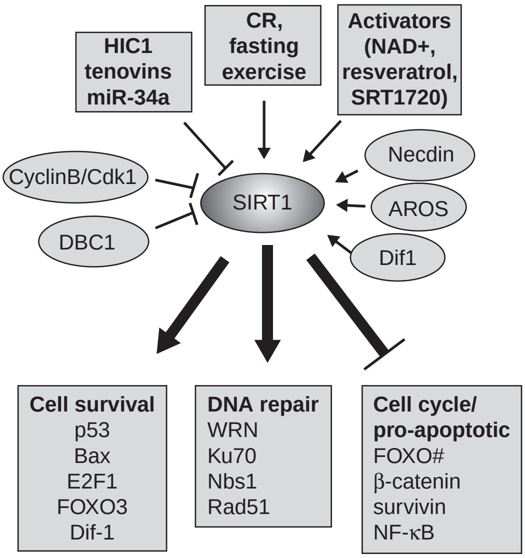

Figure 11.3 Multiple points of regulation of SIRT1. The SIRT1 gene is under the control of environmental stimuli, such as fasting and exercise, which increase Nampt and NAD$^+$ levels, as well as miRNAs, tenovins, the HIC1 transcriptional repressor, and direct phosphorylation. The SIRT1 enzyme can also be modulated by protein–protein interactions with DBC1, AROS, Dif1, and Necdin or by small molecules such as resveratrol and SRT1720 that increase activity by lowering the K_m for the substrate.

Protein Abundance

Like yeast Sir2, mammalian sirtuins are highly regulated at the posttranscriptional level, but unlike yeast, mammalian sirtuins are also transcriptionally regulated. SIRT1, SIRT3, and Nampt, for example, are induced by calorie restriction, fasting, and exercise (Brunet et al., 2004; Cohen et al., 2004b; Costford et al., 2009; Koltai et al., 2009). Of all the sirtuin gene promoters, SIRT1 is most fully characterized, being regulated by p53 (Nemoto et al., 2004), c-Myc (Yuan et al., 2009), CtBP:HIC1 corepressor (Zhang et al., 2007), TLX (Iwahara et al., 2009), E2F1 (C. Wang et al., 2006), and BRCA1 (Wang et al., 2008b). SIRT3 protein levels increase 5- to 10-fold in rodent skeletal muscle in response to caloric restriction and physical exercise (Palacios et al., 2009). SIRT3 levels are also induced by low-nutrient conditions (Hirschey et al., 2010). The regulation of the other sirtuins at the transcriptional and translational levels is less well understood.

NAD$^+$, NADH, and Nicotinamide

Alterations in NAD$^+$ levels and the NAD$^+$/NADH ratio have been shown to affect the activity of sirtuins in both yeast and mammals (Nakahata et al., 2009; Revollo et al., 2004; H. Yang et al., 2007). Though

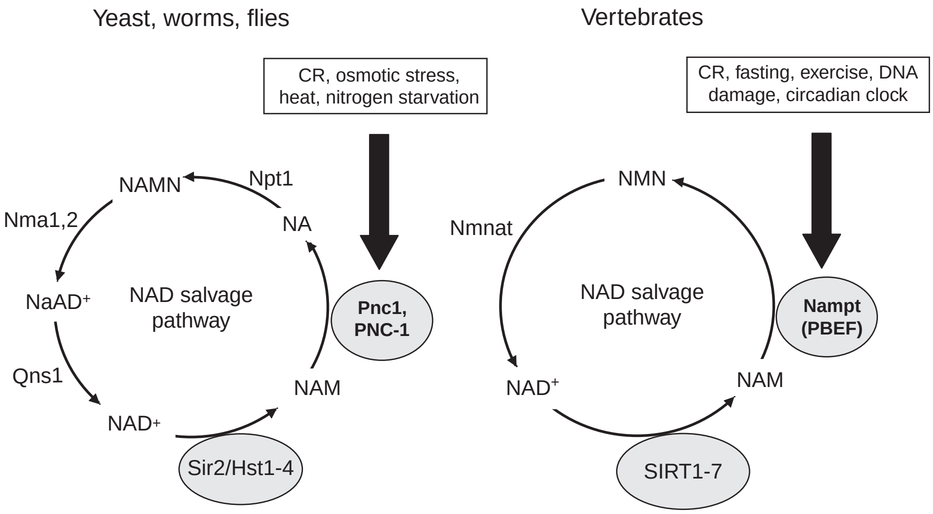

Figure 11.4 Pnc1 and Nampt are key regulators of sirtuin activity in response to energy and stress. Mammals recycle NAD from nicotinamide (NAM) in two steps rather than four, bypassing the production of nicotinic acid (NA). Pnc1 and Nampt are functionally equivalent genes that catalyze the first step in the salvage pathway and respond to stress and diet and, in turn, upregulate sirtuin activity (H. Yang et al., 2006). Pnc1 was shown to be necessary and sufficient for life-span extension by CR and stress. Like its yeast counterpart, Nampt is induced by fasting and exercise and cycles every 24 h as part of the circadian clock. Pnc1, NAM deaminase; Nampt, NAM phosphoribosyltransferase; Nmnat, nicotinamide mononucleotide adenylyltransferase.

NADH is a relatively weak inhibitor of sirtuins in vitro (Jackson et al., 2003; Schmidt et al., 2004), genetic studies in yeast indicate that NAD^+/NADH can modulate sirtuin activity (Lin et al., 2004). A product of the sirtuin reaction, NAM is also an important regulator of sirtuin activity (Bitterman et al., 2002; Sauve et al., 2005; Sauve & Schramm, 2003). The mechanism of inhibition involves NAM entering a highly conserved "C-pocket" adjacent to the NAD^+ binding site (Avalos et al., 2004; Bitterman et al., 2002) and reacting with the peptide-imidate intermediate of the reaction, thus driving the reaction in reverse (Sauve & Schramm, 2003). Adding another level of control, the sensitivity of sirtuins to NAM can also be modulated by protein–protein interactions (Tanny et al., 2004).

The Sinclair and Smith labs independently found that NAD^+ biosynthetic pathways are also critical for the regulation of sirtuins and life span in yeast (Anderson et al., 2002, 2003) (Figure 11.4). In yeast, worms, and flies, NAM is recycled back to NAD^+ in four steps, the first of which is catalyzed by the nicotinamidase Pnc1 to produce nicotinic acid. *PNC1* is upregulated in response to environmental stress such as heat and calorie restriction, leading to increased stress resistance and life span (Anderson et al., 2003; Balan et al., 2008; Gallo et al., 2004). Thus, *PNC1* connects environmental stress to life span and fits with the view that life-span extension by stress and diet is the result of an ancient survival response controlled by a few master regulatory genes. Another NAD^+ precursor, nicotinamide riboside (NR) is found in yeast and mammalian cells and, when supplied exogenously to yeast, can also extend life span (Belenky et al., 2007; Bieganowski & Brenner, 2004).

Instead of four steps, mammals regenerate NAD^+ from NAM in two steps: a NAM phosphoribosyltransferase called Nampt (visfatin or PBEF) converts NAM to nicotinamide mononucleotide (NMN) (Revollo et al., 2004; Rongvaux et al., 2002). NMN is then utilized by Nmnat1, 2, and 3 to regenerate NAD^+ in the nucleus, Golgi, and mitochondria, respectively (Berger et al., 2005). Consistent with the ability of *PNC1* to regulate Sir2 in yeast, mammalian Nampt is one of the main regulators of SIRT1 activity and provides cell protection in vivo (Revollo et al., 2004; H. Yang et al., 2006, 2007; T. Zhang et al., 2009). Interestingly, the enzyme downstream of Nampt, Nmnat-1, interacts directly with SIRT1 at promoters, suggesting that there may be local concentrations of NAD^+ or direct transfer of NAD^+ to SIRT1 (T. Zhang et al., 2009). Consistent with Nampt being a central regulator of stress, energy status, and survival, Nampt is induced by numerous environmental factors, including exercise (Costford et al., 2009; Koltai et al., 2009) and fasting (H. Yang et al., 2006, 2007), and is a central player in the 24-h circadian clock (Nakahata et al., 2009; Ramsey et al., 2009).

Interestingly, Nampt is also found in serum, and this form is known as visfatin or extracellular NAMPT (Fukuhara et al., 2005). Imai has hypothesized that the product of Nampt, NMN, acts as a signaling molecule that allows stressed or nutrient-deprived cells to communicate with other parts of the body (Imai, 2009). This concept is of considerable interest, especially given the possibility of using NMN, or a downstream molecule such as NR, as a therapeutic for type 2 diabetes or other diseases of aging (Belenky et al., 2009; Bogan & Brenner, 2008; Imai, 2009).

Sir2 from *S. cerevisiae* is highly stable in its abundance, even when cells are calorically restricted; its activity is regulated primarily at the posttranslational level. This includes the relocalization of the Sir2/3/4 complex between various silent loci, alterations in the concentrations of cosubstrates and inhibitors such as NAD^+ and NAM, and physical interaction with Sir4, which stimulates activity fivefold (Tanny et al., 2004). Interestingly, the sensitivity of Sir2 to NAM at different loci varies, suggesting that protein associations can alter the sensitivity of sirtuins to small regulatory molecules.

Protein–Protein Interactions

A surprising number of modulators have been identified for SIRT1. The activator AROS (active regulator of SIRT1) binds to the N-terminus, stimulating the activity of SIRT1 (E. J. Kim et al., 2007). Another SIRT1 activator is the maternally imprinted p53-interacting protein, expressed predominantly in postmitotic neurons, from a melanoma antigen protein family that promotes neuronal differentiation and survival (Hasegawa & Yoshikawa, 2008). The current model is that necdin downregulates p53 acetylation levels by forming a stable complex with p53 and Sirt1 to protect neurons from DNA damage-induced apoptosis.

A negative regulator of SIRT1 that also binds in the N-terminus of SIRT1 is DBC1 (for deleted in breast cancer 1; Kim et al., 2008; Zhao et al., 2008). DBC1-mediated repression of SIRT1 leads to increased levels of acetylated p53 and upregulation of p53-mediated apoptosis. Whether a mammal would benefit from deletion of DBC1 (to enhance SIRT1's metabolic function) or increased expression of DBC1 (to kill cancer cells) is unclear but analysis of the mouse DBC1 knockout should provide clues.

CHEMICAL INHIBITORS AND ACTIVATORS OF SIRTUINS

The involvement of sirtuins in critical biological processes and the possibility that they mediate some of the benefits of CR has raised the possibility of using small-molecule modulators of sirtuins to treat diseases of aging. Animal studies indicate that a molecule that activates SIRT1, for example, might be effective in treating and preventing type 2 diabetes, atherosclerosis, some forms of cancer, cataracts, osteoporosis, and a wide variety of inflammatory disorders (Lavu et al., 2008). Though it is not feasible to obtain regulatory approval for a drug that slows aging, the hope is that ultimately these medicines will be shown to prevent many diseases, thus increasing overall health span and possibly life span.

There are a variety of small-molecule modulators of sirtuins, both natural and synthetic. Inhibitors of sirtuins include the synthetic inhibitors splitomycin, tenovin-6 (Lim, 2006), salermide (Lara et al., 2009), sirtinol (Solomon et al., 2006), and sirtinol derivatives (Mai et al., 2005) and the SIRT1-specific inhibitor EX-527 (Solomon et al., 2006). SIRT1-activating compounds (STACs) include the natural molecules resveratrol, fisetin, quercetin, and the synthetic SRT1720, SRT2104, and SRT2183, and analogs of NAM (Howitz et al., 2003; Milne et al., 2007; Smith et al., 2009). NAM analogs bind in the regulatory C-pocket of sirtuins and increase the $V_{\max}$ of the enzyme. Allosteric activators such as resveratrol and SRT1720 work by lowering the K_m for the substrate and for NAD^+ (Howitz et al., 2003; Milne et al., 2007).

Although the STACs clearly activate SIRT1 in vitro via an allosteric mechanism (Borra et al., 2005; Howitz et al., 2003; Kaeberlein et al., 2005b; Milne et al., 2007; Pacholec et al., 2010), it is debated as to whether they act directly on SIRT1 in vivo. One reason for the debate is the in vitro assay. Sirtuin assays for activation have typically utilized peptide substrates that have additional groups on them, such as 7-amino-4-methylcoumarin or tetramethylrhodamine (Howitz et al., 2003; Milne et al., 2007; Nayagam et al., 2006), though these groups are not necessary to detect activation of SIRT1 by small molecules (Galonek et al., 2009). Some researchers find that SIRT1 activation in vitro requires a fluorophore on the substrate, leading them to continue to question this assay and therefore whether STACs hit their target in vivo (Funk et al., 2010; Pacholec et al., 2010). Sauve and colleagues speculate that the fluorophore reproduces biophysical properties of native substrates in cells, which provides a cognate binding site for compounds like resveratrol in vivo (Sauve, 2009). SIRT1 small-molecule activators require the SIRT1 N-terminus, raising the possibility of a common mechanism of control with DBC1 and AROS (Malik et al., 2010; Milne et al., 2007). Though debates continue about the activation mechanism, the literature strongly supports the view that resveratrol and the synthetic STACs activate SIRT1 and they give rise to the physiological and gene expression signature of CR (reviewed in Baur et al., 2010).

With regard to resveratrol and related polyphenols such as fisetin and butein, these molecules have been shown to extend life span reproducibly and robustly in a wide variety of organisms, from yeast to *C. elegans* to flies, and this requires the *SIR2* gene (Bauer et al., 2004; Jarolim et al., 2004; Viswanathan et al., 2005; Wood et al., 2004), though some labs were unable to see robust life-span extension (Bass et al., 2007; Kaeberlein et al., 2005b).

In mammals, there is abundant evidence to support the findings that STACs activate SIRT1 in vivo. There are now over 25 studies showing that STACs require SIRT1 in vivo and that they mimic SIRT1 overexpression (Banks et al., 2008; Baur et al., 2006; Boily et al., 2009; He et al., 2010; Lagouge et al., 2006; Lin et al., 2010; Nie et al., 2009; Pfluger et al., 2008; Smith et al., 2009; Sulaiman et al., 2010; Sun et al., 2007; Wang et al., 2008b; Wood et al., 2004). One compelling study in mammals linking resveratrol to SIRT1 comes from the McBurney lab and shows that the ability of resveratrol to prevent skin cancer is significantly reduced in the SIRT1 knockout mouse (Boily et al., 2009).

Treatment of mice with resveratrol prevents liver steatosis (fatty liver), increases insulin sensitivity, and delays cataracts, osteoporosis, kidney damage, and cardiovascular disease and improves performance on the rotorod and treadmill (He et al., 2010; Pfluger et al., 2008). On a high-fat diet, starting treatment from 1 year of age, resveratrol increases life span by up to 24%. Resveratrol also increases life span on a normal diet when it is given every other day but not if the mice feed ad libitum (Pearson et al., 2008).

Consistent with the effects of resveratrol and the SIRT1 transgenic mouse (Banks et al., 2008; Pfluger et al., 2008), independent studies have reported that SRT1720, which bears no structural resemblance to resveratrol, also prevents steatosis and increases insulin sensitivity in mice and rats, without any toxicity (Feige et al., 2008; Milne et al., 2007; Yamazaki et al., 2009), though a more recent paper saw no effect on glucose levels and some lethality of the compound in mice (Pacholec et al., 2010). This study also contrasts with one in which mice were fed SRT1720 from 1 year of age. These mice showed increases in insulin sensitivity and there was no sign of toxicity (Rafa de Cabo, National Institutes of Health, personal communication). In the context of a high-fat diet, SRT1720 extended both mean and maximum life span.

Consistent with a role for SIRT1 in the mobilization of fatty acids from white adipose tissue (WAT), treatment of mice on a high-fat diet with resveratrol or SRT1720 also reduces weight gain (Lagouge et al., 2006; Milne et al., 2007). A reduction in inflammation in macrophages of WAT was also observed, consistent with the lowering of blood glucose (Yoshizaki et al., 2009). Intranasal treatment with a selective STAC, SRT2172, prevented pulmonary neutrophilia and the reduction in exercise tolerance in a mouse smoking model of chronic obstructive pulmonary disease (COPD), ostensibly by blocking an increase in matrix metalloproteinase-9 (Nakamaru et al., 2009). This work indicates that SIRT1 activation could be a useful therapeutic approach to treating chronic inflammatory diseases such as COPD. Another positive benefit of SRT1720 and resveratrol is muscle type switching and increased mitochondrial respiratory activity, with a concomitant increase in grip strength and endurance.

As described in more detail below, SIRT1 and AMPK work together to modulate glucose metabolism, whereby SIRT1 regulates an AMPK kinase and AMPK increases NAD^+ for SIRT1. Upon treatment of cells and animals with resveratrol or SRT1720, both SIRT1 activation and AMPK activation are observed (Baur et al., 2006; Canto et al., 2009; Dasgupta & Milbrandt, 2007; Lin et al., 2010; Narala et al., 2008; Suchankova et al., 2009). Two groups have seen SIRT1 activation by SRT1720 or resveratrol with no increase in AMPK phosphorylation (Feige et al., 2008; Funk et al., 2010), suggesting that AMPK is not necessary for SIRT1 activation by these molecules. Multiple studies have reported that mice fed resveratrol or SRT1720 have gene expression profiles that are highly similar to those of mice on CR, arguing that these molecules are CR mimetics (Barger et al., 2008; Baur et al., 2006; Pearson et al., 2008; Smith et al., 2009).

SIRTUINS AND ENERGY METABOLISM

SIRT1 and Energy Metabolism

A growing area of sirtuin research is focused on their role in the regulation of metabolism. While the scope and detail of SIRT1 functions are not yet fully elucidated, overwhelming evidence suggests that this enzyme senses nutritional availability and relays this information to proteins that govern fuel utilization. SIRT1 is expressed in most tissues and regulates key signaling proteins involved in energy metabolism (Figure 11.5). For example, SIRT1 binds to and deacetylates a number of important transcription factors, such as peroxisome proliferator-activated receptor (PPAR) γ, PPARα, PPARγ coactivator (PGC)-1α, and the FoxO (forkhead) family of transcription factors, to drive metabolic responses such as insulin secretion, gluconeogenesis and fatty acid oxidation (Canto & Auwerx, 2008, 2009; Feige et al., 2008; D. Kim et al., 2007). Though most researchers agree that SIRT1 controls glucose production in the liver and its utilization by muscle, the field is confusing (Canto & Auwerx, 2008; Canto et al., 2009). More work with tissue-specific and whole-body knockout mice should help resolve the exact role that SIRT1 plays in this complex area of biology.

One area of increasing interest is the interplay between SIRT1 and the metabolic regulatory kinase AMPK. Upon exercise, AMPK boosts SIRT1 activity by increasing intracellular NAD^+ (Canto et al., 2009). In turn, SIRT1 deacetylates LKB1, a kinase upstream of AMPK, in a self-reinforcing loop (Hou et al., 2008). These findings help explain many of the overlapping

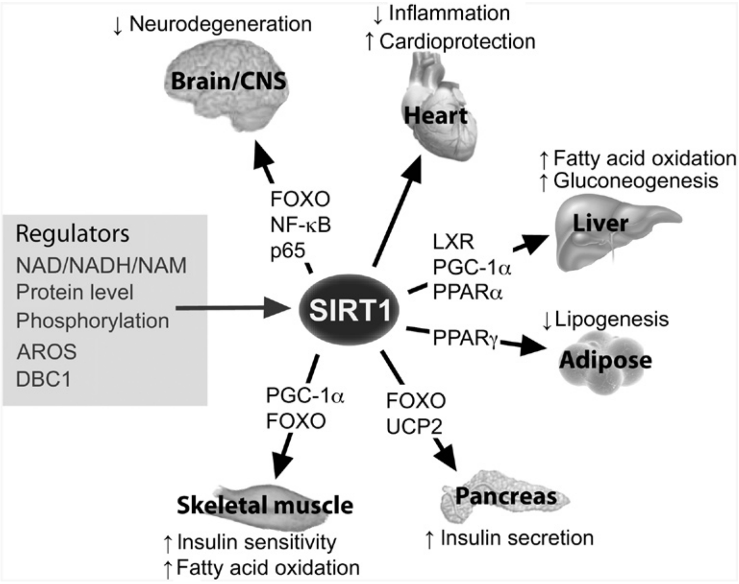

Figure 11.5 The roles of SIRT1 in age-related physiology. SIRT1 may be activated by diet or exercise. In vitro and in vivo, SIRT1 promotes the survival of neurons and protects cardiomyocytes from death. In the liver, SIRT1 promotes fatty acid oxidation and gluconeogenesis during nutrient deprivation via LXR, PGC-1α, and PPARα. In white adipose tissue, SIRT1 decreases fat storage by repressing PPARγ. SIRT1 promotes insulin secretion and pancreatic β-cell survival by suppressing UCP2 and interacting with FoxO, respectively. In skeletal muscle, SIRT1 promotes mitochondrial biogenesis through the activation of PGC-1α. NAD, nicotinamide adenine dinucleotide; NAM, nicotinamide; AROS, active regulator of SIRT1; DBC1, deleted in breast cancer 1; LXR, liver X receptor; PGC-1α, PPARγ coactivator α; PPARα, peroxisome proliferator-activated receptor α; PPARγ, peroxisome proliferator-activated receptor γ; UCP2, uncoupling protein-2; FoxO, forkhead box, subgroup O. Adapted from Haigis & Guarente, 2006.

functions of AMPK and SIRT1 activation. Importantly, pharmacological activation of SIRT1 by SRT1720 does not activate AMPK directly, but increases insulin sensitivity, improves endurance, increases respiratory quotient, and shifts muscle toward oxidative pathways, similar to the effects of resveratrol (Feige et al., 2008). Thus, the benefits of SIRT1 activation can occur in the absence of AMPK activation but clearly the two pathways act in concert to reinforce each other.

In addition to modulating insulin and glucose homeostasis in liver and muscle, SIRT1 acts on the pancreas directly. Overexpression of SIRT1 in pancreatic β cells showed that SIRT1 is a positive regulator of insulin secretion (Moynihan et al., 2005). In similar studies, whole-body SIRT1 null animals were found to have impaired insulin secretion (Bordone & Guarente, 2005; Bordone et al., 2006). SIRT1 is thought to promote insulin secretion by repressing the transcription of the UCP2 (uncoupling protein) gene, leading to an increase in cytosolic ATP/ADP ratios, a known trigger of insulin secretion (Bordone & Guarente, 2005; Moynihan et al., 2005).

SIRT1 also controls major physiological pathways in WAT. In mammals, WAT functions both to store fatty acids and to serve as an endocrine organ by secreting hormones, such as leptin and adiponectin, and inflammatory agents, such as tumor necrosis factor-α (TNFα) and resistin. With regard to its role in CR, Barzilai and colleagues argue that a reduction in WAT may be responsible for part of the life-span increase (Barzilai et al., 1998; Barzilai & Gabriely, 2001), though Masoro and others have questioned this view because they found that a decrease in fat mass is not necessary for CR to extend life span (Bertrand et al., 1980).

Though the role of WAT in CR is unclear, it is agreed that SIRT1 plays a major role in energy utilization from WAT during fasting. In cultured cells, SIRT1 has been shown to bind PPARγ and repress transcription of its target genes involved in fat storage (Picard et al., 2004). As a result, upregulation of SIRT1 in differentiated fat cells triggers lipolysis and results in decreased fat storage. Thus, an increase in SIRT1 levels could be a part of the metabolic program to mobilize fatty acids during fasting and reduce adiposity in WAT during CR. SIRT1 also contributes to the production of adiponectin by WAT by enhancing the interaction between FoxO1 and C/EBP (Qiao & Shao, 2006). As adiponectin improves insulin sensitivity, its control by SIRT1 may provide yet another mechanism for regulating metabolic homeostasis.

Studies of single-nucleotide polymorphisms (SNPs) in the human SIRT1 gene have found a significant

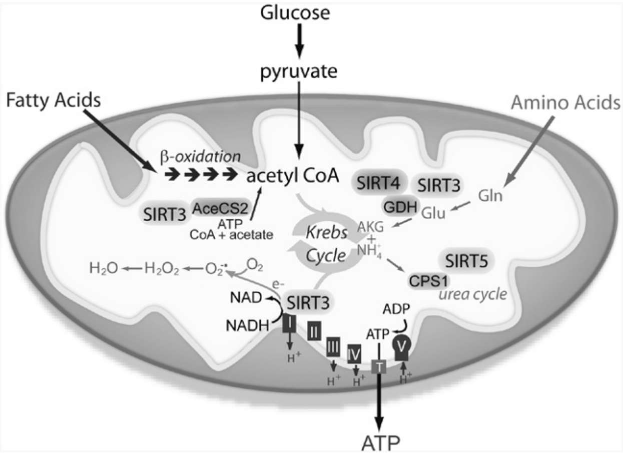

Figure 11.6 The known roles of the mitochondrial sirtuins. Mitochondria metabolize fuels such as fatty acids, amino acids, and pyruvate, derived from glucose. Electrons pass through electron transport complexes (I–IV), generating a proton gradient, which is used to drive ATP synthase to generate ATP. SIRT3 binds to complex I, regulating its activity and energy levels in the cell. SIRT3 also binds and deacetylates AceCS2 and GDH, activating their enzymatic activities. SIRT4 binds and represses GDH activity via ADP-ribosylation. SIRT5 deacetylates and activates CPS1, the rate-limiting step of the urea cycle. Abbreviations: AceCS2/ACS2, acetyl-CoA synthetase 2; GDH, glutamate dehydrogenase; CPS1, carbamoyl phosphate synthetase 1.

association between certain SNPs, obesity, energy expenditure, and insulin sensitivity (Peeters et al., 2008; Weyrich et al., 2008; Zillikens et al., 2009). This raises the possibility that human SIRT1 controls energy metabolism in ways similar to those observed in rodents.

One of the most interesting and important findings in recent years has been that SIRT1 and Nampt form an essential part of the mammalian circadian clock. CLOCK is an acetyltransferase that acetylates the BMAL transcription factor protein (on lysine 537), altering its ability to transactivate target genes with an E-box binding site. Recently, the Imai, Bass, and Sassone-Corsi laboratories identified SIRT1 as the opposing force that deacetylates BMAL1 and found that fluctuating SIRT1 activity in response to higher NAD^+ levels drives the clock (Jung-Hynes & Ahmad, 2009; Nakahata et al., 2009; Ramsey et al., 2009; Rutter et al., 2001). When NAD^+ levels rise, the CLOCK–BMAL–SIRT1 complex binds to E boxes in the promoter and an intron of Nampt, which then increases the conversion of NAM to NAD^+. This in turn activates SIRT1, which then binds to the Nampt promoter and reactivates Nampt expression, all in a 24-h rhythm. This may explain why the expression levels of SIRT1, Nampt, and NAD^+ have been seen to vary among studies in which measurements are taken at different times of the day. The discovery also has implications for the potential of NAD^+ and SIRT1-modulatory molecules in the treatment of mood and psychiatric disorders, many of which are caused or exacerbated by dysfunctional circadian rhythms (Wijnen, 2009). Whether other sirtuins regulate the circadian clock is not yet known.

SIRT3 and Energy Metabolism

SIRT3–5 are the sirtuins that reside in the mitochondria—a metabolic hot spot. Mitochondria are at the center stage for cellular energy (ATP) production and metabolism, generation of reactive oxygen species (ROS), and signaling during apoptosis. These organelles consume 85 to 95% of the oxygen used by cells in a series of enzymatic reactions that ultimately generate ATP from oxidative phosphorylation. During times when energy generation is required, mitochondrial biogenesis is often accompanied by an increase in oxidative fuel utilization to promote energy production. Mitochondria serve as a nexus for nutrient adaptation. Pyruvate, derived from glucose, is metabolized via the tricarboxylic acid (TCA) cycle; fatty acids and amino acids are also utilized in the mitochondria by fatty-acid oxidation or aminotransferase reactions, respectively. Not surprisingly, defects in mitochondrial functions have been linked to aging and can result in imbalances in metabolic homeostasis (Wallace, 2005). For instance, impaired mitochondrial function in the pancreas could inhibit the increase in ATP/ADP needed to stimulate insulin exocytosis, and defective mitochondria in muscle may lead to insulin resistance (Lowell & Shulman, 2005). Studies have revealed that mitochondrial sirtuins could be pivotal regulators of mitochondrial metabolism and integrity during aging and in response to environmental changes (Figure 11.6).

SIRT3 is the mitochondrial sirtuin most similar to SIRT1. SIRT3 is expressed in all tissues, with highest levels in metabolically active tissues such as brown adipose tissue, muscle, liver, kidney, heart, and brain (Onyango et al., 2002; Schwer et al., 2002; Shi et al., 2005). Experiments comparing membrane-bound and soluble mitochondrial fractions have suggested that SIRT3 is in the matrix and in the inner mitochondrial membrane. Moreover, electron microscopy studies have observed SIRT3 together with mitochondrial cristae, the site for oxidative phosphorylation. SIRT3 null mice do not have an overt developmental or metabolic phenotype (Lombard et al., 2007). The mice display normal body composition, normal mitochondrial number, and a typical response to fasting and cold exposure, but also display a robust biochemical phenotype—increased levels of mitochondrial acetylation (Lombard et al., 2007). This observation is significant because metabolic proteins, such as TCA cycle enzymes, fatty-acid oxidation enzymes, and subunits of oxidative phosphorylation complexes, were found to be heavily acetylated in a mass spectrometry survey of acetylation (Choudhary et al., 2009). However, it remains to be determined precisely how global acetylation impacts mitochondrial control of metabolism. It is also worth mentioning that no mitochondrial acetyltransferase has yet been identified.

SIRT3 binds and deacetylates numerous metabolic proteins in the mitochondria, including acetyl-CoA-synthetase (AceCS) (Hallows et al., 2006; Schwer et al., 2006), glutamate dehydrogenase (GDH), long-chain acyl coenzyme A dehydrogenase (LCAD) (Hirschey et al., 2010), and complex I (Ahn et al., 2008). Two groups have shown that SIRT3 deacetylates and activates AceCS (Hallows et al., 2006; Schwer et al., 2006), which forms acetyl-CoA from acetate, CoA, and ATP. AceCS activity is important under ketogenic conditions, such as prolonged fasting, when acetate released by the liver is converted into acetyl-CoA. SIRT3 also binds and deacetylates GDH, although an effect of acetylation on GDH activity has not been reported (Lombard et al., 2007; Schlicker et al., 2008). Together, these findings indicate that under conditions of energy limitation, SIRT3 funnels carbons from alternative sources into the central metabolism of the TCA cycle. Consistent with this hypothesis, SIRT3 expression in brown adipose and WAT increases during CR and decreases in genetically obese mice (Shi et al., 2005).

In addition to regulating central pathways of mitochondrial metabolism, SIRT3 is linked to mitochondrial respiration. SIRT3 binds to complex I and promotes NADH-driven mitochondrial respiration (Ahn et al., 2008). Furthermore, mitochondria from SIRT3 null livers demonstrated decreased oxygen consumption; and heart, kidney, and liver displayed a 50% reduction in basal ATP level (Ahn et al., 2008). Overexpression of SIRT3 in brown adipocytes increases oxygen consumption, reflecting heightened electron transport activity and increased uncoupling (Shi et al., 2005). Accordingly, SIRT3 overexpression results in decreased membrane potential and ROS production in these cells (Shi et al., 2005). However, these overexpression studies are difficult to interpret because they were performed using a truncated form of SIRT3 that does not localize correctly to mitochondria (Jin et al., 2009).

A recent study by the Verdin lab identified LCAD, and enzyme that performs mitochondrial fatty-acid oxidation, as a target of SIRT3 (Hirschey et al., 2010). During fasting, livers from mice lacking SIRT3 have higher levels of fatty-acid oxidation intermediate products and triglycerides, associated with decreased levels of fatty-acid oxidation, consistent with a role for SIRT3 in the regulation of mitochondrial intermediary metabolism and fatty-acid use during fasting. SIRT3 is also induced in skeletal muscle in response to CR, leading to activation of PGC-1α and CREB signaling (Palacios et al., 2009).

One mode of SIRT3 regulation is via changes in mitochondrial NAD^+ levels, which can rise as a result of increases in Nampt by up to twofold in the liver of fasted rats, with a concomitant increase in stress resistance (Sundaresan et al., 2008; H. Yang et al., 2007). Mitochondrial sirtuins are also regulated at the protein level: SIRT3 protein levels increase during CR, fasting, stress, and exercise (Hirschey et al., 2010; Shi et al., 2005; Sundaresan et al., 2008; J. J. Carmona, L. Goodyear, & D. Sinclair, 2009, unpublished data). In some studies SIRT3 is reported to translocate from the nucleus to mitochondria upon cellular stress (Nakamura et al., 2008; Scher eet al., 2007).

Given the ties between SIRT3, metabolism, and mitochondrial function, it is especially intriguing that genetic studies have linked SIRT3 to human life span. A silent G/T transversion in the conserved sirtuin core domain is associated with survivorship in elderly males, and SIRT3 contains a VNTR polymorphism found almost exclusively in males over the age of 90 (Bellizzi et al., 2005, 2007). Future biochemical studies to examine how SIRT3 SNPs modulate protein level or activity will be critical for a better understanding of these associations. Although these human genetic studies are limited in scale, these findings hint that SIRT3 may have a positive impact on human life span.

SIRT4 and Energy Metabolism

SIRT4 localizes to mitochondria of human and mouse cells and has been observed in the mitochondrial matrix (Ahuja et al., 2007; Haigis et al., 2006; Michishita et al., 2008). SIRT4 is a ubiquitously expressed gene, but its protein levels are highest in mouse kidney, heart, brain, liver, and pancreatic β cells (Ahuja et al., 2007; Haigis et al., 2006; Michishita et al., 2008). Unlike SIRT3, SIRT4 does not have a detectable NAD-dependent deacetylase activity toward canonical sirtuin targets (Ahuja et al., 2007; Haigis et al., 2006; Michishita et al., 2008). It is possible that SIRT4 is more specific in its substrate specificity than SIRT3 and future surveys may identify specific substrates that are deacetylated by SIRT4. Instead, SIRT4 has been shown to contain an NAD$^+$-dependent ADP-ribosyltransferase activity. One SIRT4 substrate has been identified; SIRT4 interacts with and represses GDH activity via mono-ADP-ribosylation (Haigis et al., 2006). GDH regulates the usage of amino acids in energy production. Isolated pancreatic islets from SIRT4 null mice exhibited higher GDH activity and had increased insulin secretion in response to glucose and amino acids. In a separate study, SIRT4 overexpression in insulinoma cells suppressed insulin secretion (Ahuja et al., 2007). SIRT4 has also been shown to interact with insulin-degrading enzyme and adenine nucleotide translocator, but the functional significance of these interactions is not known (Ahuja et al., 2007). It will be important for future studies to address whether SIRT4 affects fuel utilization in other tissues.

SIRT5

SIRT5, which localizes to the mitochondrial matrix and is ubiquitously expressed, functions as a weak NAD$^+$-dependent deacetylase. SIRT5 null mice have been generated and are developmentally normal without obvious metabolic defects (Lombard et al., 2007; Nakagawa et al., 2009). SIRT5 is translocated predominately into the mitochondrial intermembrane space or the matrix, depending on the stimulus (Schlicker et al., 2008). SIRT5 is known to interact with at least two proteins involved in cellular metabolism: cytochrome *c* and carbamoyl phosphate synthetase (CPS1; Nakagawa et al., 2009; Schlicker et al., 2008). CPS1 is the rate-limiting first step of the urea cycle; its activity is required for clearing ammonia generated by amino acid metabolism. By deacetylating CPS1, SIRT5 stimulates its enzymatic activity (Nakagawa et al., 2009). Mice lacking SIRT5 displayed elevated ammonia levels after a prolonged fast, suggesting that this sirtuin helps the liver deal with by-products of amino acid metabolism (Nakagawa et al., 2009). It remains to be seen whether loss of SIRT5 increases susceptibility to ammonia toxicity too.

SIRT6

SIRT6 functions as a corepressor of the transcription factor Hif1α, a critical regulator of nutrient stress responses (Mostoslavsky et al., 2006). In the absence of SIRT6, cells exhibit increased Hif1α activity and increased glucose uptake and lower mitochondrial respiration, in part explaining the hypoglycemia of the SIRT6 knockout mouse (Mostoslavsky et al., 2006). The mechanism of gene control appears to be direct: SIRT6 deacetylates histone H3K9 at the promoters of glycolytic genes (Mostoslavsky et al., 2006). Whether this activity is related to the base excision repair defect in the SIRT6 knockout mice is not yet known (Mostoslavsky et al., 2006).

SIRTUINS IN CELL SURVIVAL AND APOPTOSIS

SIRT1 as a Prosurvival Gene

Early evidence suggested that SIRT1 functions as an oncogene, starting with the identification of the first target of a SIRT1: the tumor suppressor p53 (Luo et al., 2001; Vaziri et al., 2001) (see Table 11.2) These early data, combined with other studies showing that SIRT1 can suppress apoptosis (Cohen et al., 2004b), prompted speculation that SIRT1 might promote cancer (Lim, 2006). Recent work, however, shows that the role of p53 extends beyond that of cell survival, including control of mitochondrial respiration (Matoba et al., 2006), which may be the primary in vivo role of SIRT1-mediated deacetylation of p53.

Cell culture experiments have provided a wealth of data showing that SIRT1 can prevent apoptosis and senescence (Brunet et al., 2004; Cohen et al., 2004a; Ford et al., 2005; Zhao et al., 2008). A study by Baylin and colleagues indicated that SIRT1-mediated silencing may play a role in tumorigenesis (O'Hagan et al., 2008; Pruitt et al., 2006). Two tumor suppressors have been identified as negative regulators of SIRT1. A recent paper identified hypermethylated in cancer 1 (HIC1), a zinc-finger/BTB domain protein regulated by p53, as a binding partner of SIRT1 that, in turn, represses the transcription of the SIRT1 gene (W. Y. Chen et al., 2005). Inactivation of HIC1 upregulates SIRT1 transcription, thereby inactivating p53, allowing cells to bypass apoptosis after DNA damage. Importantly, the authors found that the HIC1 promoter undergoes hypermethylation during aging, which may lead to upregulation of SIRT1 during aging and, therefore, susceptibility to cancer.

Another tumor suppressor that negatively regulates SIRT1 is DBC1 (Anantharaman & Aravind, 2008; Kim et al., 2008; Zhao et al., 2008). The DCB1 protein, originally identified as a protein that is absent in breast cancers, forms a stable interaction with the N-terminus of SIRT1 that inhibits SIRT1 deacetylase activity. Knockdown of DBC1 by siRNA promotes the deacetylation of p53 and allows cells to survive genotoxic stress, an effect that depends on SIRT1. These data indicate that DBC1 may promote breast cancer in part by activating SIRT1, thereby downregulating p53 and/or other tumor suppressor pathways.

SIRT1 as a Tumor Suppressor

While the case for SIRT1 as a tumor-promoting gene is a compelling one, it is by no means clear-cut.

Table 11.2 Evidence for SIRT1 having oncogenic or tumor suppressor activity

EVIDENCE FOR ONCOGENIC ACTIVITY	IN VITRO/VIVO	REFERENCES
Inactivates the p53 tumor suppressor	In vitro	W. Y. Chen et al., 2005; Luo et al., 2001; Vaziri et al., 2001
Increases cell survival	In vitro	Brunet et al., 2004; Cohen et al., 2004a; Ford et al., 2005; Zhao et al., 2008
Overexpressed in some cancers	In vivo	Bradbury et al., 2005; Hida et al., 2007; Huffman et al., 2007; Nayagam et al., 2006; Stunkel et al., 2007; Y. Zhang et al., 2009b
Inhibition can induce senescence in cancer cell lines	In vitro	Ota et al., 2006
SIRT1 promotes angiogenesis	In vivo	Potente & Dimmeler, 2008; Potente et al., 2007
HIC1 (hypermethylated in cancer) gene represses SIRT1	In vitro	W. Y. Chen et al., 2005
DBC1 (deleted in breast cancer) negatively regulates SIRT1 activity	In vitro	Anantharaman & Aravind, 2008; Kim et al., 2008; Zhao et al., 2008
Evidence for tumor suppressor activity		
Caloric restriction suppresses tumorigenesis	In vivo	Masoro, 1992
Inhibition does not induce activation of endogenous p53	In vitro/vivo	Ford et al., 2005; Huang et al., 2008; Solomon et al., 2006
Facilitates DNA break repair	In vitro	Oberdoerffer et al., 2008; Wang et al., 2008a
Overexpressing mice are protected from colon cancer in APC$^{min/+}$ background, deacetylation of β-catenin	In vivo	Firestein et al., 2008
Overexpressing mice are protected from lymphoma in p53$^{+/-}$ background; SIRT1$^{-/-}$ mice cancer prone, resveratrol protected	In vivo	Oberdoerffer et al., 2008; Wang et al., 2008a
Aged SIRT1-overexpressing mice are not cancer prone	In vivo	Banks et al., 2008; Pfluger et al., 2008
Expression unaltered or underexpressed in some cancers	In vivo	Ouaissi et al., 2008
Represses c-Myc oncogene	In vitro	Yuan et al., 2009

A number of studies have shown that neither SIRT1 knockdown, the H363Y allele, nor specific inhibition of SIRT1 in cells affects cell viability or cell growth and they are not sufficient to induce activation of endogenous p53 (Ford et al., 2005; Huang et al., 2008; Solomon et al., 2006). In the Solomon study, there was no increase in cell death despite DNA damage and increased p53 acetylation (Solomon et al., 2006). In addition, in cell culture studies, Mayo and colleagues showed that SIRT1 stimulates TNFα-induced cell death, indicating that SIRT1 can promote apoptosis, not simply suppress it (Yeung et al., 2004).

A 2009 study has highlighted a crucial feedback loop that could suppress oncogenesis in vivo (Yuan et al., 2009). The c-Myc gene encodes a proto-oncogenic transcription factor that regulates cell proliferation, cell growth, apoptosis, and stem-cell self-renewal. c-Myc binds to the SIRT1 promoter and induces SIRT1 expression, but SIRT1 then interacts with and deacetylates c-Myc, resulting in decreased c-Myc stability. This c-Myc-SIRT1 feedback loop could prevent

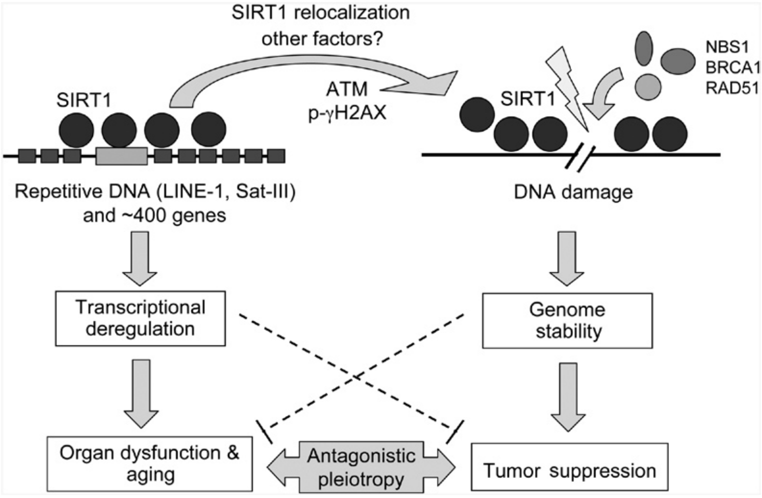

Figure 11.7 SIRT1 is a chromatin modifier that controls gene expression and promotes genome stability. SIRT1 is a histone deacetylase that targets acetylated H3K9, H3K56, H4K16, and H1K26. SIRT6 targets H3K9 at specific promoters such as NF-κB target genes and is important for packaging telomeric DNA and the stable association of WRN, the factor that is defective in the progeria known as Werner syndrome. These acetyl modifications are typically markers of active transcription and their removal leads to repressive chromatin, through processes that are still being elucidated. In response to DNA breaks or during aging, SIRT1 relocalizes away from open reading frames to the break site to alter chromatin around the break site and recruit DNA damage repair proteins such as Rad51 and Nbs1, a component of the Mre1 1/Rad50/Nbs1 (MRN) DNA break repair complex. SIRT6 is required for efficient base-excision repair and could theoretically recruit WRN to the repair site. The relocalization of chromatin modifiers is proposed to alter gene expression patterns that result in tissue dysfunction and diseases associated with aging.

cellular transformation and is consistent with a role for SIRT1 in tumor suppression.

The first study to test whether SIRT1 promotes cell survival or death in an animal was one in which SIRT1 was overexpressed in a mouse model of colon cancer, APC$^{min/+}$ (Firestein et al., 2008). In this model, loss of the remaining wild-type copy of the MIN gene results in the relocalization of β-catenin to the nucleus, activating transcription of genes such as myc and cyclin D1. CR has been shown to reduce tumor formation in this model (Masoro, 1992), as has resveratrol (Baur & Sinclair, 2006; Jang et al., 1997). Mice overexpressing SIRT1 in the small intestine and colon showed a fourfold reduction in the size, growth, and number of adenomas (Firestein et al., 2008). Deacetylation of β-catenin by SIRT1 was the favored hypothesis for the mechanism. Together these data indicated that SIRT1 activation may have potential therapeutic value against colon cancer and other tumors driven by β-catenin signaling.

Mice that are heterozygous for p53 have been used extensively to study genomic instability (Jacks et al., 1994). Given that SIRT1 downregulates p53 in cell culture experiments, SIRT1 activity might have exacerbated the p53$^{+/-}$ phenotype and shortened life span, but the opposite results were obtained. Resveratrol-treated animals had an ≈45% reduction in the frequency of fatal thymic lymphomas (Donehower et al., 1992; Jacks et al., 1994). Overexpression of SIRT1 in thymocytes also increased mean survival by ≈46% and the frequency of fatal thymic lymphomas was reduced by 45%. In a complementary study, SIRT1 null mice experienced accelerated tumorigenesis, with increased aneuploidy and chromosomal aberrations (Wang et al., 2008a).

One study found that SIRT1 is relocalized to DNA breaks in response to DNA damage, resulting in changes in gene expression that mimic aging (Oberdoerffer et al., 2008). These data support the relocalization of chromatin modifiers hypothesis, which states that the DNA damage-driven relocalization of chromatin-modifying proteins results in gene expression changes that cause aging (Imai & Kitano, 1998; Oberdoerffer et al., 2008), but further work is required to validate this model (Vijg et al., 2008) (Figure 11.7).

Resveratrol was first reported to protect mice from chemically induced skin cancers in 1997 (Jang et al., 1997). Since then, numerous studies have shown that resveratrol is a potent chemotherapeutic in mouse models of cancer. Interestingly, a recent paper by the McBurney laboratory showed that resveratrol is significantly less protective against skin cancer in the SIRT1 null mouse. Only 20% of resveratrol-treated wild-type mice developed tumors after 15 weeks, whereas 75% of SIRT1 null mice developed tumors. Thus, the majority of the protective effects of resveratrol against skin cancer require SIRT1 (Boily et al., 2009).

Other Sirtuins in Cancer

Another sirtuin studied in the context of cancer is SIRT2, a tubulin deacetylase (North et al., 2003; North & Verdin, 2007) that controls a mitotic checkpoint function in early metaphase to prevent chromosomal instability (Inoue et al., 2007, 2009). Whether SIRT2 is an oncogene or tumor suppressor is debated. Cells stably overexpressing SIRT2 have a marked prolongation of the mitotic phase of the cell cycle (Dryden et al., 2003). In glioma and glioma-derived cell lines, SIRT2 is downregulated, indicating that increased expression of SIRT2 might be beneficial in the disease (Hiratsuka et al., 2003). On the other hand, inhibition or downregulation of SIRT2 interferes with cell cycle progression and can promote cell cycle arrest in vitro (North & Verdin, 2007), indicating that inhibition of SIRT2 might be useful in treating some cancers (Heltweg et al., 2006; Y. Zhang et al., 2009a).

SIRT3 has been shown to function as a tumor suppressor. Expression of a single oncogene (Myc or Ras) in SIRT3 null mouse embryonic fibroblasts transforms them, and SIRT3 knockout mice develop ER/PR-positive mammary tumors (Kim et al., 2010; Schumacker, 2010). Similarly, SIRT3 can be proapoptotic in HCT116 cells via JNK2, a pathway independent of SIRT1 (Allison & Milner, 2007). Human breast and other human cancers have reduced SIRT3 levels, suggesting a role for SIRT3 in preventing tumorigenesis (Kim et al., 2010).

SIRT6 plays a clear role in genomic stability and may also have a role in cancer. It deacetylates H3 lysine 9 (H3K9) at telomeric chromatin, preventing end-to-end chromosomal fusions and premature cellular senescence (Michishita et al., 2008). Moreover, SIRT6 specifically interacts with GCIP (or CCNDBP1/DIP/HHM), a potential tumor suppressor on chromosome 15 that is downregulated in colon, breast, and prostate cancers (Michishita et al., 2008; Mostoslavsky et al., 2006), suggesting a possible function for GCIP–SIRT6 in tumor suppression (Ma et al., 2007). Mice lacking SIRT6 are susceptible to genomic instability resulting from a defect in base excision repair, though the precise molecular basis of this defect is not yet known (Lombard et al., 2008; Mostoslavsky et al., 2006).

SIRTUINS AND THE AGING CARDIOVASCULAR SYSTEM

Cardiovascular disease (CVD), the leading cause of death in the world, is a progressive, multistep process dependent on complex interactions between such processes as cholesterol biosynthesis, the immune system, and endothelial cell function (Malik et al., 2004). Risk factors include higher than normal levels of oxidized LDL cholesterol, vascular injury, and increased inflammation and cholesterol deposition by macrophages infiltrating smooth muscle below the vascular endothelium. For many decades, CR has been known to protect from CVD in animal (Sinclair & Howitz, 2006) and human studies (Heilbronn et al., 2006), but it has been unclear if these effects could be regulated by a single genetic pathway or a single pharmacological intervention. The evidence that the effect of CR might be mediated, at least in part, by sirtuins (Bitterman et al., 2003; Lin et al., 2000) led to speculation that they might also be useful in preventing or treating CVD (Westphal et al., 2007). Work showing that SIRT1 is cardioprotective (Alcendor et al., 2004, 2007; Hsu et al., 2008; Peeters et al., 2008) and protects against atherosclerosis (Borradaile & Pickering, 2009a; Brandes, 2008; Cardellini et al., 2009; Purushotham et al., 2009; Yu et al., 2009) has validated this notion.

SIRT1 is highly expressed in endothelial cells and controls functions critical for suppressing the development of atherosclerosis, including upregulation of endothelial nitric oxide synthase (eNOS), reduction of cell senescence in smooth muscle cells, suppression of inflammation and ROS in arteries, and increased vascular growth (Borradaile & Pickering, 2009a; Brandes, 2008). SIRT1 has also been shown to alter cholesterol biosynthesis in the liver and macrophages (Li et al., 2007) and reduce serum lipid levels (Feige et al., 2008). Together, these results are consistent with SIRT1 being a major player in the prevention of CVD by CR. As yet, there is only speculation about the potential role of the other sirtuins, SIRT2–7, in CVD.

One of the key requirements for the assembly and repair of damage to the cardiovascular system is angiogenesis. Potente and colleagues showed that mice lacking SIRT1 in the endothelium appear normal from a development standpoint but have an impaired ability to form new blood vessels in response to ischemia (Potente et al., 2007). Using endothelial cell culture and time-lapse analysis of vessel formation in developing zebrafish embryos with fluorescently labeled endothelial cells, SIRT1 was identified as a requisite factor for endothelial sprouting and vessel navigation. Whether the lack of a developmental defect in the adult SIRT1 knockout mouse is due to redundancy of sirtuins or a difference between mice and fish remains to be seen.

A major function of SIRT1 that could protect the cardiovascular system is its ability to suppress inflammation. Isolated macrophages and endothelial cells treated with SIRT1 activators have lower levels of inflammatory cytokines, including TNFα, ICAM-1, IL-6, IL-1, and inducible NOS, or iNOS (Nayagam et al., 2006; Shen et al., 2009; Yoshizaki et al., 2009). A similar anti-inflammatory response to SIRT1 activation is seen in mice and rats exposed to a high-fat diet or to cigarette smoke (Baur et al., 2006; Csiszar et al., 2008; Pearson et al., 2008). In cultured coronary arterial endothelial cells (CAECs), the protective effects of resveratrol were abolished by knockdown of SIRT1, whereas the overexpression of SIRT1 mimicked the effects of resveratrol (Csiszar et al., 2008).

Some of the cardiovascular benefits of CR and pharmacologic activation of SIRT1 seem to stem from SIRT1's ability to induce nitric oxide signaling. Endothelial nitric oxide synthase, an enzyme that generates nitric oxide, both is atheroprotective and promotes blood vessel relaxation. The first direct link between eNOS and SIRT1 was the demonstration that SIRT1 physically interacts with eNOS and deacetylates lysines 496 and 506 in the calmodulin-binding domain of eNOS, leading to enhanced eNOS activity (Mattagajasingh et al., 2007). Mice on a CR diet had lower levels of eNOS acetylation, consistent with the known increase in SIRT1 levels and eNOS in rodents on CR (Civitarese et al., 2007; Cohen et al., 2004b; Nisoli et al., 2005). Nampt/PBEF, an NAD^+ biosynthetic gene and activator of SIRT1, endows human endothelial cells with increased replicative life span and enhanced angiogenic capacity in a high-glucose environment (Borradaile & Pickering, 2009b). Conversely, inhibition of SIRT1 expression decreases NO bioavailability, inhibits endothelium-dependent vasorelaxation, and induces premature senescence of endothelial cells (Mattagajasingh et al., 2007). In agreement with the in vitro data, transgenic overexpression of SIRT1 in endothelial cells prevents the loss of vasorelaxation and lowers the number of atherosclerotic plaques in the $apoE^{-/-}$ model of atherogenesis (Zhang et al., 2008), without affecting blood lipid or glucose levels (Liang et al., 2007). Resveratrol has been shown to induce the expression of eNOS alone (Baur & Sinclair, 2006; Wallerath et al., 2002) or in combination with a statin (Penumathsa et al., 2007), albeit in one rat study it failed to induce eNOS expression (Rush et al., 2007). It is interesting to note that eNOS signaling is necessary for SIRT1 induction by CR (Nisoli et al., 2005), indicating that SIRT1–eNOS–SIRT1 signaling forms a positive feedback loop that amplifies the effects of CR and resveratrol.

Another mechanism of protection from CVD is the downregulation of angiotensin II type 1 receptor (AT1R) expression. Overexpression of SIRT1 or treatment with resveratrol reduces AT1R expression, whereas nicotinamide, an inhibitor of SIRT1, increases AT1R expression and prevents resveratrol-induced AT1R downregulation (Alcendor et al., 2007; Hsu et al., 2008). The authors concluded that the inhibition of the renin–angiotensin system by resveratrol may contribute, at least in part, to its antiatherogenic effects.

Using both cell culture and transgenic mice, the Sadoshima lab has shown that SIRT1 protects cardiomyocytes in response to infarction and ROS generated during ischemia reperfusion (Alcendor et al., 2007; Hsu et al., 2008). Interestingly, they provide evidence that hypoxia preconditioning in the heart is due to the actions of miR-199a, a microRNA that represses SIRT1 (Rane et al., 2009). Cardioprotective effects of resveratrol were also observed in a mouse model of chronic type 1 diabetes, and its effects were dependent on SIRT1 activity (Sulaiman et al., 2010). The proposed mechanism was activation of the reduced sarcoplasmic calcium ATPase SERCA2, a known protective gene against cardiomyopathy.

Ungvari and colleagues found that resveratrol treatment attenuated the deleterious effects of cigarette smoke extract (CSE) in rat arteries and cultured CAECs (Csiszar et al., 2008). The inflammatory markers (ICAM-1, iNOS, IL-6, and TNFα) were considerably reduced, as was NF-κB activation and inflammatory gene expression. In CAECs, these protective effects, including the suppression of apoptosis, were prevented by knockdown of SIRT1, whereas the overexpression of SIRT1 mimicked the effects of resveratrol. Thus, the vasoprotective and anti-inflammatory effects of resveratrol require SIRT1, making it a potential drug target for the treatment of CVD and vascular aging.

Another attribute of SIRT1 with relevance to CVD is its ability to downregulate FoxO signaling. FoxO transcription family members are essential negative regulators of blood vessel formation, with FoxO1 being the primary mediator (Paik, 2006; Paik et al., 2007; Potente et al., 2005). SIRT1 targets FoxO factors for deacetylation, thus reducing FoxO1-dependent transcriptional activity (Potente et al., 2005). SIRT1 also interacts with a component of the Notch signaling pathway, the Hairy and Enhancer of Split basic loop-helix-loop-helix repressor protein Hey2 (Takata & Ishikawa, 2003). As Potente & Dimmeler, 2008 point out, given the role of Hey2 in vascular patterning in vertebrates, the interaction between SIRT1 and Hey2 indicates that SIRT1 may function downstream of Notch and modulate Hey2 endothelial angiogenic activity (Potente & Dimmeler, 2008).

The key role of oxidized LDL cholesterol in the progression of atherosclerosis and the protective function of HDL are well known. Guarente and colleagues showed that SIRT1 deacetylates and thereby activates the nuclear receptor LXR, thereby promoting the transcription of LXR target genes involved in lipid metabolism, including the ABCA1 transporter, mediating the efflux of cholesterol from peripheral tissues (Li et al., 2007). It is tempting to speculate that one of the long-term benefits of SIRT1 activation by CR or a SIRT1 activator could be the efflux of cholesterol from peripheral tissues to slow atherosclerotic plaque formation (Potente & Dimmeler, 2008). In the liver, SIRT1 controls hepatic triglyceride synthesis by activating genes such as the sterol regulatory element binding protein, SREBP1. Consistent with this, SIRT1 knockout mice show reductions in plasma and hepatic triglyceride accumulation and decreased HDL cholesterol (Li et al., 2007).

SIRT3 has emerged as a key player in cardioprotection and muscle energetics. Furthermore, it has been shown that SIRT3 is more highly expressed in slow oxidative type I soleus muscle compared to fast type II extensor digitorum longus or gastrocnemius muscles (Palacios et al., 2009). In response to exercise, SIRT3 expression increases, leading to activation of PGC-1α and CREB signaling. Mice lacking SIRT3 are prone to cardiac hypertrophy and the addition of exogenous NAD^+ increases agonist-induced cardiac hypertrophic response through LKB1–AMPK (Pillai et al., 2010) and FoxO3-mediated increases in ROS defenses (Sundaresan et al., 2009). The nuclear form of SIRT3 is thought to promote cell survival by deacetylating Ku70, causing it to sequester the proapoptotic protein Bax away from mitochondria (Sundaresan et al., 2008).

SIRTUINS IN INFLAMMATION AND IMMUNITY

Innate and adaptive immunity comprise the major defense mechanisms possessed by higher organisms against inherent and external threats. Innate immunity provides immediate defense against infection by other organisms in a nonspecific manner and is found in unicellular organisms to humans. The adaptive immune system provides a highly specialized means to recognize and remember specific pathogens, to ramp up stronger defenses. During aging, adaptive immunity declines, while innate immunity increases inappropriately, resulting in a proinflammatory condition. The aging-associated decline in adaptive immunity, concomitant with an increase in innate immunity, sets the stage for a low-level but chronic inflammatory condition in the elderly. Increased inflammation is a central contributor in the pathogenesis of aging and several age-related degenerative and metabolic diseases. This inflammation may induce or exacerbate existing diseases, such as diabetes, cancer, atherosclerosis, and neurodegeneration.

One of the hallmarks of the CR diet is reduced inflammation (Csiszar et al., 2009; Jung et al., 2009; Phillips & Leeuwenburgh, 2005; Salminen et al., 2008). Recent studies have shown that sirtuins interact with regulators of immune responses and may directly impact inflammation in vivo. These advances suggest that sirtuin-based therapies may also attenuate inflammatory diseases. Sirtuins have been identified as novel regulators of the immune system, and numerous studies show that SIRT1 suppresses inflammation in multiple tissues (Csiszar et al., 2006, 2008; Nayagam et al., 2006; Pfluger et al., 2008; Shen et al., 2009; S. R. Yang et al., 2007; Yoshizaki et al., 2009).

One of the master regulators of both adaptive and innate immunity is the NF-κB system, which comprises receptors and signaling molecules with functions that have been highly conserved throughout evolution. NF-κB is a pleiotropic transcription factor that responds to a range of danger signals, including immune attacks, hypoxia, oxidative stress, and genotoxic stress. NF-κB forms complexes with a number of other proteins, including Rel family members (RelA/p65, c-Rel, and RelB) and NF-κB components p50 and p52. In the basal state, NF-κB complexes are retained in the cytosol via binding to the inhibitory IκB family of proteins. Upon activation, IκB proteins are phosphorylated and targeted for ubiquitination and subsequent degradation. The removal of IκB frees NF-κB complexes to translocate into the nucleus and trigger expression of target genes, which are largely proinflammatory. Upregulation of the NF-κB binding domain is strongly associated with aging, while NF-κB activity is strongly diminished by CR (Jung et al., 2009).

SIRT1 has emerged as a potent inhibitor of the NF-κB system, providing a mechanistic link between inflammation, diet, and aging (Salminen et al., 2008). After translocation to the nucleus, the p65 subunit is acetylated by p300 to enhance DNA binding. SIRT1 binds and deacetylates RelA/p65, inhibiting the transcriptional activity of NF-κB (Yeung et al., 2004), suggesting that SIRT1 inhibits NF-κB signaling in vitro.

Uveitis, or inflammation of the middle layer of the eye, is estimated to be responsible for approximately 10% of the blindness in the United States. Resveratrol pretreatment has also been shown to provide significant and dose-dependent suppression of leukocyte adhesion to retinal blood vessels in endotoxin-induced uveitis mice (Kubota et al., 2009). Levels of MCP-1, ICAM-1, 8-OHdG, and nuclear translocation of NF-κB are significantly reduced by resveratrol administration, coincident with increased SIRT1 activity in retinal pigment epithelium chondroidal cells (Kubota et al., 2009).

The only other sirtuin linked to the regulation of immune responses is SIRT6. This sirtuin interacts with the RelA/p65 component of the NF-κB complex and is recruited to some promoters of NF-κB target genes (Kawahara et al., 2009). SIRT6 deacetylates histone H3 lysine 9 at these promoters and represses the expression of NF-κB targets. Interestingly, an independent study found that SIRT6 regulates TNFα production (Van Gool et al., 2009). Strikingly, hyperactive NF-κB signaling may be responsible for the short life span and degenerative phenotype of SIRT6 null mice, as haploinsufficiency of RelA rescues the premature aging phenotype of SIRT6 knockout animals (Kawahara et al., 2009).

In addition to the direct regulation of NF-κB complexes by SIRT1 and SIRT6, sirtuins may activate NF-κB signaling indirectly by regulating other factors, such as FoxO proteins, which converge on insulin signaling and stress pathways. For example, FoxO3a suppresses NF-κB signaling. Its overexpression can inhibit the TNFα-induced activation of NF-κB, and FoxO3a can promote apoptosis by suppressing NF-κB activity (reviewed by Peng, 2007). Finally, FoxO3a null mice display multitissue inflammation that was postulated to stem from the activation of NF-κB signaling (L. Lin et al., 2004). Interestingly, several studies have reported that SIRT1 and SIRT2 interact with and deacetylate FoxO proteins in worms and in mammalian systems (Brunet et al., 2004; Motta et al., 2004; Viswanathan et al., 2005), indicating that there might be an additional layer of NF-κB signaling control. A 2009 study showed that in response to increase NAD^+ levels, SIRT6 downregulates TNFα production at a posttranscriptional step (Van Gool et al., 2009).

Studies of mouse models have generally supported the notion that SIRT1 plays a key role in suppressing inflammation. SIRT1-overexpressing mice have lower levels of lipid-induced hepatic inflammation when challenged by a high-fat diet (Pfluger et al., 2008). These animals demonstrate decreased NF-κB activity, which results in diminished expression of proinflammatory cytokines, such as TNFα and IL-6. Modulation of SIRT1 activity also affects lipid accumulation in adipocytes, which has an impact on the etiology of metabolic diseases such as obesity and insulin-resistant diabetes. Consistent with this, liver-specific SIRT1 null mice show increased signs of inflammation and NF-κB signaling when fed a high-fat diet (Purushotham et al., 2009). In contrast to these findings, a study using SIRT1 whole-body null mice demonstrated that these animals accumulate immunoglobin complexes in the kidney and liver, but do not show alterations in innate immune responses (Sequeira et al., 2008).

Smoking also increases inflammation, and the interaction between SIRT1 and RelA/p65 is diminished by cigarette smoke, resulting in increased acetylation and activation of NF-κB proinflammatory responses in macrophages (S. R. Yang et al., 2006). As discussed above, resveratrol treatment attenuates the deleterious effects of CSE in rat arteries and cultured coronary arterial endothelial cells, effects that are prevented by knockdown of SIRT1 (Csiszar et al., 2008). Interestingly, coronary arterial endothelial cells cultured with serum from CR mice showed decreased ROS generation, NF-κB activation, and induction of inflammatory genes. Moreover, these changes were diminished by siRNA knockdown of SIRT1. Thus, the antioxidant and anti-inflammatory effects of CR on the vasculature may be mediated via a SIRT1-dependent mechanism (Csiszar et al., 2009). Along similar lines, the lungs of smokers and patients with COPD have decreased levels of nuclear SIRT1, compared with nonsmokers (Nakamaru et al., 2009; Rajendrasozhan et al., 2008).

There is also some evidence that SIRT1 modulates the immune response during infection. NF-κB signaling has been implicated in the hyperactive immune response that helps to promote HIV-1 infectivity and replication (Blazek & Peterlin, 2008). The Tat protein of HIV-1, which is required for the transcriptional activation of the HIV-1 virus is a target for SIRT1 deacetylation (Kwon et al., 2008; Pagans et al., 2005), allowing Tat to bind TAR RNA and transcriptional elongation factors (Kwon et al., 2008). Tat also seems to regulate SIRT1 activity, blocking its ability to deacetylate p65 (Kwon et al., 2008). In doing so, Tat hyperactivates NF-κB target gene expression in wildtype, but not in SIRT1 null cells. Thus, SIRT1, Tat, and NF-κB act in a circuit in which SIRT1 promotes Tat, and Tat neutralizes the negative effect of SIRT1 on NF-κB signaling. Whether these cell culture findings have in vivo relevance is not yet known.

There is increasing interest in the potential roles of SIRT1 in immunity. A 2009 study showed that SIRT1 negatively regulates T cell activation and plays a major role in clonal T cell anergy. Loss of SIRT1 function in vivo increases T cell activation and a breakdown of $CD4^+$ T cell tolerance, whereas upregulation of SIRT1 expression led to a decrease in T cell activation (J. Zhang et al., 2009). On one hand, as the authors suggest, activators of SIRT1 may be useful for the treatment of autoimmune diseases (J. Zhang et al., 2009), but they might also negatively impact T-cell-mediated immunity, something undesirable in the elderly or frail.

SIRTUINS IN THE AGING BRAIN

By the early 2000s, SIRT1 was already known to suppress apoptosis in a variety of cell types (Bitterman et al., 2002; Brunet et al., 2004; Cohen et al., 2004b; Giannakou & Partridge, 2004; Vaziri et al., 2001), but it was the study by Milbrandt and colleagues that provided the first evidence that SIRT1 is neuroprotective (Araki et al., 2004). Their study showed that SIRT1 was necessary for the protection of neurons against Wallerian degeneration (WD), which is essentially the dieback of axons following a nerve crush (Araki et al., 2004; Raff et al., 2002). Wld^S mice have greatly increased resistance to WD because of a mutation that amplifies the NAD^+ biosynthetic gene Nmnat fused to a ubiquitin assembly protein, Ufd2 (Coleman & Perry, 2002; Conforti et al., 2000). Milbrandt's paper proposed that increased NAD^+ production by Nmnat protein and subsequent SIRT1 activation were necessary for neuroprotection. While the ability of SIRT1 to provide neuroprotection in a variety of contexts is now undisputed, there continues to be debate about how

Nmnat-1 protects cells and whether SIRT1 is involved (Conforti et al., 2007; Zhai et al., 2006). For example, SIRT1 has been found to be dispensable for the protective effects of NAD$^+$ and there are conflicting data on whether Nmnat alone can protect neurons in vivo or if catalytic activity is required (Avery et al., 2009; Conforti et al., 2007; Sasaki et al., 2009; Watanabe et al., 2007; Yahata et al., 2009), although in vivo data support the Milbrandt model (Sasaki et al., 2009). A study showing that resveratrol abolishes the resistance of Wld^S mice to WD proposed that SIRT2 activation was responsible (Suzuki & Koike, 2007), though this result is confusing given that resveratrol does not activate SIRT2 in vitro (Milne et al., 2007). The work also seems to run counter to a study showing that inhibition of SIRT1 provided neuroprotection in a mouse model of Parkinson disease (Outeiro et al., 2007).

Although the role of SIRT1 and SIRT2 in WD is unclear, SIRT1 does provide neuroprotection in the context of a variety of cell stresses, both in vitro and in vivo. In 2004, Brunet, Greenberg, and colleagues were the first to show SIRT1 mediated neuroprotection using cerebellar granule neurons, which was traced to SIRT1's ability to target FoxO3 for deacetylation, thus dampening FoxO-mediated cell death (Brunet et al., 2004). In the same year, Sinclair and colleagues showed that SIRT1 targets the C-terminus of Ku70 for deacetylation, which promotes the binding of Bax to Ku70, thus preventing Bax from initiating apoptosis (Cohen et al., 2004a,b). Tsai and colleagues reported that SIRT1 protects neurons from apoptosis resulting from overexpression of mutant SOD1-G37R, an allele linked to amyotrophic lateral sclerosis (ALS) (D. Kim et al., 2007). They also showed protection from the p25 mutant version of cyclin-dependent kinase 5, a suspected contributor to axonopathies and Alzheimer disease (AD) in humans. In a p25-transgenic mouse model of AD and ALS, resveratrol and SIRT1 provided protection from neurodegeneration and the decline in cognition (D. Kim et al., 2007). Human SIRT1 and resveratrol were also shown by Pasinetti and Sauve to reduce the Aβ peptide content of neurons in the Swedish Tg2576 mouse model of AD, which expresses mutant human cDNA for APP (Qin et al., 2006, 2008). The same paper proposed that the neuroprotective effects of CR might be due to changes in the NAD$^+$/NAM ratio and subsequent SIRT1-mediated activation of ROCK1, a Rho kinase that induces the protective, nonamyloidogenic α-secretase pathway (Qin et al., 2006).

Optic neuritis is caused by inflammation of the optic nerve resulting in a loss of vision, usually due to swelling and destruction of the myelin sheath covering the optic nerve, and is a precursor to multiple sclerosis. Shindler and colleagues found that the activation of SIRT1 by injection of resveratrol or a precursor to NAD$^+$, nicotinamide riboside, into the eyeballs of mice significantly attenuated the loss of retinal ganglia cells, and this neuroprotective effect was blocked by sirtinol, a SIRT1 inhibitor (Shindler et al., 2007). Another eye disease, age-related macular degeneration (AMD), is the leading cause of severe vision loss in the elderly. Polymorphisms in the complement factor H (CFH) gene that reduce activity of CFH increase the risk of AMD. Overexpression of SIRT1 has also been shown to attenuate FoxO3 recruitment to the regulatory region of the CFH gene and to reverse H_2O_2-induced repression of CFH gene expression, raising the possibility that SIRT1 activation may ameliorate or slow the progression of AMD (Wu et al., 2007).

PERSPECTIVE

It was only at the beginning of the 21st century that *SIR2* was shown to be a deacetylase that extents yeast life span. Since then, researchers have discovered that *SIR2* is but one example of a large and highly conserved family of enzymes that control key cellular processes such as fuel adaptation to low-nutrient conditions, mitochondrial biogenesis and function, DNA break repair, neuronal survival, circadian clocks, and the maintenance of a youthful pattern of gene expression, to name a few.

Still, much remains to be elucidated. For example, differences in the findings from mouse studies present a complicated scenario that brings to light the fact there is still much unknown regarding SIRT1 function, even though many of its substrates have been identified. Some of the confusion in interpreting these studies comes from conflicting data about when SIRT1 is active. For example, is SIRT1 induced in liver by fasting or CR? Are earlier studies on SIRT1 difficult to interpret now that we know SIRT1 and its regulator Nampt are key components of the circadian clock that vary over a 12-h cycle (Asher et al., 2008; Nakahata et al., 2008, 2009; Ramsey et al., 2009)? NAD$^+$ and nicotinamide are important physiological regulators of sirtuins, even in mitochondria (H. Yang et al., 2007), yet it is still not clear how NAD$^+$ is synthesized in various cellular compartments. Is it generated from NAM or is it transported as NMN through the membranes? Finally, while a growing literature of work links SIRT1 with physiological pathways involved in aging, we are only beginning to glimpse how SIRT2–7 function.

Despite the rapid advances in sirtuin biology, there are still very few tools for accurately measuring SIRT1 activity on a native substrate in vitro or assessing sirtuin activity in vivo. Currently, investigators use sirtuin protein level, NAD$^+$ concentration, and the acetylation state of sirtuin substrates as readouts. When used in combination, these methods can generate a rough snapshot. However, this snapshot may not be entirely accurate because sirtuins have overlapping targets, as in the case of SIRT1–3 and FoxO proteins. Sirtuin activity may also be regulated by posttranslational modification or by inhibitory proteins, as in the case of DBC1 for SIRT1 (27, 62). Thus, the above studies highlight a need for assays that can provide a quantitative readout for the specific activities of SIRT1–7.

A major question that remains is whether SIRT1 activation is safe enough to be used as a therapy for human diseases such as diabetes or CVD. Clearly CR has some drawbacks, including decreased fertility and osteoporosis, but these negative effects have not yet been reported for SIRT1-overexpressing mice (Elliott & Jirousek, 2008; Lagouge et al., 2006; Lavu et al., 2008; Milne et al., 2007). One area to watch for is the effect of SIRT1 on immunity, in light of the finding that SIRT1 can suppress T cell activation (J. Zhang et al., 2009).

Although there are significant hurdles to overcome, in contrast to the time when the mammalian sirtuins were first discovered, there is little doubt that these enzymes have greatly increased our knowledge about the elegant mechanisms that coordinate energy intake with organismal health and survival. In the coming decades we will know whether it is possible to take full advantage of this knowledge to discover medicines that safely harness the ability of sirtuins to marshal the body's natural defenses against disease.

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Inflammation in Aging Processes: An Integrative and Ecological Perspective

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CHAPTER CONTENTS

Introduction 275
Overview of Inflammatory Responses 275
Systemic Manifestation of Inflammation and Aging 278
Tissue Inflammatory Changes during Aging 280
Inflammation in Atherosclerosis and Alzheimer Disease 281
Cancer 282
Bone and Joints 283
Blood Glucose Elevations in Inflammatory Processes of Human Aging 283
Diet, Metabolism, and Exercise 284
Genetics 285
Environmental Influences: An Ecological Perspective 286
Conclusions 287
Acknowledgments 287
References 287

INTRODUCTION

This review surveys inflammation during aging and chronic disease in an integrative and ecological perspective that emphasizes generalizable changes, interactions of inflammation with oxidative damage, and environmental influences. Inflammation may be considered a core process of human aging because of its involvement in baseline aging and in the major degenerative diseases of later life, atherosclerosis, Alzheimer disease, and cancer. Blood levels of C-reactive protein (CRP), interleukin-6 (IL-6), and other proinflammatory cytokines are risk indicators of cardiovascular events and mortality. Even in the absence of specific pathological lesions, inflammatory gene expression increases during aging in humans and animal models, in mammals, and in several invertebrate models. Nonetheless, inflammatory mediators are essential to normal function and health, as illustrated by the importance of IL-6 secretion to normal metabolic activities, e.g., IL-6 is released by skeletal muscle in proportion to exercise intensity, while IL-6 also regulates insulin sensitivity of adipocytes (Finch, 2007, p. 58).

Many inflammatory changes are highly plastic and influenced by nutrition and physical activity. Inflammation may prove central to therapeutic interventions for specific diseases as well as to general antiaging strategies. Moreover, the global spread of obesity and the increasing inflammatory loads from polluted air and water may be limiting factors in future increases in life expectancy. This integrative perspective on inflammation in aging considers all levels of function and causality, from the atomic to the cellular to the environmental. A leading question is the role of endogenous vs extrinsic factors in the perpetuation of chronic elevations in acute-phase innate immune responses. Outlining these broad domains and links in a concise review necessarily precludes in-depth review of most topics and neglects mention of a great amount of excellent relevant science.

OVERVIEW OF INFLAMMATORY RESPONSES

Inflammatory responses are part of the host immune defenses against pathogens and tissue responses to injury and operate at all levels of biological organization

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(A)

Atomic/biochemical

ROS oxidation of DNA, lipids, proteins
glycooxidation (AGE formation)

Cellular

Activation of macrophages by endotoxins, oxidized proteins and lipid, and AGEs
autogenous cytoinflammation from replicative cell senescence

Systemic

Humoral elevations of CRP, IL-6 and cytokines
redox shift in glutathione
induction of fever by IL-6 and TNFα; increased basal metabolic rate
sickness behaviors: lethargy and loss of appetite

Environmental

Infectious pathogens that causes systemic inflammation and focal lesions
Ambient inflammogens from combustion of fossil fuels and tobacco

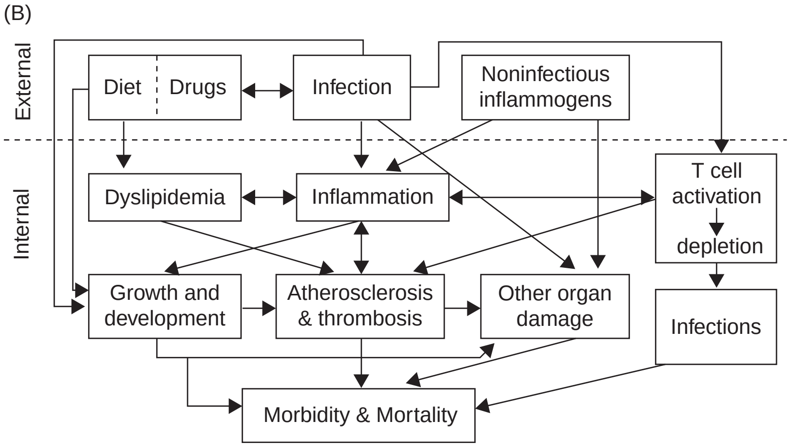

Figure 12.1 Schema of inflammatory responses. (A) Biological levels. (B) Organ levels. Schema showing external influences on organ systems leading to morbidity and mortality at later ages from chronic inflammatory conditions. (From Finch, 2007, p. 5, and Crimmins & Finch, 2006.)

(Figure 12.1), from atomic free radicals to behavior. Inflammatory responses can be focal (a few cells) or systemic. The temporal organization of host immune responses is described in two phases: acute (<1 week) and chronic (>1 week, possibly lifelong). The acute phase of inflammation largely involves the "innate" immune responses, whereas subsequent phases may include "adaptive" immune responses of antigen-selected T and B cells, but with continuing mediation by various innate immune factors. These highly evolved processes remove and regenerate damaged tissues while minimizing infection.

The "cardinal signs" of inflammation known for millennia still give instructive descriptions of the two responses to traumatic injury: redness and swelling with heat and pain[1]. The redness represents increased local blood flow; the swelling, or edema, is associated with increased tissue fluid, leukocyte infiltration, and cell proliferation; the heat is produced by local mitochondria or fever due to increased whole-body metabolism. In addition to local responses to focal injury, acute-phase systemic inflammatory responses are governed through neuroendocrine and autonomic activation involving the hypothalamic–pituitary–adrenal cortex axis and the vagal nerve afferents to the brain stem and hypothalamus. Pain as a cardinal sign of inflammation is part of a suite of changes that minimize aggravation to the injured region, along with "sickness behaviors of lethargy and loss of appetite." Inflammation can be energetically expensive: basal metabolism increases 10% per centigrade degree of fever (Finch, 2007, p. 5; Waterlow, 1984). Inflammation thus involves an integrative system of focal responses and responses throughout the body.

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[1] The four cardinal signs of Celsus, *rubor et tumor cum calor et dolore;* in *De Medicina,* written circa 50 CE by Cornelius Celsus, a Roman encyclopedist. A fifth sign, *functio laesa* (disturbed function), is often attributed to Galen, 150 years after Celsus, but was most probably introduced in the mid-19th century by Rudolph Virchow (*Cellular pathologie,* 1858) (Majno, 1975).

At the molecular level (Figure 12.1A), systemic infections and traumatic wounds can trigger rapid innate immune responses with hepatic secretion of IL-6 and TNFα, which mediate systemic energy metabolism and adaptive immunity. The liver secretes CRP, an ancient protein of innate immunity with many activities: CRP assists in pathogen clearance by binding to bacterial endotoxins, e.g., the gram-negative lipopolysaccharide (LPS), and CRP activates the complement cascade by direct binding to C1q to produce anaphylactic peptides (C3a, C4a, C5a) that enhance macrophage production of reactive oxygen species (ROS) (Hage & Szalai, 2007; Szalai et al., 1995). However, during chronic inflammation, CRP can also aggregate in tissues to form amyloids with complex fibrils that are found in many chronic inflammatory diseases (see below). Scavenger receptors on hepatocytes and other cells are activated by pathogen-associated molecular patterns (PAMPs), which are endotoxin epitopes shared by groups of pathogens (Chou et al., 2008; Ranjan et al., 2009; Vance et al., 2009). Additionally, tissue amyloids may accumulate during chronic inflammation from the acute phase hepatic secretion of CRP, serum amyloid A, and serum amyloid P (SAP), all of which can bind microbial pathogens (Finch, 2007, pp. 59–60). Notably, the amyloid β-peptide of senile plaques and blood platelets also potently inhibits growth of *Staphylococci*, *Candida*, and other common human pathogens (Soscia et al., 2010).

Macrophages, as part of the initial host defense system, rapidly respond to PAMPs by phagocytosis and killing of microorganisms via generation of ROS. During the acute phase, adaptive immune responses may be induced with antigen-specific clonal responses of B and T cells. However, there is no real dichotomy between innate and adaptive mechanisms, which synergize at many levels with multiple pleiotropies. For example, IL-6 is a mediator of fever during acute-phase innate responses to LPS, while IL-6 also mediates adaptive immune response by stimulating somatic mutation of immunoglobulin genes (Wu et al., 2009).

The huge complexity of immune responses is being considered in the context of "integrative systems" approaches, which range from cellular level analysis of transcription networks during in vitro macrophage responses to LPS (Tegnér et al., 2006) to systemic level analysis of inflammation with in silico modeling (Vodovotz et al., 2010; Daun et al., 2008; Gardy et al., 2009; Zak & Aderem, 2009; Finch, 2007, pp. 318–323). Hundreds of genes and multiple signaling pathways are involved. Energy allocation is regulated through insulin–IGF, mTOR, and other pathways that are of inflammatory cascades. The ergonomics of inflammation may explain how food restriction attenuates fever in response to LPS (Inoue et al., 2008) and shortens the duration of footpad edema by 50% (Klebanov et al., 1995). The sensitivity of innate immune responses to energy reserves has obvious relevance to manipulations of aging by caloric restriction and exercise (see Diet, Metabolism, and Exercise, below) that future studies may consider in a fully quantifiable framework.

Inflammation-associated cellular–molecular damage is recognized as a major feature in aging. Bystander damage by ROS to neighboring cells and molecules is an important source of oxidative damage during aging that interacts with the endogenous damage from free radicals proposed by Harman over 50 years ago (Finch, 2007, pp. 60–65). The activation of macrophages and neutrophils increases secretion of ROS, which can cause oxidative bystander damage to DNA and proteins within a cell and to neighboring cells and extracellular proteins. Immune activation in response to specific antigens can also have bystander effects through secretion of interferon-γ and other cytokines that influence the differentiation of neighboring T cells (Fletcher et al., 2005; Finch, 2007, p. 63). These complex cascades are attenuated by antioxidant systems, such as glutathione, cytokines with anti-inflammatory activities (IL-4, IL-10, and TGF-β), and resolvins (endogenous enzymatically derived ω-3 fatty-acid products).

The oxidized molecules from bystander damage are recognized by macrophages through RAGE (receptor for advanced glycation end products, AGEs); these transmembrane receptors can stimulate further inflammatory reactions, as discussed below. RAGE has broad ligand binding and is a mediator of systemic oxidative stress and inflammatory responses (Cai et al., 2008a,b; Lin et al., 2009) through ROS production by NAD(P)H oxidases and electron transport (Gao & Mann, 2009; Herold et al., 2007). Microbial pathogens also activate RAGE (Chou et al., 2008).

Foci of chronic inflammation also typically stimulate local cell proliferation, which in some instances progresses to cellular (proliferative) senescence. On the other hand, inflammation inhibits stem cell proliferation in adult brain (Mathieu et al., 2009) and heart (Abarbanell et al., 2009; Herrmann et al., 2009). Because tissue damage activates gene responses shared with the acute phase, most degenerative diseases of aging involve innate immune responses. Lastly many degenerative processes in aging can be viewed as non-adaptive inflamation (Rae et al., 2010) with extensive antagonistic pleiotropy.

SYSTEMIC MANIFESTATION OF INFLAMMATION AND AGING

Blood levels of the acute-phase responses CRP, IL-6, and TNFα tend to increase during aging in humans, as in the InChianti study of community dwelling elderly (Ferrucci et al., 2005) (Figure 12.2A). Chronically elevated acute-phase proteins are risk indicators for high mortality: in the National Health and Nutrition Examination Survey (NHANES III), those over 60 with elevated CRP of >0.30 mg/dl serum had 2.7 times higher mortality than those below this threshold. Interpretation is complex, because elevated blood CRP, IL-6, and other acute-phase responses are also risk indicators of cardiovascular events by themselves (e.g., Danesh et al., 2008; Ridker, 2009) and in conjunction with LDL and other lipid risk indicators. For example, those in the top two tertiles of IL-6 and LDL showed a 10-fold higher risk of cardiovascular events (Luc et al., 2003). IL-6 elevations are among a host of other intercorrelated risk indicators of cardiovascular events and mortality (Crimmins et al., 2008; Goldman et al., 2006; Sattar et al., 2009). The InChianti elevations of CRP and IL-6 (Figure 12.2A) were mainly attributed to cardiovascular disease and morbidity (Ferrucci et al., 2005). Blood IL-6 also tends to increase during aging in rodent models (Longo & Finch, 2003; Panda et al., 2009). Although cardiovascular disease is very mild or absent in lab rodents, detailed histopathology is needed to evaluate possible links between IL-6 elevations and the presence of tumors and renal degeneration of individual animals.

Many aspects of chronic elevations of acute-phase responses are unresolved. First, we do not know the sources of the elevated blood inflammatory markers. In the acute-phase response, most of the CRP and IL-6 is attributed to increased hepatic secretion. However, arterial cells also make and secrete various acute-phase proteins: CRP by vascular smooth muscle cells (Guo et al., 2009) and IL-6 by the foam

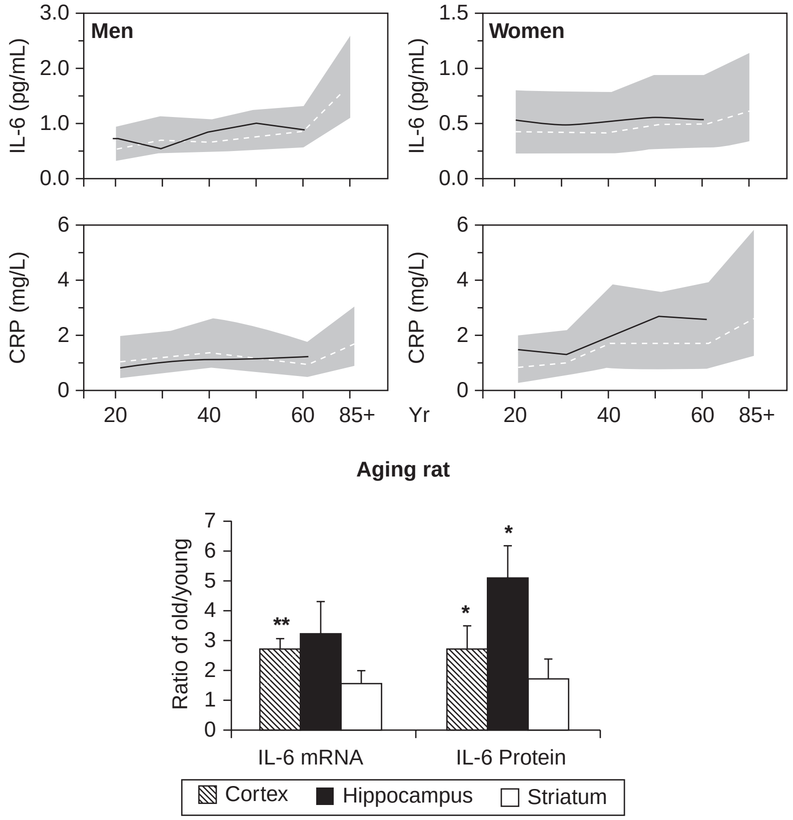

Figure 12.2 Aging and IL-6. (A) Serum IL-6 and CRP in community-dwelling Italians (InCHIANTI Study), means by decade. Dotted line, total sample; shaded area, 95% confidence interval; continuous line, means of healthy individuals <85 years, without morbidity and at low risk for cardiovascular disease (Ferrucci et al., 2005). Thus, most change is attributable to cardiovascular risk factors and morbidity. (B) Glia from aging male rats have increased levels of IL-6 mRNA and protein in primary culture of mixed glia (astrocytes plus microglia). Data are shown as ratios by age (old, 24 months:young, 3 months) for three brain regions (Xie et al., 2003).

cells (macrophages with lipid inclusions) of atherosclerotic plaques (Groeneweg et al., 2006), as well as adipose tissues (see Diet, Metabolism, and Exercise, below). The progression of arterial lipid accumulation (Figure 12.3A) and of advanced atherosclerosis during aging (Figure 12.3B) could thus contribute directly to the blood levels of CRP and IL-6. Other longitudinal studies of vascular disease in diverse populations would further inform about diet, alcohol, and other factors in relationships of blood inflammatory markers to age-related pathology.

Second, we must ask if these increases indicate increased low-grade infections with aging. As is well documented for influenza, the elderly have increased vulnerability to infections (Elliot & Fleming, 2008). Senescence in the adaptive immune system is associated with an "immune risk phenotype" of elevated blood IL-6, and TNFα, and higher mortality during seasonal influenza (Finch, 2007, pp. 19–21; McElhaney & Effros, 2009; Trzonkowski et al., 2003). Lifelong antigenic exposure gradually accumulates highly differentiated memory cells (CD4$^+$ T cells) at the expense of naïve CD4$^+$/CD28$^+$ T cells (Gruver et al., 2007). CMV and other persistent viral infections may prove to have broad importance to outcomes of aging (Gress & Deeks, 2009; Pawelec et al., 2010). Obesity and diabetes type 2 also predispose to chronic low-grade infections.

Moreover, the acute-phase response can induce many of the lipid changes that are also associated with cardiovascular risk: modified HDL ("acute-phase HDL") from dissociation of paronoxenases (PON1–3) and transferrin that decreases antioxidant activity, oxidized LDL, higher triglycerides, and inhibition of reverse cholesterol transport (Finch, 2007, pp. 84–86). I suggest that elderly populations should be characterized for acute-phase HDL in relation to endogenous blood endotoxin levels. After eating, there is a minor surge of postprandial endotoxins from gut bacteria that is mediated by chylomicrons and enhanced by fatty diets (Ghoshal et al., 2009). Gut aging changes may enhance postprandial endotoxemia through

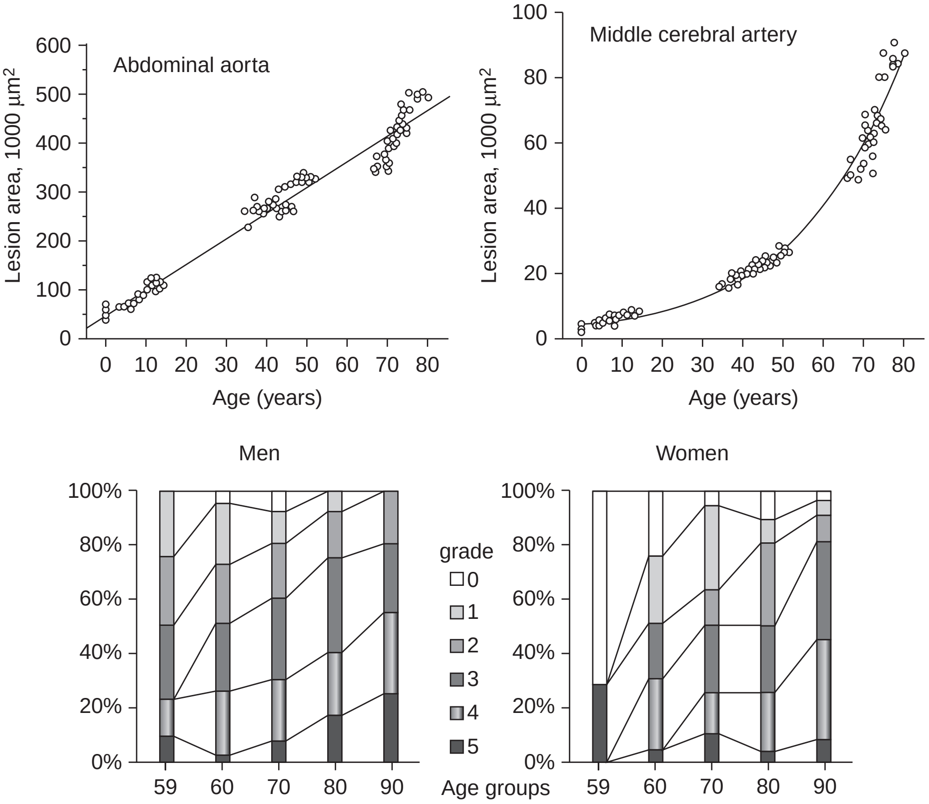

Figure 12.3 Atherosclerosis progresses across the life span. (A) Arterial lipid accumulation in the abdominal aorta and middle cerebral artery from birth through old age: a multisite autopsy collection (D'Armiento et al., 2001). (B) Cardiovascular atherosclerosis at necropsy of elderly in the Hisayama community, ages 69–90+ (80% of deaths, 1988–1996) (Nakashima et al., 2009). The severity of atherosclerotic grade (scaled 0–5) increased progressively with age; by age 90, nearly all had advanced lesions. This study is among the few autopsy series of a community that includes the majority of deaths at later ages.

increased intestinal permeability (Mullin et al., 2002) and crypt cell dysplasia (Martin & Kirkwood, 1998).

TISSUE INFLAMMATORY CHANGES DURING AGING

Messenger RNA prevalence analysis by microarrays has defined a consistent profile of increased inflammatory gene activity during normal aging in most tissues in the absence of clinical-grade pathology (Finch, 2007, pp. 107–112; Park et al., 2009; Schumacher et al., 2008). Brain, heart, and other organs of healthy aging rodents have 50% or more increases in mRNA for IL-6 and C1q complement and other inflammatory factors. In the human brain during normal aging, in the absence of Alzheimer disease, we found activated complement proteins in the diffuse amyloid deposits (Zanjani et al., 2005). A generalized change in middle age is the increase in activated microglia, which are monocyte–macrophage-like cells derived from bone marrow: in vivo and in vitro, microglia from aging rodent brain express and secrete more IL-6 (Xie et al., 2003; Sierra et al., 2007; Sparkman & Johnson, 2008) (Figure 12.2B). IL-10, an anti-inflammatory, is also increased (Sparkman & Johnson, 2008). Despite greater production of IL-6, the microglia from aging rat brain produce fewer ROS upon stimulation by LPS (Xie et al., 2003; Sparkman & Johnson, 2008). Glial activation arises during middle age in lab rodents on normal diets in the complete absence of recognizable degenerative disease (Morgan et al., 1999). The lab mouse and rat are important models for nonclinical aging because the standard strains on normal diets do not develop atheromas or Alzheimer-like neurodegeneration. Many of these same markers are further increased in degenerative diseases of aging (see the next three sections and Table 12.1). The mild inflammatory changes in many tissues may be a substrate for chronic degenerative diseases with inflammatory components.

A major challenge is to identify the signaling pathways and transcriptional–translational networks that mediate the inflammatory components of aging. The insulin–IGF and mTOR signaling of "longevity pathways" (Longo & Finch, 2003; Chapters 2 and 9 of this book) also mediate inflammation (Finch, 2007, pp. 7, 202–209; Salvioli et al., 2009) as well as atherosclerosis (Finch, 2007, p. 7) and other chronic diseases. The AMP kinases and the hexosamine pathway may also be involved through their roles in glucose and energy regulation (Finch, 2007, pp. 315–323). Transcriptional regulation by NF-κB in many inflammatory pathways suggests an important role in chronic inflammation during aging (Jung et al., 2009). At the translational level, microRNAs may be important to inflammatory pathways in aging (Bhaumik et al., 2009; Davidson-Moncada et al., 2010; Liang et al., 2009). The remarkable generality of inflammatory aging changes in humans and animal models could be the basis for a

Table 12.1 Comparison of inflammatory factors in atherosclerosis and Alzheimer disease

	ATHEROMA	SENILE PLAQUE
Cells		
Astrocytes	0	++
Mononuclear cells		
Macrophage	+++ (Foam cell, macrophage; CD68)	++ (Microglia; CD68)
T cell	++ (CD3 CD4/Th1)	0
Mast cells	++	0
Platelets	++	0
Neovascularization	++	+
Proteins		
Amyloids		
Aβ	? (Macrophages with ingested platelets)	++
CRP	++	+ (Neurites)
SAP	+	+
Complement C5b-9	+	+
Cytokines: IL-1, IL-6	+	+
Metals	**Fe**	**Cu, Fe, Zn**

For references, see Finch (2005) and Finch (2007, pp. 51 and 78). The sources of inflammatory proteins may be systemic or local cells, e.g., neurons express mRNA for CRP, complement C1q, and other acute-phase proteins (Akiyama et al., 2000; Rozovsky et al., 1994; Yasojima et al., 2000).

comprehensive theory of aging and age-related disease (Chung et al., 2009; Finch, 2007; Franceschi et al., 2007; Salvioli et al., 2009).

Another source of inflammatory factors is autogenous DNA damage during cell senescence. As described by Campisi and collaborators, replicative cell senescence (Hayflick model) causes increased secretion of IL-6, metalloproteinases, and other inflammatory factors (senescence-associated secretory phenotype) in association with DNA damage that causes cell cycle arrest (Rodier et al., 2009). The primary role of DNA damage in cell senescence was shown by blocking telomere erosion by transfection with *htert*, which delayed the inflammatory secretions and cell cycle arrest; reciprocal experiments increased DNA damage by low-dose X-rays and accelerated these changes. Other studies by Kirkwood and von Zglinicki document the importance of mitochondria DNA damage in increased production of ROS during cell senescence via a checkpoint pathway involving the cytokine TGF-β (Passos et al., 2010). I suggest the these generalizable phenomena be designated as "autogenous cytoinflammation." These findings imply that inflammation-related DNA damage to bystander cells can cause further local inflammatory cascades through cell senescence pathways that are already documented in atherosclerosis and cancer, as discussed below. Because chronic inflammation typically stimulates cell proliferation, e.g., in the gut (Abreu, 2010) and lung (Bauer et al., 2009), chronic inflammatory conditions of aging will also drive cell senescence, with local tissue bystander consequences.

Aging changes are also observed in the acute phase of inflammatory processes during aging that may be considered part of immunosenescence. In response to endotoxin (LPS), aging rats had greater elevations of IL-6, but smaller induction of T-kininogen and other acute-phase responses; the high incidence of hepatic abscesses suggests a weakened host defense (Gomez et al., 2008). At the cellular level, neutrophils from aging rats had decreased phagocytic activities and oxidative burst (Butcher et al., 2001; Lord et al., 2001; Schroder & Rink, 2003; Panda et al., 2009), as did microglia, as noted above. Nonetheless, the blood levels of circulating neutrophils tend to increase, in contrast to declines in monocytes, Thus, the declining resistance of the elderly to infections may be understood in terms of both innate and adaptive immunity.

INFLAMMATION IN ATHEROSCLEROSIS AND ALZHEIMER DISEASE

Atherosclerosis has been long recognized as an inflammatory process (Ross, 1999; Galkina & Ley, 2009). In Virchow's view (*Cellular Pathologie,* 1858) "…inflammation of the arterial coat was the starting point of the so-called atheromatous degeneration" (Langheinrich & Bohle, 2005). It is generally agreed that arterial inflammatory changes begin before birth and progress across the human life span (Figure 12.3). Prenatal human arteries have microscopic foci of macrophages associated with oxidized lipids and proteins that may be the seeds of the low-grade macroscopic atheromas that are found almost universally in young adults (D'Armiento et al., 2001). The postnatal accumulation of lipids is linear with age in the aorta, but may be exponential in cerebral arteries. Activated macrophages, accumulation of inflammatory proteins and tissue amyloids, and oxidative damage are progressive in atheromas from early to later ages (Table 12.1). The accumulation of advanced atheromas continues into later ages, as shown in a community necropsy study from Japan (Figure 12.3B) (Nakashima et al., 2009). Moreover, even in arterial segments lacking gross fatty infiltration or focal pathology, there are inflammatory changes (Wang et al., 2007, 2009). As stressed by Lakatta et al. (2009) "Arterial aging and subclinical arterial disease are fundamentally intertwined at macroscopic and molecular levels."

Atherosclerotic plaques also accumulate "senescent" cells, defined by markers of replicative senescence (senescence-associated β-galactosidase) and telomere shortening (Edo & Andrés (2005)). As noted above, replicative senescence induces inflammatory gene expression. True to Celsus's signs, atheromas are hotter than flanking arterial segments by up to 3 °C; temperature elevations are proportionate to macrophage density and may indicate plaque instability (Madjid et al., 2006; Tan & Lip, 2008; Toutouzas et al., 2007). The heat is generated by the induction of UCP2, which uncouples mitochondrial ATP production from respiration to generate radiant energy (Van De Parre et al., 2008).

Atherosclerotic lesions may harbor infectious pathogens, e.g., cytomegalovirus (CMV) and *Chlamydia pneumoniae* (Finch, 2007, pp. 115–121). CMV itself directly binds to vascular endothelial cells through epidermal growth factor receptors to stimulate proliferation and angiogenesis during atheroma development (Bentz & Yurochko, 2008). Moreover, in CMV-seropositive coronary patients, myocardial dysfunction was associated with excessive telomere shortening in $CD8^+CD28^-$ T cells (Spyridopoulos et al., 2009). However, over several decades of study, links of specific pathogens to cardiovascular diseases have been less consistent than those of *Helicobacter pylori* in gastrointestinal cancer (see Cancer, below) and may vary because of sporadic events or pathogen clearance.

Relationships of blood vascular risk indicators (CRP, LDL-cholesterol, oxidized lipids) to the progression of atherosclerosis remain controversial. About 35% of heart attacks in the Framingham study occurred despite initially normal blood cholesterol

(Castelli, 1996). The statins, which inhibit cholesterol synthesis by blocking HMG-CoA reductase, have shown remarkable efficacy in reducing the risk of heart attack. However, lower blood LDL may not be a sufficient explanation, because even in those with low cholesterol, statins lower both blood CRP and heart attack risk (Jupiter Study, Ridker et al., 2009). The emerging broad anti-inflammatory activities of statins may involve signaling pathways sensitive to isoprenoids derived from mevalonate, a cholesterol precursor that is also lowered by statins. These complex pleiotropies beyond blood cholesterol are being addressed in the Cardiovascular Inflammation Reduction Trial (Ridker, 2009).

Alzheimer disease is widely recognized as involving inflammatory processes (Akiyama et al., 2000). The senile plaque (neuritic plaque) consists of extracellular fibrillar aggregates of the amyloid β-peptide (Aβ) with microglia and local neuritic degeneration with hyperphosphorylated tau. Senile plaques contain many inflammatory factors, most of which are also found in atheromas (Table 12.1). While fibrillar amyloid deposits of Aβ are restricted to the brain and cerebrovasculature, SAP (acute-phase response) accumulates in amyloid deposits of both brain and heart. Iron and other redox-active metals in arterial and brain plaques that promote ROS formation through Fenton chemistry are a further substrate for local oxidative stress The extensive similarity of atheromas and senile plaques with shared inflammatory proteins and activated monocytes (foam cells, atheroma; microglia, senile plaque) suggests parallel mechanisms in pathogenesis. Note the progressive exponential accumulation of lipids in cerebral arteries (Figure 12.3B), which may anticipate the accelerating incidence of cerebrovascular lesions with aging (Cole & Vassar, 2009).

Cardiovascular disease and Alzheimer disease share many risk factors. Genetically, both share the *apoE4* allele, which increases cardiovascular event risk by about twofold and Alzheimer disease risk by more than fivefold in *E4/E4* homozygotes (Finch, 2007, pp. 357–368; Mahley et al., 2007; Roses et al., 2007; Poirier, 2008). A familial study of risk factors in middle age showed increased white blood cell production of IL-6 and other proinflammatory cytokines in Alzheimer-prone families, independent of the *apoE4* allele (van Exel et al., 2009). Obesity and diabetes are also shared risk factors. In the respective rodent or private models, high-cholesterol diets accelerate, while caloric restriction retards, progression of atheromas (Guo et al., 2002) or Alzheimer brain Aβ deposits (Patel et al., 2005; Wang et al., 2005; Quin et al., 2006a,b), discussed further under Diet, Metabolism, and Exercise. Despite the major benefits of statins for lowering blood cholesterol and risk of cardiovascular events, statins did not alter incidence of dementia or cognitive decline in two large randomized control trials (HPS 2002, simvastatin; PROPSER, pravastatin) (McGuiness et al., 2009). Resveratrol is a new candidate drug for treating atherosclerosis (Marzetti et al., 2009; J. Wang et al., 2009) and Alzheimer disease (Albani et al., 2010; Karuppagounder et al., 2009), which may inhibit inflammation and oxidative damage by activating SIRT1 (Sulaiman et al., 2010) on a longevity pathway (see Chapter 11 of this book) and/or inhibiting NF-κB signaling (Chen et al., 2005).

CANCER

Cancer is deeply connected to inflammation through two processes: local inflammation acting on initially healthy proliferating cells and local inflammatory responses to neoplasia. Both processes involve synergistic mechanisms of DNA damage, ROS production, oxidative stress, and tissue remodeling that promote accumulation of mutations, angiogenesis, and tissue invasiveness (Coppé et al., 2008; Lazennec & Richmond, 2010; O'Connor et al., 2010). Local inflammation, by stimulating cell proliferation and causing DNA damage from ROS, increases the chance of oncogenic mutations. A classic example is the association of *H. pylori* infections with gastrointestinal cancers, which are the second-ranked cause of malignant deaths (Finch, 2007, pp. 154–155). *H. pylori*, a common mildly pathogenic bacterium, attaches extracellularly and causes localized mucosal cell proliferation. Infiltrating monocytes produce ROS that cause telomere shortening and DNA damage (8-OHdG) (Farinati et al., 2008; Kuniyasu et al., 2003; O'Connor et al., 2010). Suppression of *H. pylori* by the use of NSAIDS, as well as improving public health and hygiene in the 20th century, has reduced the prevalence of gastrointestinal cancers. As another example, mice deficient in granulocyte–macrophage colony-stimulating factor and interferon-γ developed lymphomas and solid tumors concurrent with chronic inflammation and bacterial infections; a link of tumors to infection was shown by the suppression of both by the antibiotic enrofloxacin (Enzler et al., 2003).

Many other occurrences of neoplasia are linked to local and systemic effects of airborne combustion products from fossil fuels, tobacco smoke, and other noninfectious inflammogens (Finch, 2007, pp. 156–157 and 209–211). In addition to the bystander damage from ROS produced by activated monocytes during inflammation, cell senescence also causes tissue bystander damage from inflammatory "secretory phenotypes" that arise during proliferative senescence independent of exogenous ROS (see Tissue Inflammatory Changes during Aging). Moreover, replicatively senescent cells can enhance cancer metastasis by altering the microenvironment through secretion of metalloproteinases and inflammatory cytokines (Coppé et al., 2010).

BONE AND JOINTS

Osteopenia of normal aging with increased risk of fractures and clinical osteoporosis begins before 30 in both sexes and precedes major decreases in blood levels of sex steroids (Riggs et al., 2008). Peak bone mass, low body weight, and weight loss are risk factors for osteoporotic fractures (Papaioannou et al., 2009; Winsloe et al., 2009), which is relevant to bone fracture risk during caloric restriction (Mardon et al., 2008). After menopause, obesity may be protective for osteoporosis through endogenous estrogens derived from aromatase in adipose tissue (Albala et al., 1996; Haffner & Bauer, 1992).

Bone turnover is mediated by mechanisms shared with inflammatory processes, again with prominent roles for ROS (Banfi et al., 2008), macrophages, and cytokines (IL-6, IL-6, TNFα) (Mundy 2007; Axmann et al., 2009; McLean, 2009). Elevated blood IL-6 was associated with greater osteopenia in longitudinal studies (Ding et al., 2008). The balance between new bone formation and resorption involves the osteoclast, which is derived from circulating macrophage–monocytes. The cytokine RANKL (receptor activator of NF-κB ligand) mediates osteoclast formation and bone matrix remodeling through ROS, mediated by the differential regulation of NADPH oxidase (Nox) isoforms (Sasaki et al., 2009). In hyperlipidemic mice, oxidized lipids contributed to osteopenia by enhancing RANKL production in T cells (Graham et al., 2009). The protection against bone loss and fractures by estrogens also involves RANKL and Nox (X. Chen et al., 2008). Unexpectedly, statins also show anabolic benefits to bone (Tang et al., 2008). The apparent conjunction of bone and heart health in drug responses (Anagnostis et al., 2009) was also noted for the benefits of resveratrol in aging mice (Pearson et al., 2008).

BLOOD GLUCOSE ELEVATIONS IN INFLAMMATORY PROCESSES OF HUMAN AGING

Blood glucose elevations are directly linked to inflammatory changes in many aspects of aging, by driving the formation of AGEs, which in turn is directly linked to vascular disease. As described in the first edition of this handbook (Andres & Tobin, 1977), many studies have shown mild elevations of fasting blood glucose after age 40. The progressive glucosemia of normal aging is emerging as a canonical feature of aging paralleling the increase in systolic blood pressure and atherosclerosis. New links between glucosemia and mortality risk and longevity have emerged from longitudinal analysis of the Framingham Study (Yashin et al., 2009, 2010). The total Framingham population, diabetics included, shows a strong trend for progressive increase in fasting glucose (Figure 12.4A). Nondiabetics also show strong linear increases up through the oldest ages (Figure 12.4B). It seems potentially important that mortality risk at later ages becomes increasingly sensitive to blood glucose (based on a Cox hazards model) (Yashin et al., 2009). These findings suggest that cardiovascular disease risk scales with modest glucosemia below conventional thresholds of glucose intolerance (Ko et al., 1998).

Blood glucose levels are directly linked to inflammation: glucose causes spontaneous oxidative damage to proteins by nonenzymatically forming covalent bonds with free $-\varepsilon NH_2$ groups to form AGEs by complex chemistry involving Maillard and Amadori reactions (Lee & Cerami, 1990; Finot, 2005; Nursten, 2005). For example, glycated hemoglobin (HbA_{1c}) can reach 30% of total hemoglobin in uncontrolled diabetes: HbA_{1c} is a reliable indicator of glucose levels during the prior 2–3 months because of turnover of erythrocytes with a half-life of 4 months. The proportionality of blood HbA_{1c} to blood glucose, verified in a multinational study (Little & Sacks, 2009), is consistent with glucose as the chemical driver of HbA_{1c} formation with first order chemical kinetics. Corresponding to the progressive increases in glucose, the levels of HbA_{1c} increase linearly with age, e.g., in nondiabetics of the Framingham Study (Figure 12.4C) and in NHANES (Pani et al., 2008). However, a full accounting of HBA_{1c} during aging requires data on erythrocyte turnover at later ages.

While increased HbA_{1c} alone does not alter oxygen binding, other AGE adducts have direct consequences for vascular disease because erythrocytes from diabetics have greater stickiness to arterial endothelial surfaces. Erythrocyte adhesion would favor thrombosis, as well as transfer of erythrocyte cholesterol and iron into the atheroma (Kolodgie et al., 2003). The erythrocyte adhesion involves endothelial RAGE activation by AGE adducts in erythrocyte membrane proteins and lipids, which induce adhesion molecules, cytokines, and ROS (Grossin et al., 2009; Lai et al., 2004) and alter deformability and other rheologically important characteristics (Cho et al., 2008). I suggest that erythrocytes from elderly nondiabetics with elevated HbA_{1c} (Figure 12.4C) will also be prothrombotic, which may explain why small additional increments in blood glucose in elderly nondiabetics cause excess mortality above younger norms. Another mechanism linking the age creep in blood glucose to vascular events is through the activation of RAGE by AGE on proteins and lipids (see Overview of Inflammatory Responses). RAGE activation is implicated in cardiovascular disease at many levels and is intensified by chronic hyperglycemia in clinical diabetes (Yan et al., 2007).

These links of AGE to inflammation and vascular disease further specify Cerami's hypothesis that AGE formation is fundamental to aging (Cerami et al., 1987;

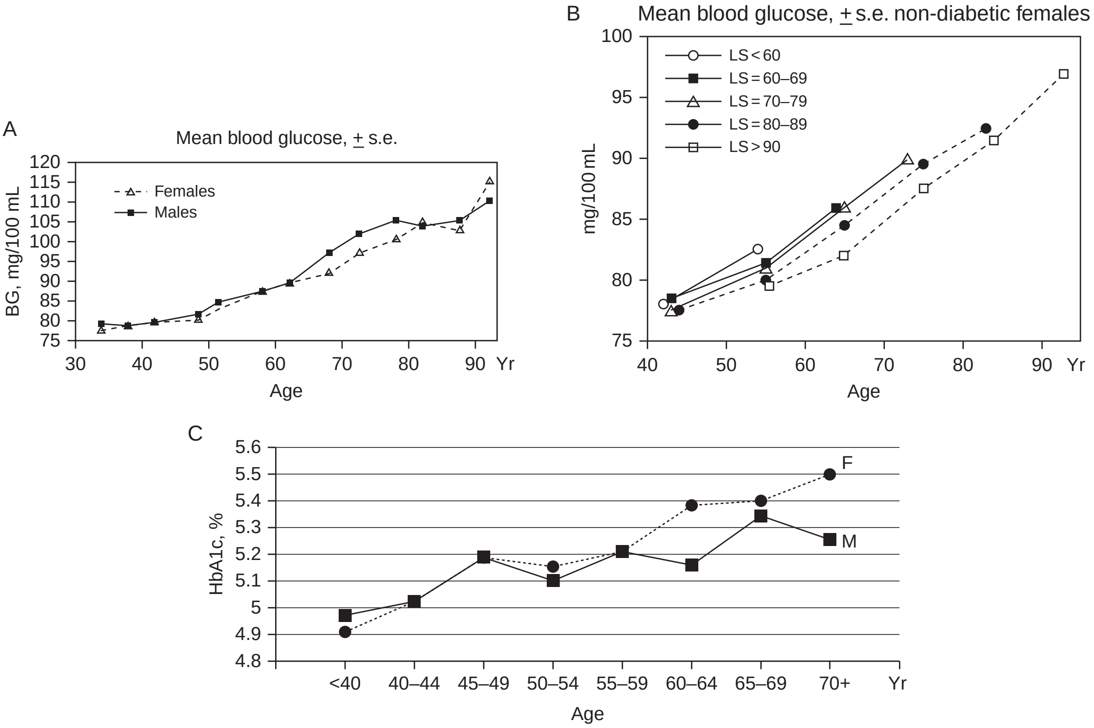

Figure 12.4 Longitudinal trends of blood glucose and hemoglobin A_{1c} from the Framingham Heart Study, approximating fasting (Yashin et al., 2009a,b). (A) Blood glucose, total sample, shows 5% increase per decade after age 40 (calculation by C.E.F.). (B) Blood glucose, excluding type 2 diabetes and stratified by life span (LS); the longest-lived (LS > 90) show slightly delayed age-related increases in blood glucose. (C) Hemoglobin A_{1c} in nondiabetics (Pani et al., 2008).

Lee & Cerami, 1990). Further links of AGE to mortality acceleration at later ages may emerge through associations of type 2 diabetes and obesity with certain cancers (colonic, hepatic, and pancreatic cancer; Ogunleye et al., 2009; Hevener et al., 2010) and with leukocyte telomere shortening (Salpea et al., 2010), an indicator of immunosenescence. The browning of food by cooking also produces AGEs, which can further add to the inflammatory load, as discussed below.

DIET, METABOLISM, AND EXERCISE

Diet has major systemic influences on inflammation through the levels of energy intake, energy storage in fat depots, and ingested AGEs produced during cooking. As a general principle, innate immune responses are regulated by the energy available (Finch, 2007, pp. 56–58). Fasting or caloric restriction, in addition to limiting the febrile response, as noted in the Introduction, attenuates other acute-phase responses. A systemic mechanism in caloric restriction may be elevation of corticosteroids, which is a broad gluconeogenic homeostatic response to partial starvation to maintain sufficient levels of blood glucose (Patel & Finch, 2002). Lowering of blood glucose by caloric restriction attenuated production of AGEs, e.g., *N*-ε-carboxymethyllysine and methylglyoxal derivatives (Ulrich & Cerami, 2001), which are proinflammatory. The oxidative load also is diminished by caloric restriction in most tissues, e.g., 10–30% reduction of carbonyl and dityrosine content (Chapters 13 and 21 in this book; Chung et al., 2009; Forster et al., 2000).

Gene expression profiling studies consistently show that caloric restriction attenuates the increased expression of cytokine and complement factor genes during aging in brain, heart, and liver (Park et al., 2009; Schumacher et al., 2008; Swindell, 2009). Caloric restriction also attenuates atherosclerosis, cancer, and Alzheimer disease in rodent models (Inflammation in Atherosclerosis and Alzheimer Disease, above; Chapter 9; Finch, 2007, pp. 210–211). In inbred mice, the genotype can influence response to caloric restriction. Among 41 recombinant inbred strains, the majority did not show increased life span on caloric restriction; these effects cannot be attributed to early deaths of nonadapted individuals because maximum and mean life spans were well correlated (Liao et al., 2010). Future studies may consider inflammatory gene variants, which were implicated in the lack of response of the DBA/2J inbred strain, which has an inactive complement C5 peptide (Finch, 2007, p. 227) among other major differences in innate immunity (Mills et al., 2000).

In contrast to low lean body mass and low inflammatory tone under caloric restriction, obesity is understood as a proinflammatory state with chronic activation of acute-phase responses (Karalis et al., 2009; Korner et al., 2009; Lee et al., 2009; Redinger, 2009). Blood CRP and IL-6 are strongly correlated with the degree of obesity across a broad range of the body mass index (e.g., Khaodhiar et al., 2004). White adipose depots contain numerous macrophages, which secrete proinflammatory cytokines (Galic et al., 2009; Maury & Brichard, 2010). Visceral fat in particular secretes adipokines and IL-6 (Fontana et al., 2007). Adipocytes from diabetics show increased cytokine production, telomere shortening, and other markers of senescence (Minamino et al., 2009). The increased blood inflammatory profile in obesity is linked to insulin resistance and diabetes, to cardiovascular disease, and to cancer (Hevener et al., 2010). In moderately obese patients, diet restriction lowered blood CRP and IL-6 (Salas-Salvadó et al., 2006), with correspondingly lower incidence of cardiovascular events (Lee & Aronne, 2007). Nonetheless, there is an "obesity paradox" in which some obese patients with cardiovascular disease have a better prognosis than the nonobese (Lavie et al., 2009).

Dietary AGEs can contribute to systemic inflammation, glucose dysregulation, and vascular disease. Cooking produces AGEs as part of the chemistry of browning (see above). Vlassara and colleagues (2009) have shown in clinical studies that increasing the dietary AGE content caused rapid impairments in glucose tolerance, with concomitant elevations of blood CRP indicative of systemic inflammation (Uribarri et al., 2007). Moreover, mice on caloric restriction given food heated to increase AGE content had increased oxidative stress (blood AGEs, isoprostanes, GSH:GSSH ratios), impaired glucose tolerance, and shortened life span in association with fibrosis of heart and kidney and elevations of myocardial RAGE and p66^{shc} (Cai et al., 2008a,b). AGEs can act directly on pancreatic cells to impair insulin secretion through induction of inducible nitric oxide synthase (Zhao et al., 2009).

Physical activity also influences systemic inflammation. Although exercise induces IL-6 release by skeletal muscle, which might be considered proinflammatory, this cytokine also increases blood levels of anti-inflammatory cytokines (IL-1 receptor antagonist and IL-10) and stimulates fat oxidation (Mathur & Pedersen, 2009). For example, induced treadmill exercise of old rats decreased renal lipid oxidation (malondialdehyde) and increased blood IL-10 (anti-inflammatory cytokine) (Asghar et al., 2007). Exercise also inhibits the accumulation of macrophages in fat depots (Woods et al., 2009). The benefits of exercise are recognized as reducing the risk of many chronic conditions of aging (Bruunsgaard, 2005; Finch, 2007, pp. 211–223; Mathur & Pedersen, 2008) and may extend to preclinical Alzheimer disease (Baker et al., 2010). We may anticipate many new therapeutic targets in metabolism that engage the multifarious pathways of innate immunity.

GENETICS

Among genes that influence longevity (see Chapter 10), some pleiotropic alleles also influence inflammatory responses. An expanding example is the apolipoprotein (apoE), a blood cholesterol carrier that mediates reverse cholesterol transport to the liver and independently mediates cholesterol transport to neurons (Mahley et al., 2007). The *apoE4* allele is associated with higher risk of coronary artery disease and Alzheimer disease than the most prevalent *apoE3* allele (see Inflammation in Atherosclerosis and Alzheimer Disease). *apoE4* carriers also incur more damage from head injury and stroke, for which drugs are being developed (James et al., 2009; Tukhovskaya et al., 2009). Not surprisingly, *apoE4* is also associated with life-span shortening: after age 80, the prevalence of the *apoE4* allele drops sharply, because of early mortality in proportion to *E4* allele dose (Schächter et al., 1994; Choi et al., 2003; Ewbank 2004; Dato et al., 2007). The *apoE* allele system is unique to humans and is hypothesized to have evolved during the shift from plant-based diets of the great apes to the meat-rich diets of humans (Finch, 2010; Finch & Stanford, 2004; Finch & Sapolsky, 1999). The apoE protein of great apes, while having the residues characteristic of apoE4 (R112, R158), is predicted to function like apoE3 (C112) (Finch & Stanford, 2004; Finch, 2010), because another difference (T61) should render peptide folding to resemble the apoE3 protein functionally (Raffai et al., 2001). Resistance to infection may have been a factor in the origins and preservation of *apoE4*, because its carriers have less fibrotic liver damage from hepatitis C infections (Fabris et al., 2005; Wozniak et al., 2002). In other circumstances, *apoE4* carriers have greater postsurgical elevations of TNFα in humans (Grünenfelder et al., 2004; Drabe et al., 2001), while transgenic mice with targeted replacement of human *apoE3* and *E4* show similar differences in cytokine response (Vitek et al., 2007, 2009). This fragmentary evidence suggests complex and conditional pleiotropies of inflammatory system genetics during human evolution (Finch 2007, Chapters 1 and 6).

Other longevity gene candidates that are emerging from screens for cardiovascular risk factors in general populations and for enrichment in centenarians have pleiotropies with links to inflammation and oxidative stress and include, in addition to *apoE*, alleles of *APOC3* (Atzmon et al., 2006), *FoxO3A* (Flachsbart et al., 2009), *IGF-1R* (Suh et al., 2008), *IL-6* (Capurso et al., 2007), *IL-10* (Lio et al., 2002), *PARP-1* (Walston et al., 2009), and *PON1* (Lescai et al., 2009). So far, the common polymorphisms of inflammatory genes have not shown strong associations with mortality at advanced ages, e.g., in Danish nonagenarians (Dato et al., 2010).

Invertebrate aging models also show genetic influences on life span (this Handbook, 6th edition; Ford & Tower, 2006; Henderson et al., 2006) in association with host defense changes (Finch, 2007, pp. 318–329). In the nematode *Caenorhabditis elegans,* the *age-1* and *daf-2* mutants that increase life span also increase resistance to bacterial pathogens (Garsin et al., 2003; Garigan et al., 2002) and expression of antibacterial host defense genes (Kurz & Tan, 2004; Troemel et al., 2006). Aging flies (*Drosophila melanogaster*) show increased expression of Toll receptors, antimicrobial peptides, and other host defense genes (Landis et al., 2004; Pletcher et al., 2002). Trade-offs between immune activation and life span in various *C. elegans* genotypes (Libert et al., 2006) are modified by caloric restriction (Libert et al., 2008).

ENVIRONMENTAL INFLUENCES: AN ECOLOGICAL PERSPECTIVE

The external environment has powerful lifelong influences on aging and chronic disease (Figure 12.1B) that underlie the progressive increases in life span since 1800 (Finch & Crimmins, 2004; Finch, 2007, 114–117). The role of adequate nutrition in adult health and longevity was emphasized in the "technophysiological evolution" theory of Fogel & Costa (1997) and the Barker theory of fetal origins of adult disease (Barker, 1998). Additionally, infections and inflammogens influence many aspects of aging and chronic disease during the historical increase in life span (Finch & Crimmins, 2004, 2005; Crimmins & Finch, 2006). A cohort analysis of four European countries for which data were available in the 18th and 19th centuries showed strong statistical associations of early age mortality in birth cohorts with their later mortality at age 70 (Crimmins & Finch, 2006). Because neonatal mortality is largely due to infections, Crimmins and I hypothesize that adults who survived high-mortality environments before the 20th century carried inflammatory and infectious loads throughout life that accelerated aging and shortened life expectancy at later ages. Evidence for pathogens in vascular lesions was reviewed under Inflammation in Atherosclerosis and Alzheimer Disease. However, early exposure to infections need not require persistence of the pathogen. Further analysis of the 1918 influenza epidemic has yielded a new link between coronary disease and prenatal infection that does not depend on the persistence of pathogens in the vascular system (Mazumder et al., 2010). Men exposed during mid- or later gestation during peak flu mortality in the fall of 1918 had 25% excess ischemic heart disease 60 years later, relative to men born in the 3 months just before or after. Because H1N1 does not cross the placenta in rodent models (Shi et al., 2005), these gestational effects are attributed to maternal stress, e.g., elevated corticosteroids or IL-6, which can accelerate arterial degeneration postnatally (Mazumder et al., 2010). In sum, the progressive twofold increase in life expectancy at all ages during the past 200 years is associated with progressive reductions in the inflammatory and infectious load with improved hygiene and public health. Improving nutrition year-round in the industrialized countries was also a major factor in reducing infections during the past 200 years.

Environmental effects on aging extend to laboratory mice. The life span of the C57BL/6J mouse, inbred since 1937 at The Jackson Laboratory, began to increase in the 1950s because of improvements in animal husbandry that reduced the load of infections (Figure 12.5), e.g., from *Mycoplasma, Salmonella,* ectromelia (mouse pox), MHV, and Sendai virus (Finch, 2007, pp. 136–142). More extreme is the case of the pituitary dwarf mouse currently noted for exceptional longevity. However, just 2 decades ago, pituitary dwarfs were considered models of accelerated aging because of short life span, <6 months, and cataracts and wasting (Finch, 2007, p. 340; Fabris et al., 1988).

Airborne inflammogens from combustion products are major causes of accelerated aging. The best documented is exposure to tobacco smoke, direct or indirect, which accelerates atherosclerosis through inflammatory mechanisms involving oxidative stress (Armani et al., 2009; Stephens et al., 2008). These adverse effects on arterial functions extend to passive exposure to smoke in utero observed in neonates (Gunes et al., 2007) and in children exposed postnatally (Kallio et al., 2009; Wigle et al., 2008). Fossil fuel combustion also generates toxic particles that are associated with higher cardiovascular mortality. Epidemiological studies show intraurban gradients of cardiovascular disease and mortality that correspond to dose-dependent exposure (Pope et al., 2009). For example, cardiovascular mortality increased by ≥25% (*RR* of 1.24–1.6) for each 10 mg/m^3 increase in 2.5-μm particles in Los Angeles County over a threefold range (Jerrett et al., 2005; Künzli et al., 2010). Mouse models show corresponding acceleration of atherosclerosis from 3 months of exposure to peak freeway traffic levels of ambient particles (Araujo et al., 2008). The effects of air pollution include neuroinflammatory responses, which overlap with some aspects of Alzheimer disease (Block & Calderón-Garcidueñas, 2009). Young adults exposed to the severe pollution of Mexico City had activated microglia and diffuse amyloid deposits, with indications of greater susceptibility in *apoE4* carriers (Calderón-Garcidueñas et al., 2008). Last, airborne inflammogens are anticipated to increase in most regions of the world, particularly from four sources: vehicular hydrocarbon fuels; coal

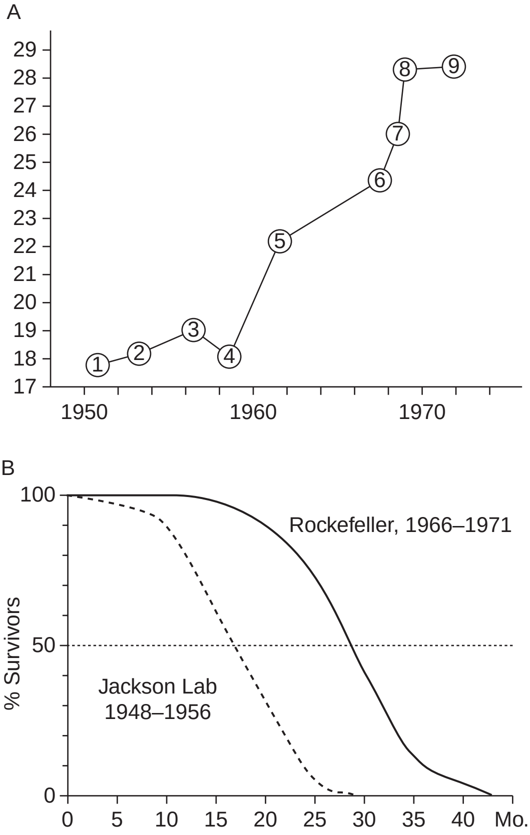

Figure 12.5 The increasing life span of the laboratory mouse with husbandry improvements that reduced the infectious load in C57BL/6J male mice, e.g., from *Mycoplasma, Salmonella,* ectromelia, MHV, and Sendai virus. (A) Mean life span from The Jackson Laboratory, Oak Ridge National Laboratory, and other sources cited in Finch, 2007, p. 137. (B) Survival curves from The Jackson Laboratory (1948–1956) (Russell, 1966) and Rockefeller University (1966–1971) (Finch, 1972).

in power generation; burning of trash, and fires in grasslands and forests.

CONCLUSIONS

The fundamental role of inflammation in aging processes is now widely recognized, particularly for arterial disease, which begins before birth. The strong age trends for elevated blood glucose and glycated proteins starting in midlife are hypothesized to be a fundamental driver of vascular disease and the acceleration of risk for vascular events. Pre- and postnatal environmental factors influence the progression of arterial degeneration, obesity, and hyperglycemia, but also airborne inflammogens from tobacco smoke and air pollution. These myriad pre- and postnatal environmental influences on arterial disease during aging give some insight into the low heritability of life spans in humans, which is <35% in identical twins, as well as in mice, flies, and worms maintained under highly uniform environments (Finch & Tanzi, 1997; Finch & Kirkwood, 2000; Hjelmborg et al., 2006).

The major effects of statins on decreasing the rate of heart attacks may be mediated by anti-inflammatory mechanisms as well as by improving cholesterol indicators. The prospective apoE4 protective drugs being developed (see Genetics, above) might also benefit health generally in the later years by reducing the risk of arterial events and Alzheimer disease (Mahley & Huang, 2009). Drugs being developed as caloric restriction mimetics may also have anti-inflammatory activities, e.g., resveratrol (Csiszar et al., 2009; Issuree et al., 2009). Of importance to life in the real world, resveratrol also inhibits wound healing (Bråkenhielm et al., 2001), as does caloric restriction (Finch, 2007, pp. 199–200; Hsieh et al., 2005). Despite these bright prospects, we must be concerned with global transmission of infectious diseases, which can accelerate aging processes, as observed for the 1918 influenza, and the increase in airborne inflammogens from combustion products. Thus, the future of human aging may depend as much on our global ecology as on advances in medicine.

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Chapter | 13 |

Protein Homeostasis and Aging

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CHAPTER CONTENTS

Introduction: Proteome Maintenance 297
Protein Homeostasis 298
Molecular Chaperones in Quality Control 299
  Cytosolic Chaperones in Folding and Refolding 300
  Chaperones in the Organelle Response to Stress 301
The Ubiquitin/Proteasome System and its Changes with Age 302
  The Catalytic Machinery 302
  The Ubiquitinization Code 304
The Autophagic/Lysosomal System 305
  Types of Autophagy 305
  Physiological Functions of Autophagy 306
  Autophagy in the Removal of Soluble Proteins 306
  Handling of Protein Aggregates by Autophagy 307
  Autophagy in Aging and Longevity 307
Concluding Remarks and Pending Questions 309
Acknowledgments 311
References 311

INTRODUCTION: PROTEOME MAINTENANCE

All proteins inside cells are subjected to continuous renewal to preserve their functionality (Ciechanover, 2005; Knecht et al., 2009). Added to this basal maintenance, cells are exposed to a changing extracellular environment that requires rapid cellular adaptation through modification of their proteome. Furthermore, agents from both inside and outside the cell can damage intracellular proteins that need to be removed before they contribute to more intracellular damage. To fulfill this maintenance and intracellular clean-up, all cells count on a precise and efficient system of quality control (Morimoto, 2008; Willis et al., 2009). The main activities of this system are surveillance, to identify the altered proteins; repair, in those instances in which damage is reversible; and, removal through breakdown whenever repair is not possible. Chaperones and proteolytic systems are the main components of the protein quality control systems inside the cells.

Maintaining a “healthy” proteome is essential for cell survival. In fact, accumulation of intracellular proteins underlies the basis of severe human disorders, often catalogued as protein conformational disorders or aggregopathies (Morimoto, 2006). In each of these disorders, the pathogenic protein and where it accumulates are different, but in all instances the accumulation of the toxic protein products leads to loss of cellular function and often cell death. Alterations in protein homeostasis are also a common feature of most tissues in old organisms (Morimoto & Cuervo, 2009). Two factors contribute to the age-dependent alteration of the proteome: increased generation of cytotoxic agents, due to age-related changes in cellular metabolism, and progressive decline of the quality control systems. Most of the cytotoxic agents that generate protein damage do not originate from the extracellular environment but instead from inside the cells. Malfunctioning of organelles, alterations in cellular metabolism, and poor performance of buffering systems all lead to an intracellular increase in harmful

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components such as free radicals and different amino-reactive species (Bulteau et al., 2006; Cakatay et al., 2003; Grune et al., 2003; Huang & Manton, 2004; Soskic et al., 2008; Viteri et al., 2004). However, even when exposed to similar cytotoxic agents, cells from a young organism are able to preserve the integrity of their proteome better than cells from an old organism, suggesting that not only an increase in protein damage but also a compromised ability to handle this damage may be behind the alterations in protein homeostasis observed in old organisms. A better understanding of the complex systems that contribute to quality control of proteins has been essential in identifying their defective functioning in aging cells. In addition, genetic manipulations in experimental models aimed at modifying the activity of these surveillance mechanisms have revealed that proper functioning of these systems is required for the increase in the life span of long-lived mutants and that their malfunctioning often leads to life-span shortening. The mechanisms responsible for maintenance of cellular homeostasis have thus been revealed as essential for longevity.

In this chapter, we first elaborate on the need for precise mechanisms to maintain protein homeostasis and then review the main components of the quality control systems—chaperones and proteases—and how they change with age. The impact that proteotoxicity has on health span is discussed in the last part of this chapter.

PROTEIN HOMEOSTASIS

The cellular proteome is highly dynamic. Proteins undergo major conformational changes for their maturation, for mobilization through cellular compartments, and during their assembly into functional units (Sakahira et al., 2002; Willis et al., 2009) (Figure 13.1). A percentage of de novo synthesized cytosolic proteins fold spontaneously; however, others require the assistance of chaperones and chaperonins. The same is true for proteins synthesized in the endoplasmic reticulum (ER), most of them destined for secretion, that reach their folding state upon interacting with the chaperones

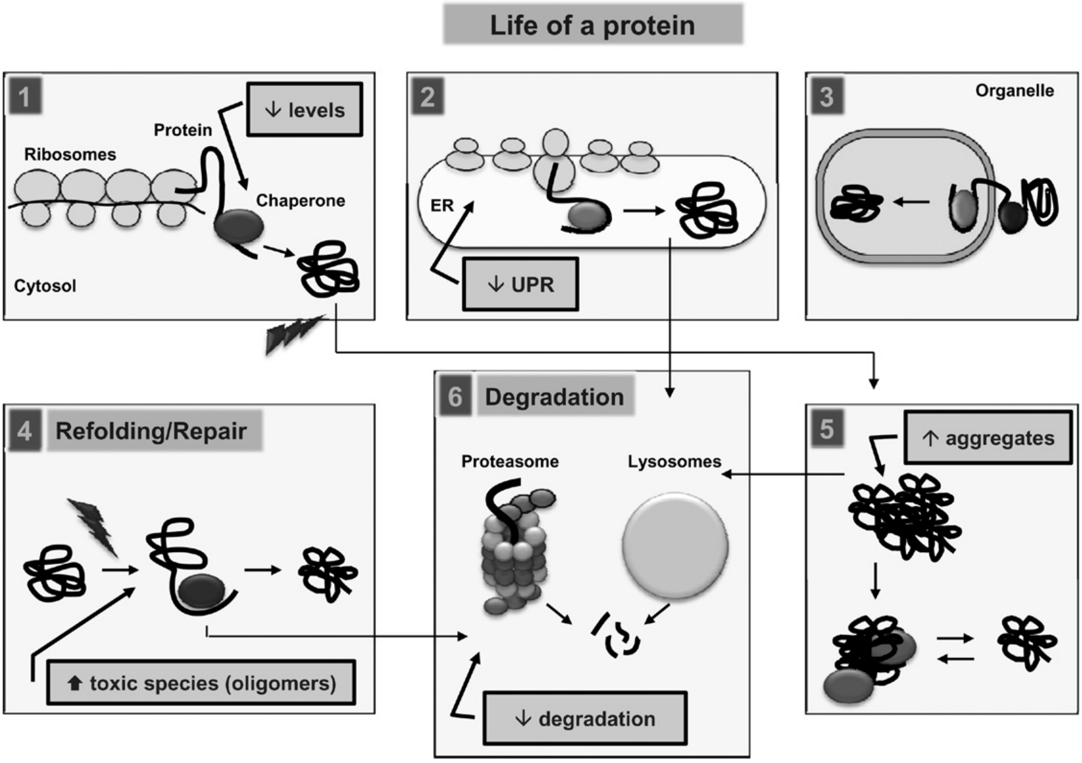

Figure 13.1 Chaperone requirements through the life of a protein inside cells and changes with aging (in gray boxes). Chaperones assist (1) folding of newly synthesized proteins in the cytosol and (2) in the endoplasmic reticulum (ER) and (3) unfolding of previously folded proteins for trafficking across organelle membranes. (6) Failure to fold will destine both cytosolic and ER luminal proteins for degradation. Chaperones also assist the (4) refolding or (5) disaggregation of damaged proteins. However, if the damage is irreversible molecular chaperones mediate the degradation of the altered proteins by the proteolytic systems (6).

resident in the lumen of this organelle (Scheper & Hoozemans, 2009). But protein folding is not limited to de novo synthesized proteins. Previously folded proteins may be required to unfold to cross organelle membranes through translocation complexes, such as in the case of the subset of mitochondrial proteins that, after being synthesized in the cytosol, need to cross the double membrane of the mitochondrion to reach the lumen where they exert their normal function. In these cases, the translocated protein needs to refold again inside the organelle to recover functionality. Partial unfolding/refolding is also required for the assembly of proteins into multiprotein complexes. Last, refolding is also required for mature proteins that unfold as a consequence of the effect of harmful agents (Bukau et al., 2006; Frydman, 2001; True, 2006) (Figure 13.1).

Folding and unfolding of the protein backbone required in all these instances often expose areas of the protein normally hidden from the rest of the cellular components because of their proaggregating nature (i.e., stretches of hydrophobic amino acids). If conformational changes could occur in an isolated compartment in the absence of interactions with any other proteins, exposure of their hydrophobic regions would have no negative consequences. However, that is not the case. Folding and unfolding of proteins occur in the cellular cytosol or in the lumen of organelles where protein density is very high and the chances of fortuitous interactions among molecules are high. The main function of molecular chaperones is to prevent these undesired interactions between proteins in the midst of conformational change (Bukau et al., 2006; Frydman, 2001; True, 2006). Independent of the nature of the protein, the immediate consequences of protein misfolding or unfolding if chaperones do not intervene will be self-association to form irreversible oligomeric structures and often their association with other nearby proteins, giving rise to protein aggregates.

Misfolding can lead to protein toxicity or proteotoxicity due to several factors (Balch et al., 2008). First is the loss of function of the modified protein and often of the other proteins that coaggregate. Second, as reviewed in detail in later sections, protein aggregates act often as cellular sinks not only for other proteins but also for cellular chaperones that bind to the unfolded proteins in their attempts to prevent growth of the aggregates (Ravikumar et al., 2002). Third, cellular toxicity can be attributed to the formation of abnormal protein complexes (self-oligomers, fibers, and aggregates). Aggregates are probably the least toxic of these structures, since studies show that often there is no direct correlation between the amount of intracellular aggregates and cell viability (Liberek et al., 2008; True, 2006). In fact, under certain conditions, formation of protein aggregates can be protective against the more toxic effect of oligomers. However, even these aggregates may not be completely innocuous because of the above-mentioned “sink effect.” In addition, aggregates also occupy cellular space and can potentially clog the proteolytic systems because of their resistance to proteolytic cleavage. The severity of this proteotoxic effect is also determined by the cellular location of the unfolded protein. For example, protein aggregates in the cytosol can be amenable for degradation by the autophagic system, whereas aggregates in the nucleus usually persist there (Martin-Aparicio et al., 2001; Tanaka et al., 2004). Protein misfolding in the lumen of organelles, such as the endoplasmic reticulum and mitochondria, elicits an organelle-specific response that increases chaperone content and thereby facilitates protein folding (Scheper & Hoozemans, 2009). Accumulation of unfolded proteins in other organelles may be resolved only by complete degradation of the organelle.

The ability of cells to maintain protein homeostasis depends on the cellular conditions. For example, diminished energy production will reduce the cell’s homeostatic capability because quality control mechanisms require energy. Also, an increase in the intracellular content of even a single pathogenic protein can indirectly reduce the stability of a subset of the proteome by limiting chaperone availability for the maintenance of proper folding (Ben-Zvi et al., 2009; Brignull et al., 2007; Gidalevitz et al., 2006).

MOLECULAR CHAPERONES IN QUALITY CONTROL

Molecular chaperones, also known as heat-shock proteins, serve both as sensors of alterations in protein quality and as active or passive facilitators of protein folding (Bukau et al., 2006; Liberek et al., 2008; Morimoto, 2008; True, 2006). Chaperones are often called heat-shock proteins (Hsp’s) because of the fact that levels of most of these proteins increase under conditions of stress, heat shock being the first stress identified. However, not all the chaperones are inducible. Cells contain a broad repertoire of chaperones that are constitutively expressed and that contribute to the maintenance of the proteome under basal conditions (Frydman, 2001; Spiess et al., 2004). Different families of chaperones have been identified based on their molecular weight (Hsp100, Hsp70, Hsp60, and small heat-shock proteins (sHsp’s)). Each chaperone family contains both constitutive and inducible chaperones that can also localize in different cellular compartments. Independent of the type of chaperone, all of them contain two distinctive regions, a substrate binding domain that interacts with the protein that needs folding assistance and a nucleotide-binding region at which cycles of ATP hydrolysis modulate the affinity of the chaperone for the substrate protein (Frydman, 2001; Spiess et al., 2004). Most chaperones act passively by binding to the unfolded protein to cover proaggregating regions and allowing spontaneous refolding (Figure 13.1). However, a subset of cytosolic chaperones, known as chaperonins, plays a more active role in folding, providing a favorable environment for protein folding (Spiess et al., 2004). To that purpose, chaperonins offer an internal chamber secluded from the cytosol where the substrate protein is internalized for folding.

Cytosolic Chaperones in Folding and Refolding

The main cytosolic chaperones involved in quality control are the Hsp70/Hsp90 family and the sHsp's, of which Hsp60 is the main chaperonin (Frydman, 2001; Sakahira et al., 2002). Hsp70 assists in folding of native proteins, some of them while still coming out of the ribosome. The constitutive form of this family, Hsc70, is more involved in the assistance of proteins in assembly and disassembly and in selective targeting of some proteins for degradation in lysosomes (Frydman, 2001; Sakahira et al., 2002). Hsp70 often acts in a coordinated manner with Hsp90, which, rather than complete protein folding, allows stabilization of proteins in transient conformations. Chaperone activity is often modulated by cochaperones, proteins that do not directly interact with the substrate protein but bind to the chaperone and modify its nucleotide binding activity or change its conformation (Frydman, 2001; Sloan et al., 2009). Some of the cytosolic chaperones are rather promiscuous and recognize very common regions such as the hydrophobic stretches recognized by Hsp70. Other chaperones, as it is the case for Hsp90, display higher selectivity for a particular subset of proteins and recognize specific motifs in the substrate proteins (Frydman, 2001; Sloan et al., 2009). Cytosolic chaperones not only mediate protein folding, but in those instances in which folding is not possible—because of either irreversible damage or cellular conditions unfavorable for folding—the same chaperones are often responsible for targeting the unfolded protein for degradation through either the proteasome or the lysosomes (Esser et al., 2004).

Changes in the content of cytosolic chaperones with age have been extensively reported in almost all organisms and tissues (Figure 13.1). These changes result, for the most part, from impaired upregulation of their expression during stress. The extent of changes in the stress-induced synthesis of heat-shock proteins is chaperone-, tissue-, and organism-specific.

Decreased transcript and protein levels of Hsp70 have been described both in senescent cells in culture and in many tissues from old organisms (Fargnoli et al., 1990). These changes in Hsp70 with age are almost undetectable in cells from human centenarians (Ambra et al., 2004; Marini et al., 2004) or in models of caloric restriction, the most efficient intervention to slow down aging (Heydari et al., 1993; Pahlavani et al., 1995). In vivo studies on the response to stress of specific organs have revealed that the changes with age are not uniform but that some regions of a particular organ are more affected (Hall et al., 2000).

The attenuation of the chaperone response to stress during aging has been shown to result from decreased binding of the heat-shock transcription factor (HSF-1) to the Hsp70 gene promoter (Ambra et al., 2004; Heydari et al., 2000; Locke & Tanguay, 1996; Singh et al., 2006). This finding may provide an explanation as to why an A/C polymorphism in the promoter region of Hsp70 has been reported to be associated with life span in humans (Altomare et al., 2003). The reasons behind the decreased binding of HSF-1 with age remain unknown but it could originate from age-related changes in the several modulatory HSF-1 binding proteins (Morimoto, 1998). Hsp90, for example, binds to HSF-1 and stabilizes it in an inactive form, whereas two histone deacetylases, the histone deacetylase 6 (HDAC6) and Sirt1, are involved in HSF-1 activation (Boyault et al., 2007; Westerheide et al., 2009). Because deacetylase activity has also been associated with longevity, these molecules may be the main players coupling protein quality control and longevity pathways. The carboxyl terminus of Hsc70-interacting protein (CHIP), a cytosolic cochaperone, may also contribute to the changes in HSF-1 regulation with age. CHIP interacts with both Hsp70 and Hsp90 and mediates their tagging for degradation (Marques et al., 2006). Dysregulation of the Hsp–CHIP interactions in old organisms has been suggested and could reduce the degradation of Hsp90, resulting in functional inactivation of HSF-1 through its cytosolic retention (Jana et al., 2005; Min et al., 2008).

Strong genetic connections have been established between cytosolic chaperones and longevity. For instance, increased induction of several cytosolic chaperones such as Hsp70 and sHsp's has led to enhanced stress resistance and increased longevity in both uni- and multicellular organisms, such as *Drosophila melanogaster, Caenorhabditis elegans,* or budding yeast (Kurapati et al., 2000; Lithgow et al., 1995; Shama et al., 1998; Tatar et al., 1997). Conversely, inactivation of Hsp genes has been shown to decrease life span (Morrow et al., 2004). HSF-1 overexpression (Hsu et al., 2003) and knockout (Garigan et al., 2002) also result in lengthening and shortening of life span, respectively. These findings all support the notion that a better capacity to adapt to stress makes a major contribution to life-span extension. However, the beneficial effect of upregulated chaperones is not universal. For instance, some studies have shown differences in the extent of the effect of overexpressing a single chaperone in different organisms. Thus, expression of Hsp16 results in proportionally higher life-span extension in worms than in flies (Morley & Morimoto, 2004). Furthermore, as highlighted above, differences in chaperone content between young and old organisms are most evident upon induction compared to basal conditions. In fact, several studies have reported higher constitutive levels of chaperones in tissues from old organisms that can be reversed by caloric restriction (Cao et al., 2001; Weindruch et al., 2001). Consequently, a chronic increase in chaperone levels may not be the ideal solution to aging. In fact, continuous presence of high amounts of chaperones could lead to increased malignancy, due to their stimulation of cell proliferation and cell survival (Soti & Csermely, 2007). Maintaining low basal levels of chaperones, but restoring their inducibility in response to stress, may be more beneficial instead.

In contrast to the vast literature on the effects of chaperones on life span of cultured cells and invertebrates, studies in mice have been sparser. Of particular interest have been studies in the mouse knockout of CHIP, which displays reduced longevity, increased oxidative stress, and accelerated cellular senescence (Min et al., 2008). HSF1 knockout mice fail to activate a heat-shock response and are hypersensitive to endotoxemia but, surprisingly, do not show premature aging under laboratory conditions (Xiao et al., 1999). The unnatural stress-free environment in which these animals are maintained may be the reason for the lack of effect on longevity. The contribution of chaperones to longevity in mammals may be more complex than in invertebrates. For example, changes in Hsp mRNA in caloric-restricted mice are different depending on the organ (Ehrenfried et al., 1996; Heydari et al., 1993; Pahlavani et al., 1996). Similar organ-specific changes in Hsp mRNA levels have been described in mice with increased life span through manipulations in their growth hormone/ insulin growth factor pathways (Swindell et al., 2009).

Chaperones in the Organelle Response to Stress

Quality control of organelle-resident proteins is essential for proper organelle functioning. Although, as described in later sections, organelles can be degraded as a whole in lysosomes, this does not need to be the fate for those organelles in which only a subset of proteins is altered. For these instances, organelles contain their own chaperones that ensure refolding of the faulty proteins or target them for degradation outside the organelle. In addition, organelle chaperones also assist in refolding of resident proteins as they gain access into the organelle through the translocation complexes in their membrane.

Quality control of de novo synthesized proteins in the endoplasmic reticulum is attained through exquisite chaperone-mediated mechanisms in which chaperones recognize particular sugar groups in the nascent polypeptide chain even before it is detached from the ribosome and assist in its folding (Frydman, 2001; Ron & Walter, 2007; Scheper & Hoozemans, 2009). Release from the chaperone is mediated by sugar trimming; however, if proper folding is not attained the protein is then recognized by an enzymatic system that adds back the sugar residue, allowing a new cycle of recognition/binding by the luminal chaperones. Although this mechanism applies to almost all proteins translated into the ER lumen, specific chaperones are also available to assist secretory proteins that are particularly difficult to fold (Frydman, 2001). Conditions under which the amount of unfolded proteins in the ER surpasses the chaperone folding capacity activate a safety mechanism known as the unfolding protein response (UPR) (Ron & Walter, 2007; Scheper & Hoozemans, 2009). The immediate consequence of UPR activation is the attenuation of protein translation to decrease the rate at which new unfolded proteins reach the ER lumen. The UPR also enhances expression of ER luminal chaperones, which increases the rate of protein folding (Ron & Walter, 2007; Scheper & Hoozemans, 2009). Those proteins that fail to fold after repeated chaperone attempts face the same fate as described for unfolded proteins in the cytosol and are targeted for degradation. In this case, degradation occurs outside the organelle, thus requiring retrotranslocation of the unfolded protein from the ER lumen into the cytosol. Once there, most proteins undergo ubiquitinization and are degraded by the proteasome through a process known as ER-associated degradation (Meusser et al., 2005). Although, so far, lysosomal degradation has been shown only for whole regions of the ER (Yorimitsu et al., 2006), it is plausible that some of the proteins retrotranslocated from the ER lumen could be targeted to lysosomes for degradation.

Levels of several ER chaperones have been shown to decrease with age in various tissues in rodents and in senescent human fibroblasts in culture (Choi & Kim, 2004; Erickson et al., 2006; Hussain & Ramaiah, 2007; Naidoo et al., 2008; Paz Gavilan et al., 2006; Rabek et al., 2003). For some of the ER chaperones, in addition to the drop in protein levels, a decrease in their activity with age has also been reported (Nuss et al., 2008). Functional decline could be a consequence of chaperone damage. For example, an age-associated increase in oxidation of key ER resident chaperones has been recently reported in mouse liver (Nuss et al., 2008). The ability to activate an efficient UPR under conditions of compromised ER homeostasis is also reduced in old organisms (Gavilan et al., 2009; Naidoo et al., 2008; Paz Gavilan et al., 2006). Inefficient UPR in old organisms originates from both failure to activate the expression of some of the ER chaperones and inappropriate persistent upregulation of others (Wang et al., 1996; Zinszner et al., 1998).

Experimental imbalance of the UPR, similar to that observed in old organisms, has been shown to sensitize cells to stressors and eventually to precipitate cell death (Ikeyama et al., 2003; McCullough et al., 2001).

Although the information on changes in mitochondrial chaperones with age is still limited, increased oxidative damage has been described for mitochondrial (mt) Hsp70 in old organisms (Jin et al., 2007; Shan et al., 2007). In genetic studies, better resistance to stress and increased life span have been observed in invertebrates overexpressing mitochondrial chaperones such as mt-Hsp70 and the sHsp Hsp22 (Morrow et al., 2004; Yokoyama et al., 2002). Furthermore, flies selected for increased longevity display Hsp22 transcriptional upregulation (Kurapati et al., 2000). Conversely, knockdown of these same proteins shortens life span (Kimura et al., 2007; Morrow et al., 2004). However, more studies are needed to elucidate the mechanisms by which these chaperones exert their beneficial effects and to determine their contribution to cellular aging.

THE UBIQUITIN/PROTEASOME SYSTEM AND ITS CHANGES WITH AGE

The ubiquitin/proteasome system (UPS) contributes to protein quality control by degrading some of the proteins that fail to fold or refold in the cytosol. The UPS also degrades unfolded proteins normally resident in organelles that are retrotranslocated into the cytosol to avoid organelle stress (Yorimitsu et al., 2006). Targeting to the catalytic component where breakdown takes place is mediated by chaperones that often recognize a degradative tag in the substrate protein (Figure 13.2). Because of the rapid degradation that characterizes the UPS, this proteolytic system has an essential role under conditions requiring rapid change in a subset of the cellular proteome, such as regulation of cell cycle, cell division, transcription, and cell signaling (Goldberg, 2007; Murata et al., 2009; Navon & Ciechanover, 2009).

The Catalytic Machinery

Proteolysis in the UPS takes place in the proteasome, a multicatalytic protease characterized by its barrel shape; substrate proteins are internalized by one side of this barrel and are then broken down in small peptides released on the other side of the barrel (Navon & Ciechanover, 2009). The proteasome is formed by four sets of seven subunits that assemble in the form of four rings. The α subunits form the two inner rings, whereas β subunits are placed in the two external rings (Figure 13.2). This complex is named the 20S proteasome based on the centrifugal force required for its sedimentation. The 20S proteasome performs three major types of proteolytic activities—trypsin-like, chymotrypsin-like, and peptidylglutamyl peptide-hydrolyzing activity or caspase-like (Cheng, 2009). Specific subunits of the proteasome can be interchanged, giving rise to various subpopulations of 20S proteasomes coexisting in the same cell. The activity of the catalytic barrel is modulated by various protein complexes that form the regulatory subunits. The most common are the 19S and the 11S (Murata et al., 2009; Navon & Ciechanover, 2009). Association of the 20S with a 19S regulatory subunit forms the 26S proteasome, the type of proteasome more closely linked to quality control. The 22S proteasome, or immunoproteasome, resulting from the combination of the 20S core with the 11S regulatory subunit, was initially thought to be fully dedicated to antigen presentation. However, further studies support the notion that the 22S proteasome may also be involved in selective breakdown of other cytosolic proteins and may thus contribute to protein homeostasis (Ferrington et al., 2005; Husom et al., 2004).

The 19S regulatory complex contains various subunits that participate in the recognition of the degradative tag, removal of this tag for its recycling, unfolding of the substrate protein, opening of the catalytic chamber, and active delivery of the substrate into this chamber (Murata et al., 2009; Navon & Ciechanover, 2009) (Figure 13.2). Most of the proteins that integrate the 19S are ATPases, explaining why proteasome degradation is an energy-dependent process.

A decrease in the catalytic activity of the proteasome with age has been described in various tissues and organs, but as in the case of the chaperones, these changes are not universal. Loss of proteasome function with age has been described in human muscle (Ferrington et al., 2005; Husom et al., 2004), lens (Viteri et al., 2004), lymphocytes (Carrard et al., 2003; Ponnappan et al., 2007), and epidermal cells such as keratinocytes (Petropoulos et al., 2000) and fibroblasts (Hwang et al., 2007; Sitte et al., 2000). A similar functional decline has been found in aged tissues of other mammals (mice, rats, and bovines), such as liver (Conconi et al., 1996; Keller et al., 2000; Shibatani et al., 1996), spinal cord (Keller et al., 2000), lens (Shang et al., 1997), heart (Bulteau et al., 2002; Keller et al., 2000), retina (Louie et al., 2002), kidney (Keller et al., 2000), and some brain regions (Keller et al., 2000; Zeng et al., 2005). Often, age-dependent changes in each of the proteolytic activities of the 20S are not synchronous. Functional decline in the proteasome is prevented by caloric restriction in many tissues in rodents (Dasuri et al., 2009; Hepple et al., 2008; Lee et al., 1999; Lee et al., 2008; Shibatani et al., 1996; Zhang et al., 2007), and it is not noticeable in fibroblasts from healthy centenarians in culture (Chondrogianni et al., 2000). The contribution of decreased proteasome

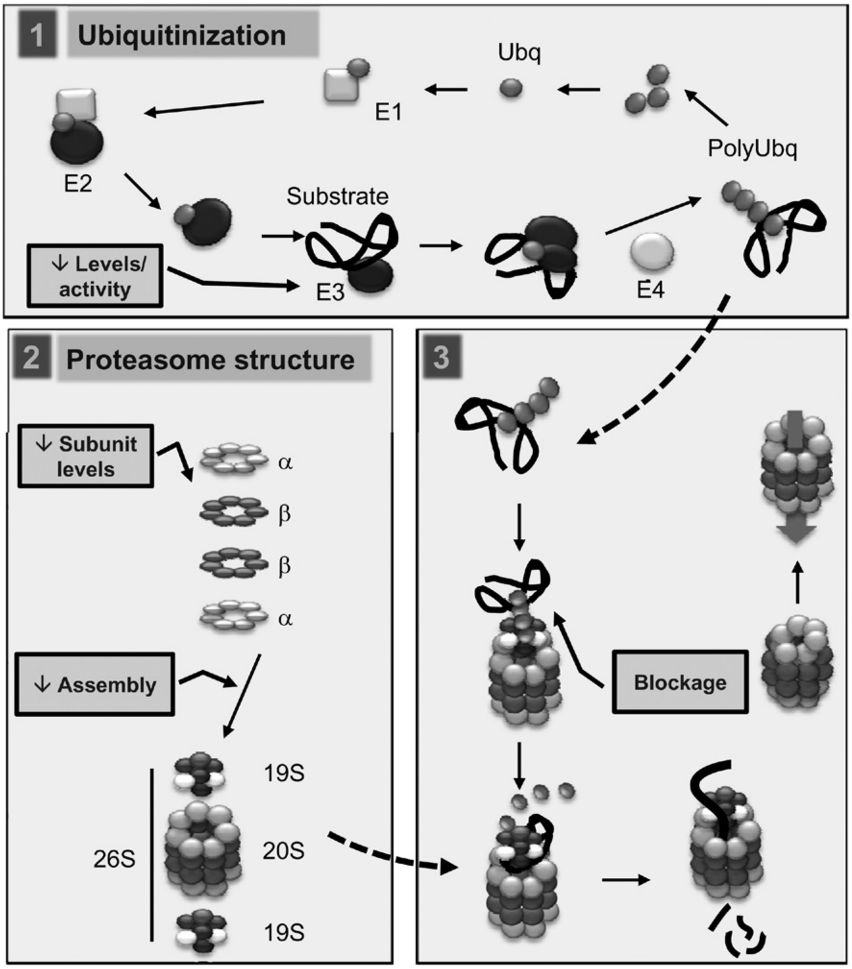

Figure 13.2 Schematic model of the ubiquitin/proteasome system and changes with aging (in gray boxes). (1) Most proteins are targeted for degradation through the covalent attachment of four or five ubiquitins to a lysine residue. Ubiquitinization requires the coordinated action of catalytic enzymes (E1, E2, and E3) that act sequentially to activate the ubiquitin (E1) and ligate it (E2) to the substrate presented by the E3. (2) The proteolytic component, the 26S proteasome, has a catalytic core (the 20S), formed by four rings containing two types of catalytic subunits (α and β), and a regulatory complex (the 19S). (3) Polyubiquitin chains are recognized by components of the regulatory subunit, of which deubiquitinases reverse the covalent conjugation, releasing free ubiquitin for recycling. The substrate is unfolded by unfoldases in the regulatory lid and ATPases in this complex provide the energy required for the extrusion of the substrate protein into the catalytic barrel or 20S proteasome that is often present in a latent state with the gates closed. Alternatively (right), hydrophobic patches in the substrate protein can cause the gate to open.

activity to replicative aging is experimentally supported by the fact that partial proteasome inhibition in young primary fibroblast cultures triggers an irreversible senescence-like phenotype (Chondrogianni & Gonos, 2004; Chondrogianni et al., 2003; Torres et al., 2006). Interestingly, overexpression of a single proteasome subunit, the β5 subunit, in cultured cells is enough to overcome the functional decline of this protease in senescent fibroblasts and to extend the life span of these cultured cells by 15–20% (Chondrogianni et al., 2005). Similar effects can be achieved by overexpression of the proteasome chaperone POMP, which assists in proteasome assembly (Chondrogianni & Gonos, 2007; Witt et al., 2000). Overexpression of regulatory subunits such as the 19S component Rpn11 has been shown to extend fly life span as well (Tonoki et al., 2009). These studies suggest that artificial activation of the proteasomal system may be an effective antiaging strategy, at least in vitro and in some model invertebrates (Chondrogianni & Gonos, 2008).

Several factors underlie the age-dependent decrease in proteasome-mediated degradation (Figure 13.2).

Some of the changes occur directly in the proteasome, such as changes in levels of its subunits, problems in assembly, or posttranslational modifications of the subunits. Other changes in proteasome activity are imposed by cellular changes with age. For example, increased levels of cross-linked or aggregated substrates can get stuck into the catalytic barrel, interfering with proteasome function, or a global energetic compromise can diminish the efficiency of this ATP-dependent proteolytic complex.

Some, but not all, proteasome subunits have been found to be downregulated in aged tissues (Bulteau et al., 2000; Keller et al., 2000; Petropoulos et al., 2000) as well as in replicative senescent fibroblasts (Chondrogianni et al., 2003; Hwang et al., 2007; Merker et al., 2000). Transcriptional downregulation is behind the lower levels of some of these subunits (Ly et al., 2000). However, because age-dependent downregulation does not evenly affect α- and β-type subunits during aging (Chondrogianni et al., 2003; Keller et al., 2000)—decrease in β-type subunits occurs sooner—an excess of "free"/not assembled α subunits is common in senescent cells (Chondrogianni et al., 2003). Even in aging tissues in which subunit levels are preserved, proteasome dysfunction could originate from defects in assemblage (Vernace et al., 2007). Last, as for any other cytosolic resident protein, posttranslational modifications, such as oxidation, ubiquitination, glycation, glycoxidation, and conjugation with lipid peroxidation products, have been reported to target specific subunits and to alter proteasome activities in vivo (Bulteau et al., 2000; Carrard et al., 2003; Keller et al., 2000) and in vitro (Bulteau et al., 2001b).

Malfunctioning of the proteasome could also be secondary to changes in the substrate proteins themselves. Although oxidized proteins are frequent substrates for the proteasome (Grune et al., 1997), extensively oxidized proteins fail to be degraded and can instead become inhibitors of this protease (Bulteau et al., 2001a; Okada et al., 1999; Sitte et al., 2000). Lipofuscin and ceroids—fluorescent pigments that form through cross-linking of proteins and lipid peroxidation products—inhibit proteasome activities in vitro and also in whole cells when loaded with these pigments (Sitte et al., 2000). Even cross-linked proteins, before incorporating into lipofuscin, can inhibit the proteasome, further slowing down the removal of other modified and damaged proteins (Friguet & Szweda, 1997; Friguet et al., 1994; Sitte et al., 2000). Conversely, the lower content of lipofuscin and cross-linked proteins in the nucleus has been proposed to explain the better preservation of the functionality of nuclear proteasomes with age (Merker et al., 2003).

In contrast to the frequent decrease in constitutive proteasome, levels of the immunoproteasome have been described to increase with age in tissues such as muscle (Ferrington et al., 2005; Husom et al., 2004) and in human senescent fibroblasts (Ferrington et al., 2005; Stratford et al., 2006). Although increased levels of immunoproteasomes may occur as a compensatory mechanism in response to the downregulation of constitutive proteasomes, further studies are required to clarify the relationship between both complexes, their changes with age, and the contribution of these changes to the aging phenotype.

The Ubiquitinization Code

Tagging of substrate proteins for proteasome degradation is attained through covalent conjugation of one of their lysine residues with a small protein—ubiquitin—which has the ability to self-assemble to form polyubiquitin stretches of four or five ubiquitins that are recognized by subunits of the 19S complex (Tsukamoto & Yokosawa, 2009). Conjugation of ubiquitin to the targeted protein is catalyzed by several enzymes known as E ligases. E1 binds to ubiquitin and modifies it for linkage. E3 enzymes present the substrate for covalent linkage, which is actually performed by the E2 enzymes (Figure 13.2). Polyubiquitinization is attained through successive cycles, as described above, or directly by the action of E4, which can add ubiquitin molecules to ubiquitin already conjugated to proteins without the participation of E1, E2, or E3 (Finley, 2009; Goldberg, 2007). Covalent attachment of ubiquitin can occur at different lysine residues in this molecule, giving rise to different types of linkage designated by the lysine used. K48 is the most common linkage, almost fully dedicated to tagging for protein degradation through the 26S proteasome (Ravid & Hochstrasser, 2008). Although the code is not completely deciphered, other linkages such as K63 seem to mediate degradation through other proteolytic systems or even functions other than degradation, such as subcellular compartmentalization, signaling, or conformational stabilization (Kirkin et al., 2009).

Aging is associated with a decrease in ubiquitination activity as well as in the amounts of free and conjugated ubiquitin (Jahngen et al., 1990; Scrofano et al., 1998a). Expression of two ubiquitin-conjugating enzymes (Ruotolo et al., 2003) and an E3 ligase (Hawse et al., 2004) is downregulated in human lens with age. Not only basal levels but also stress-induced upregulation of E1 and E2 are compromised in liver of old rodents (Scrofano et al., 1998b). Changes in gene expression seem also to be behind the reported decrease in ubiquitin with age (Chen et al., 2006). In addition, molecular misreading during translation can generate dysfunctional ubiquitin that is resistant to degradation (Tsirigotis et al., 2001). Whether aging affects other steps in ubiquitinization such as the ability of E3 to recognize substrates, the type of ubiquitin linkage, or the activity of deubiquitinases remains to be analyzed.

THE AUTOPHAGIC/LYSOSOMAL SYSTEM

The other major proteolytic system involved in quality control is a whole organelle, the lysosome. Lysosomes are single-membrane vesicles that contain a powerful enzymatic machinery capable of breaking down not only proteins but also all other types of macromolecules such as lipids, sugars, and nucleic acids (Hochshild, 1970). For this reason, lysosomes contribute not only to protein quality control but also to turnover of organelles, thus having a more global effect on total cellular homeostasis. This degradation of intracellular components in lysosomes is known as autophagy (He & Klionsky, 2009; Mizushima et al., 2008).

Types of Autophagy

Proteins can reach the lysosomal lumen for degradation through various pathways, namely, macroautophagy, microautophagy, and chaperone-mediated autophagy (He & Klionsky, 2009; Mizushima et al., 2008). Each of these autophagic pathways has dedicated molecular components, is differentially regulated, and can perform unique additional tasks (Figure 13.3).

Proteins can be sequestered along with other cytosolic components inside double-membrane vesicles or autophagosomes through macroautophagy. The autophagosome acquires the enzymes required for degradation of their luminal content through fusion with lysosomes. Formation of autophagosomes requires conjugation of a subset of proteins to other proteins or to lipid molecules to generate a limiting membrane that seals on itself and surrounds the cargo to be degraded (Ohsumi & Mizushima, 2004). Almost 35 different proteins, generically known as autophagy-related proteins, or Atg's, participate in macroautophagy (Klionsky et al., 2003). In addition to the protein conjugation cascades involved in autophagosome formation, other Atg's form part of complexes required for macroautophagy induction, such as the beclin/Vps34 complex (Itakura et al., 2008). Other protein complexes prevent macroautophagy activation, specifically the mTOR kinase complex (Noda & Ohsumi, 1998).

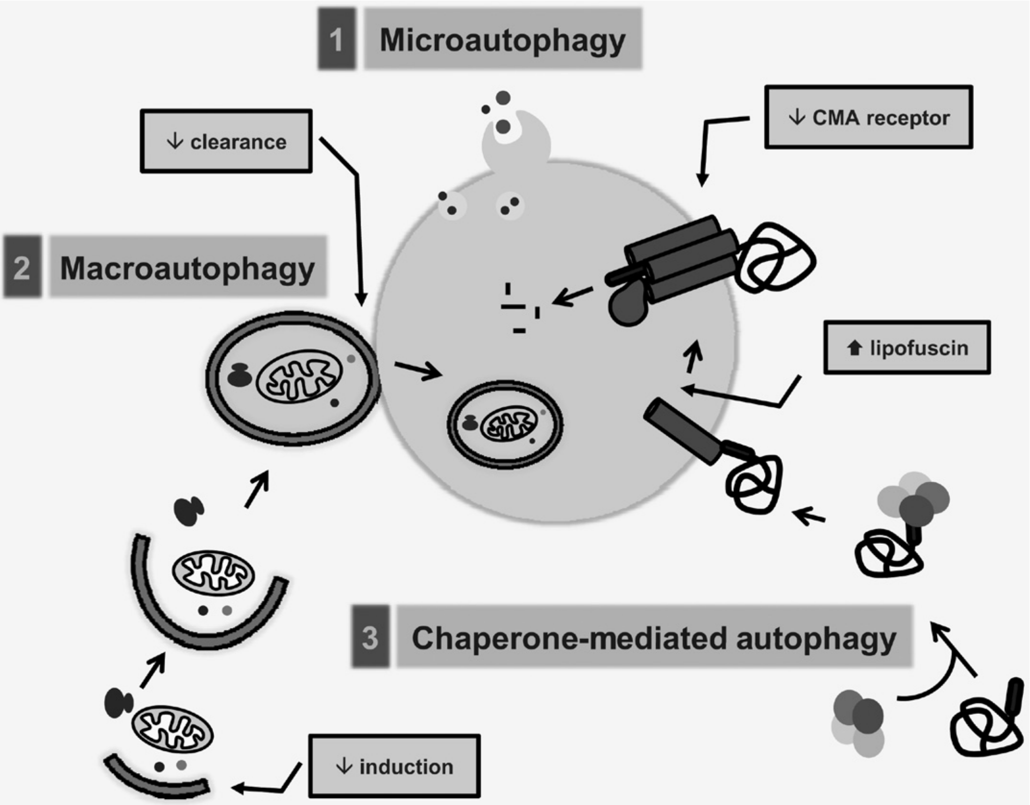

Figure 13.3 Intracellular autophagic pathways and changes with age (in gray boxes). Three different mechanisms contribute to the delivery of cytosolic cargo to lysosomes: (1) microautophagy, (2) macroautophagy, and (3) chaperone-mediated autophagy (CMA).

Specific Atg's are involved in autophagosome/lysosome fusion, recycling of autophagosome components, and even cargo selection—as it is the case, for example, for Atg32, which has been shown to be necessary for mitochondrial autophagy (Tolkovsky, 2009). Recognition of cargo is actually one of the aspects of macroautophagy subjected to intense investigation. Various cargo-recognition proteins have been now identified. These have the ability to bind at the same time components in the substrate and components of the autophagic machinery (Tolkovsky, 2009). Thus, these molecules can bring the autophagic machinery to the cargo and form autophagosomes in situ. The first identified protein of this nature was p62, initially shown to mediate degradation of protein aggregates in the cytosol (Bjorkoy et al., 2005) and more recently revealed to help recognize organelles for degradation (Kim et al., 2008). Other cargo-recognizing proteins such as NBR1 seem so far to be limited to recognition of protein aggregates (Waters et al., 2009).

A similar sequestration of cytosolic cargo for degradation takes place in microautophagy, but in this case cargo is directly trapped in lysosomes through invaginations of their membrane (Marzella et al., 1981; Mortimore et al., 1988) (Figure 13.3). Studies in yeast have shown that microautophagy shares some components with macroautophagy but it also requires a subset of proteins specific for this process. Although microautophagy was initially identified in mammals, most of the advances in the molecular dissection of this pathway have been in yeast (Dubouloz et al., 2005). However, so far mammalian homologs of the yeast genes involved in microautophagy have not been identified. This has limited the understanding of mammalian microautophagy because there are no currently available assays to quantify this process in mammals.

The third type of autophagy described in mammals, chaperone-mediated autophagy (CMA), is radically different from micro- and macroautophagy. In CMA, single soluble cytosolic proteins are targeted for degradation, but instead of being sequestered they are made to cross the lysosomal membrane to access the luminal proteases (Cuervo, 2009; Dice, 2007) (Figure 13.3). Only proteins containing a specific motif in their amino acid sequence (Dice, 1990) can be recognized by a cytosolic chaperone, Hsc70, which brings the substrate to the lysosomal membrane (Chiang et al., 1989). The substrate binds to a lysosomal receptor, the lysosome-associated membrane protein type 2A (LAMP-2A) (Cuervo & Dice, 1996), and after unfolding (Salvador et al., 2000), the substrate is translocated into the lysosomal lumen assisted by a lysosome-resident chaperone (Agarraberes et al., 1997). In contrast to other autophagic pathways conserved from yeast to mammals, CMA does not occur in yeast, as the limiting component of this pathway, LAMP-2A, is not present there.

Physiological Functions of Autophagy

All types of autophagy fulfill two main functions inside cells: acting as an alternative source of energy and participating in cellular quality control (He & Klionsky, 2009; Mizushima et al., 2008). Breakdown of cellular proteins and lipid deposits when nutrients are scarce provides the essential components, amino acids and free fatty acids, necessary for de novo synthesis of proteins and to generate energy (Mizushima et al., 2004; Singh et al., 2009). The capability of the autophagic system to remove both proteins and organelles allows continuous turnover of all cellular components and the elimination of defective ones before they can interfere with normal cellular functioning.

In addition to these general functions, the various autophagic pathways also participate in other specialized cellular functions. Macroautophagy contributes to cellular remodeling and differentiation, important, for example, in embryogenesis or during wound healing (He & Klionsky, 2009; Mizushima et al., 2008). Macroautophagy serves also as a mechanism of defense against extracellular pathogens that reach the cytosol and it mediates the presentation of intracellular antigens as part of the innate and acquired immunity (Deretic, 2009; Levine & Klionsky, 2004). CMA also has specific functions, for example, it contributes to antigen presentation (Zhou et al., 2005), controls cellular growth in kidney through the degradation of Pax-2 (Sooparb et al., 2004), maintains neuronal viability by degrading MEF2D (Yang & Mao, 2009), and regulates part of the transcriptional response to stress by modulating levels of the transcription factor c-fos and the inhibitor of the NF-κB transcription factor, IκBα (Cuervo et al., 1998).

Autophagy in the Removal of Soluble Proteins

Autophagy contributes to protein quality control by facilitating removal of proteins while still present as soluble single units or when organized into insoluble cytosolic aggregates. Although macroautophagy does not allow selective removal of individual proteins, bulk degradation of a part of the proteome by this pathway takes place continuously in all cells and it is required for proteome maintenance. Thus, animal models in which essential autophagy genes have been knocked out in various organs display a higher content of soluble polyubiquitinated proteins and of oxidized and damaged protein products (Hara et al., 2006; Komatsu et al., 2006; Kuma et al., 2004). It is unlikely that soluble polyubiquitinated proteins can be removed selectively by macroautophagy. Instead, the balance between the soluble and the aggregated pool of polyubiquitinated proteins may be altered in these tissues secondary to the capability of macroautophagy to remove protein aggregates (described below). Selective degradation of altered cytosolic proteins in lysosomes can be more efficiently achieved via CMA, by which removal of an altered protein in the cytosol can be attained without perturbing unaltered neighboring proteins. However, CMA cannot be utilized for the removal of all damaged proteins in the cells, as only those containing a targeting motif recognizable by Hsc70 can be targeted to lysosomes by this pathway (Dice, 1990). Amino acid sequence analysis and pulldowns with antibodies that recognize the CMA-targeting motif have revealed that approximately 30% of the proteins in the cytosol contain this motif. It is possible that a higher number of proteins could become CMA substrates under particular circumstances, because the motif is based on the physical properties of the constituent amino acids rather than on the specific amino acid sequence. Thus, posttranslational modifications that result in changes in the amino acid charge could, at least theoretically, convert a protein containing four of the five required amino acids into a CMA substrate. The contribution of CMA to the removal of oxidized cytosolic proteins has been experimentally demonstrated in cellular and animal models. CMA substrates when oxidized are more readily amenable to CMA degradation, probably because of improved recognition by the chaperone in those cases in which the targeting motif becomes exposed after partial unfolding. Partial unfolding may also facilitate translocation across the lysosomal membrane (Kiffin et al., 2004). Oxidized cytosolic proteins can be detected in the lumen of lysosomes isolated from cells and animals exposed to oxidant agents (Kiffin et al., 2004). Furthermore, in support of the hypothesis that activation of CMA is required for an efficient cellular response to oxidative damage, blockage of CMA in cultured cells renders them more susceptible to oxidative stress (Massey et al., 2006). CMA may also contribute to the continuous removal of oxidized proteins under basal conditions. Supporting this idea is the observation that restoration of normal CMA activity in livers of old mice, as described in more detail below, dramatically reduces the amount of oxidized proteins in this organ (Zhang & Cuervo, 2008). It is likely that other undesired posttranslational modifications that result in exposure of the CMA-targeting motif in proteins can also favor their elimination via CMA. In vitro studies have revealed that protein denaturation, truncation, and abnormal synthesis (by introduction of an amino acid analog) accelerate their targeting and lysosomal uptake via CMA. Whether a similar process takes place in vivo requires further investigation.

Handling of Protein Aggregates by Autophagy

CMA is unable to degrade proteins when they organize in irreversible oligomeric structures or aggregates, because complete unfolding of the substrate protein is required for translocation inside lysosomes. In contrast, a growing number of studies support the notion that many protein aggregates are amenable to degradation by macroautophagy. In fact, upregulation of macroautophagy in certain aggregopathies benefits the cell, prevents cell death, and contributes to slowing down disease progression (Ravikumar et al., 2004; Sarkar et al., 2009). However, despite the initial proposals that stimulation of macroautophagy could be used as treatment in all these diseases, recent studies indicate that not all protein aggregates can be removed by macroautophagy and that, even for those amenable to degradation, there is a window in which such treatments may be beneficial. Thus, certain protein aggregates cannot be recognized by the autophagic system and remain in the cytosol despite efficient upregulation of macroautophagy (Wong et al., 2008). Current research efforts are focused on identifying mechanisms that mediate aggregate recognition by the autophagic system and designing approaches that makes aggregate removal by macroautophagy universal. However, even if all aggregates are recognized by the autophagic system, upregulation of the system may be beneficial only in the early stages of the disease, because as the pathology progresses, the autophagic system itself could be compromised. In this case, further induction of macroautophagy may not have the expected beneficial effect and could be even detrimental. For example, a decrease in the ability to clear up autophagosomes by fusion with lysosomes has been described in advanced stages of some proteinopathies (Boland et al., 2008). Further induction of macroautophagy under these circumstances would just increase accumulation of undegraded autophagosomes, worsening the autophagic clog.

Autophagy in Aging and Longevity

The lysosomal system undergoes marked morphological changes in most tissues of old organisms (reviewed in Cuervo et al., 2005). Expansion of the lysosomal compartment, changes in the levels and activity of lysosomal enzymes, and accumulation of undegraded products inside lysosomes in the form of lipofuscin are common characteristics of aging cells. Deposits of lipofuscin inside lysosomes interfere with their normal functioning by decreasing the acidification of this compartment, which is necessary for optimal functioning of most lysosomal enzymes. Also by-products and toxic species generated by the cross-linked products that form the lipofuscin may damage the resident enzymes and result in the loss of lysosomal function (Figure 13.3). Cells respond to decreased lysosomal degradation effectiveness by synthesizing new lysosomal enzymes, but because they are delivered to compartments filled with lipofuscin, full proteolytic capability is not recovered

(Terman, 1995; Terman & Brunk, 1998). Compromise of lysosomal function with age will negatively affect all pathways that use this organelle as the terminal compartment, including all types of autophagy. However, defects in other steps of these pathways have also been described in old organisms and some of them may appear before the functional failure of lysosomes (Figure 13.3). Thus, a defect in macroautophagy induction in response to nutritional stress has been described in liver of old rodents (Bergamini & Kovacs, 1990). Insulin exerts an inhibitory effect over macroautophagy, whereas glucagon stimulates this pathway. In the periods in between meals, the decrease in circulating insulin levels and the concomitant increase in glucagon lead to induction of macroautophagy. However, in old animals, even in the absence of nutrients, the stimulatory effect of glucagon is counterbalanced by the age-related increase in basal insulin-independent signaling through the insulin receptor (Bergamini & Kovacs, 1990). Oxidative changes to the insulin receptor may be behind this abnormal signaling. Although still not explored in detail, it is anticipated that persistent insulin signaling may also interfere with the ability to stimulate macroautophagy in old organisms in response to stressors other than nutritional deprivation. Direct changes with age in the Atg's that participate in macroautophagy induction also contribute to the reduced activation of this pathway. For example, lower levels of beclin-1, the main component of the macroautophagy induction complex, have been described in aging human brain (Shibata et al., 2006). Changes with age in the levels of other Atg's have been analyzed in rodents and have revealed marked organ-dependent differences (Wohlgemuth et al., 2007). Although the decrease in macroautophagy with age occurs in almost all the tissues, different defects could be behind the observed decline. In fact, in addition to the problem with the induction of macroautophagy, failure of lysosomes to clear up autophagosomes efficiently has been described in livers of old rats (Terman, 1995). Problems in autophagosome mobilization inside cells, in their fusion with lysosomes, or in the breakdown of the autophagic cargo once fused with lysosomes could be behind the observed accumulation of autophagosomes in these old livers. Although formation of autophagosomes at least isolates the damaged intracellular components—including proteins—from the rest of the cytosol, thus preventing their harmful effects, the accumulation of autophagosomes can also become detrimental for cells. The inability to eliminate the autophagosomes once formed will contribute to worsening intracellular trafficking. In addition, persistence of these autophagosomes for longer periods than the usual 10 to 20 minutes eventually results in destabilization of their membranes and subsequent release of their cargo back into the cytosol. Consequently, any intervention aimed at inducing macroautophagy to enhance intracellular clearance must be done in a context in which autophagosome clearance is intact and functional, to avoid their accumulation upon induction.

Even though the reasons for decreased macroautophagic function with age remain elusive, genetic connections between macroautophagy and aging have been clearly established (reviewed in Cuervo, 2008). Upregulation of macroautophagy has been described in almost all long-lived mutants in invertebrates (i.e., those defective for components in the insulin signaling pathway, Hars et al., 2007; Melendez et al., 2003; those with knocked out mTOR, a nutrient sensing kinase, Hansen et al., 2008, or the tumor suppressor p53, Tavernarakis et al., 2008; those overexpressing sirtuin-1, Morselli et al., 2010; and those mutants with feeding defects that mimic caloric restriction, Hansen et al., 2008; Hars et al., 2007; Jia & Levine, 2007; Melendez et al., 2003; Tóth et al., 2008). Moreover, inactivation of essential macroautophagy genes decreases the life span of these otherwise long-lived mutants. These results indicate that functional macroautophagy is required to attain longevity in these mutants.

Of particular interest is the requirement for intact macroautophagy in both insulin-defective mutants and those mimicking caloric restriction, since these interventions have been shown to have an additive effect, suggesting that different mechanisms operate in their life-span extension (see Chapters 1 and 9 in this book). Thus macroautophagy is required for both mechanisms. In contrast with the strong macroautophagy dependence of these longevity phenotypes, there has been some controversy regarding the involvement of the macroautophagy pathway in normal life span. The origin of this disagreement has been the contrasting results obtained when inactivating macroautophagy in control organisms. Whereas studies in flies and some of the worm analyses revealed shortening of life span in macroautophagy-deficient mutants of otherwise wild-type animals (Hars et al., 2007; Simonsen et al., 2007; Tavernarakis et al., 2008; Tóth et al., 2008), other studies in worms did not find differences between control and autophagy-deficient models regarding life span (Hansen et al., 2008; Jia & Levine, 2007; Melendez et al., 2003). It is possible that the different nutritional conditions among these studies could account for their higher or lower dependence on macroautophagy. Also, they may have differed in the degree of autophagic impairment attained and the ability to activate compensatory mechanisms. However, regardless of the effect on life span, it is anticipated that all the macroautophagy-defective models will have compromised functionality due to the alterations in cellular homeostasis. In addition, overexpression of various Atg proteins has been shown capable of extending life span and providing resistance to various stressors in flies (Simonsen et al., 2007), further reinforcing the beneficial effect of enhancing autophagic function.

Although longevity studies have not been performed in mammals with altered macroautophagy yet, the severe consequences of blockage of macroautophagy in various organs have been well characterized in the various tissue-specific autophagy knockout mouse models (Hara et al., 2006; Komatsu et al., 2006). The dramatic neurodegeneration observed, for example, in the animals with compromised neuronal autophagy has already provided experimental support to the proposed aggravating effect of aging in these disorders. The effect of genetic upregulation of macroautophagy on life-span extension has not been analyzed yet either. Recent exciting studies in mouse models exposed to chronic treatment with rapamycin, a well-characterized stimulator of macroautophagy, have revealed a pronounced extension of life span and health span in these mice (Harrison et al., 2009). However, the multiplicity of cellular pathways affected by the treatment (since rapamycin blocks mTOR, the major nutrient-sensing kinase complex in mammals) makes it premature to attribute the observed beneficial effects to upregulation of macroautophagy alone. Further work in these animals is needed to determine the contribution of each of the cellular pathways modified by the treatment.

Genetic studies to determine possible connections between CMA and aging have been limited by the fact, as described above, that CMA has not been characterized yet in organisms other than mammals. However, changes in the activity of CMA with age and the reasons behind those changes have been well characterized. A decrease in CMA activity with age has been reported both in senescent cells in culture and in many organs in old rodents (Cuervo & Dice, 2000; Dice, 1982). Functional decline of this pathway starts usually in midlife and CMA function continues to decrease gradually with age (i.e., it starts around 12–14 months of age in mouse models with 26–28 months mean life span). A detailed analysis of the changes with age in the various steps and components of CMA has revealed that the main steps affected in old organisms are the binding and uptake of substrates to lysosomes, whereas substrate targeting and degradation by the lysosomal enzymes remain intact until late in life. The primary defect behind decreased CMA in most models analyzed to date is a decrease in the number of receptors for this pathway, LAMP-2A, at the lysosomal membrane (Cuervo & Dice, 2000; Dice, 1982) (Figure 13.3). This decrease occurs gradually with a starting point actually earlier than the observed functional decline in CMA. Usually, lysosomal levels of LAMP-2A begin to decrease by 9–10 months in mouse liver, but normal CMA functioning is preserved for a couple of additional months through a compensatory increase in the total number of lysosomes capable to perform CMA (those containing in their lumen the chaperone required for substrate translocation) (Cuervo & Dice, 2000; Dice, 1982). The reduced levels of LAMP-2A with aging are not due to decreased transcription or problems with lysosomal delivery of this receptor. Instead, a reduced stability of LAMP-2A molecules once they reach lysosomes is behind the decreased levels of the protein observed in tissues from old organisms. Changes in the lipid composition of the lysosomal membrane are in part responsible for the reduced stability of LAMP-2A with age (Kiffin et al., 2007).

Blockage of CMA in whole animals has not been performed yet. However, reduced resistance to stress observed in cultured cells impaired for this pathway (Massey et al., 2006), combined with the close connection between longevity and cellular resistance to stress, suggests that functional CMA is required for normal life span. In addition, recent work from our laboratory indicates that decreased CMA activity in old organisms contributes to their alterations in cellular homeostasis and functional loss. Specifically, using a genetic mouse model expressing an exogenous copy of LAMP-2A in liver, we have been able to correct for the age-related decrease in levels of LAMP-2A and consequently prevent the age-dependent decline in the activity of this pathway (Zhang & Cuervo, 2008). Livers from our old transgenic mice with preserved CMA activity show a marked improvement in their cellular homeostasis and in their response to stress. In addition, functional analysis revealed maintenance of liver function to levels similar to those observed when the mice were young (Zhang & Cuervo, 2008). Although further studies are required to determine the critical components that are behind the observed beneficial effect, work in these animals supports the idea that CMA dysfunction contributes to the phenotype of aging.

CONCLUDING REMARKS AND PENDING QUESTIONS

Protein quality control and maintenance of proteome homeostasis are tightly interconnected to the cellular response to stress. Alterations in protein homeostasis underlie the basis of severe human diseases of very different characteristics for which aging is an aggravating factor. All these age-related diseases have in common the presence of deposits of toxic protein products in the respective affected tissue (Figure 13.4). There has been growing interest in trying to enhance the mechanisms that contribute to protein quality control as a possible antiaging intervention and as treatment for these age-related disorders. The potential of this intervention resides in its universal

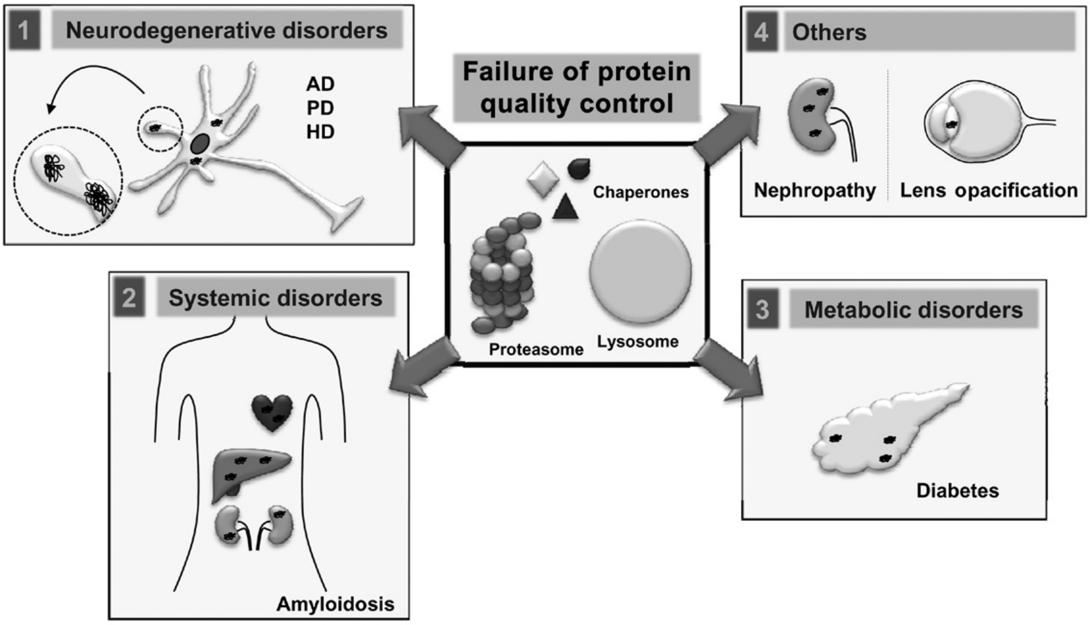

Figure 13.4 Altered protein quality control and age-related disorders. Alterations in the activity of the quality control mechanisms—chaperones, proteasomes, and lysosomes—have been described as the basis of common human diseases of higher incidence in the elderly population. Some of these disorders and the organs where proteotoxicity manifests are highlighted in this model. AD, Alzheimer disease; PD, Parkinson disease; HD, Huntington disease. Protein aggregates are represented in dark gray.

nature, because independent of the harmful agent or misbehaving protein all cells count on common mechanisms to respond to cellular stress and proteotoxicity. Keys to this protection are the components of the protein quality control—chaperones and the proteolytic systems. Better molecular characterization of each of these pathways and extensive genetic evidence supporting the idea that manipulations in essential components of these pathways result in changes in longevity and life span further support the applicability of modifications in the activity of these pathways to regulate aging.

One of the most exciting outcomes of the various manipulations aimed at enhancing the activity of the quality control system is the fact that changes in the activity of only one of these mechanisms have a beneficial effect beyond that expected by correcting a single arm of this antistress response. For example, overexpression of a single chaperone or a single subunit of the proteasome is enough to increase life span and improve the response to stress. Likewise, correction of the age-dependent defect in CMA has beneficial effects that cannot be directly attributed to CMA activity, such as improved organelle homeostasis (Zhang & Cuervo, 2008). Better protein quality could indirectly preserve organelle integrity because of the direct contact between organelles and the soluble-protein milieu in the cytosol. In addition, the reported existence of a pool of meta-stable proteins inside the cells prone to "misbehave" as a result of changes in cellular homeostasis could also explain why maintaining these proteins "in check" for a longer time could have many beneficial effects.

Although genetic manipulations have been a major asset to provide proof-of-principle support to the potential of regulating quality control mechanisms to modify longevity, better pharmacological agents are needed before these methods can be implemented to humans. Thus, despite the very promising results with the mTOR inhibitor rapamycin (Harrison et al., 2009) and more recently with a natural amine (spermidine) (Eisenberg et al., 2009), these agents still target components involved in multiple cellular processes. Design of small molecules customized for modulation of more specific steps or components of the protein quality control machinery should be sought in the near future. In addition, studies on the molecular characterization of these pathways should also start, emphasizing the interconnections among these systems. It is no longer possible to study these mechanisms outside the context of the complex cellular network that contributes to maintain proteostasis. It is likely that tissue and organ-specific peculiarities in these interconnections could be behind the differences in the abilities of different organs and species to adapt to stress and ultimately in their different rates of aging.

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Chapter | **14** |

Terminal Weight Loss, Frailty, and Mortality

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CHAPTER CONTENTS

Introduction 321
Aging and Body Weight 321
Frailty 322
Unintentional Terminal Weight Loss 323
Decreased Food Intake and Terminal Weight Loss 325
Factors Other than Decreased Food Intake in Terminal Weight Loss 326
What is Known and What Needs to be Done 327
References 328

INTRODUCTION

Body weight, blood pressure, and body temperature are the most commonly made measurements in clinical medicine. In my experience, they are measured during every annual or semiannual examination of patients by primary care physicians and by specialists for reasons as diverse as assessing a patient for possible inguinal hernia surgery by a surgeon or for a memory problem by a neurologist. These measurements are usually made by an assistant just prior to the physician seeing the patient.

The primary care physician uses the body weight data to assess whether young and middle-aged patients are overweight; i.e., body weight is used as a crude index of adiposity. The overweight patient is usually advised to lose weight, sometimes following the measurements of body mass index (BMI) and the waist–hip circumference ratio to determine the amount and distribution of body fat. This advice relates to the strong evidence that excess body fat, particularly visceral fat, predisposes the patient to a range of diseases and to premature death (Cameron & Zimmet, 2008).

Although obesity remains a problem in the elderly, the geriatrician is also greatly concerned by the occurrence of a loss of body weight. Indeed, loss of body weight in older adults is associated with an increased risk of disability, nursing home placement, and mortality (Launer et al., 1994; Locher et al., 2007; Newman et al., 2001; Payette et al., 2000). It is this association that is the subject of this chapter.

AGING AND BODY WEIGHT

In the United States and other industrialized nations, the body weight of humans increases until about the sixth decade of life and remains at that level until about age 70 years, after which it slowly decreases (Holloszy & Kohrt 1995). Since this life-span pattern of weight change is based on data generated by cross-sectional studies, the effect of aging may be confounded by selective mortality and the cohort effect, problems inherent to cross-sectional studies. However, many relatively short-term longitudinal studies indicate that the cross-sectional findings provide a reasonably valid assessment of the effects of aging on body weight. The increase in body weight does not involve an increase in fat-free mass (FFM), since it remains stable until about 40 years of age, after which it decreases with advancing age (Holloszy & Kohrt 1995). Moreover, the increase in body fat is not due to an increased food intake (Hallfrisch et al., 1990); thus, it probably results from the increasingly sedentary lifestyle commonly associated with advancing adult age.

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A similar pattern of age-change in body weight has been found in cross-sectional studies of the major rodent models used in aging research (Turturro et al., 1999; Yu et al., 1982). Such data on 115 male F344 ad libitum-fed rats and 115 male F344 food-restricted rats are presented in Figure 14.1 (Yu et al., 1982). In the case of that study, body weight was measured longitudinally over the lifetime of each of the rats used in the generation of Figure 14.1. Thus, in that study, confounders of the cross-sectional findings were readily assessed and it was found that they did not influence the conclusions about age changes in body weight based on the cross-sectional analysis.

It should be also be noted from Figure 14.1 that the general pattern of the age change in body weight for the food-restricted rats is similar to that of the ad libitum-fed animals.

This finding is significant since the food-restricted rats lived much longer than the ad libitum-fed rats and exhibited a different disease pattern, including many deaths that could not be related to disease processes based on gross pathology and histopathology (Maeda et al., 1985).

As part of the National Institute on Aging-sponsored Biomarkers of Aging Program, cross-sectional data were reported on body weight over the life span of four mouse strains (C57BL/6NNia, DBA/2JNia, B6D2F1, and B6C3F1) and three rat strains (BN/RijNia, F344/NNia, and F344 × BNF1) commonly used in aging research (Turturro et al., 1999). Both ad libitum-fed and dietary-restricted male and female mice and rats of each of the strains were studied. In each case, the general pattern of age-associated weight change was similar to that reported in Figure 14.1 for male F344 rats.

In humans, skeletal muscle mass is significantly decreased by the sixth decade of life and is markedly less than that of young adults by the seventh decade (Doherty, 2003). This is the age range when human body weight is at its maximum. The increase in fat mass between young and middle age explains this paradox. The FFM of old endurance-trained athletes is less than that of young endurance athletes and the difference is as great as that between young and old sedentary people (Pollock et al., 1987). This finding indicates that aging results in the loss of muscle mass even in the absence of a sedentary lifestyle.

The rat model differs from humans in some particulars. A loss in muscle mass occurs only late in the life of F344 × BNF_1 hybrid rats and it is not marked until near the end of life (Lushaj et al., 2008). Although loss of fat mass also occurs late in the life of the shorter-lived male inbred F344 rats (Bertrand et al., 1980), it precedes the loss of lean mass (Yu et al., 1982). The latter finding is consistent with the near end of life loss of muscle mass reported by Lushaj and colleagues (2008) for the long-lived F344 × BNF_1 rats.

FRAILTY

Frailty, which usually involves loss of body weight, is a geriatric syndrome prevalent in older adults, with an

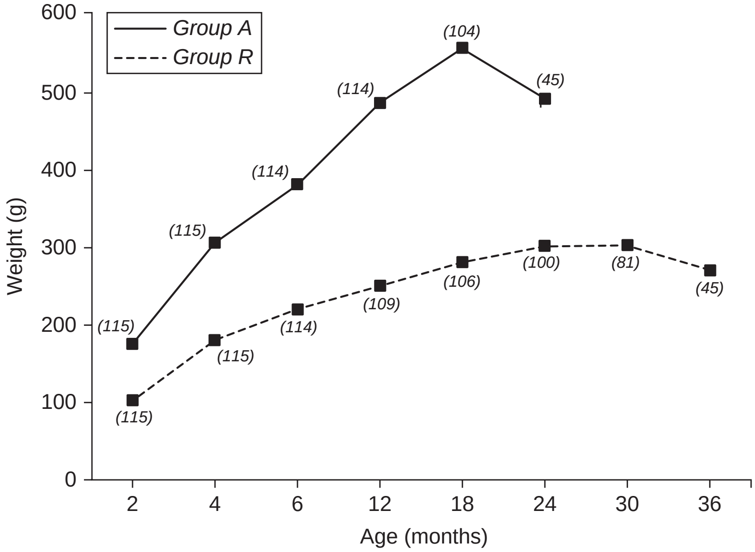

Figure 14.1 Changes in body weight during the life span of male F344 rats. (Reprinted from Yu et al., 1982, copyright Oxford University Press, reproduced by permission of the publisher.) The solid line refers to the ad libitum-fed rats (Group A) and the broken line to the rats fed 60% of the ad libitum intake (Group R). The data are mean values for the number of animals living (number in parentheses) at the given chronological age.

estimate of 7% of those over age 65 years and 20% of those over age 80 years being frail (Fried et al., 2001; Wilson, 2004). It is hypothesized that frailty represents a syndrome of loss of resilience and increased vulnerability to stress (Bergman et al., 2007; Fried & Walston, 1998). Surprisingly, a generally agreed upon definition of frailty has been elusive (Rockwood, 2005).

In 2001, a landmark paper (Fried et al., 2001) provided the following definition that the authors hoped would serve as a standard: *Frailty is a clinical syndrome in which three or more of the following are present: unintentional weight loss (10 lb in the past year); self-reported exhaustion; weakness (reduced grip strength); slow walking speed; low physical activity.* This study, using data from the Cardiovascular Health Study longitudinal cohort, found that individuals defined as frail using these criteria are at a significantly increased risk of falls, worsening disability, hospitalization, and death. Many studies have later applied these criteria in other epidemiologic cohort studies, although often in modified form because of variation in data availability for the cohort being studied; these studies have cross-validated the findings that older adults identified as frail using these criteria have a significantly increased risk for a multitude of adverse outcomes (Bandeen-Roche et al., 2006; Ottenbacher et al., 2005; Woods et al., 2005).

The landmark paper of Fried et al. (2001) pointed out that frailty is distinct from comorbidity or the presence of multiple age-associated diseases. It was also made clear that frailty is not a synonym for disability or inability to perform one or more "activities of daily living." In fact, the paper showed that while frailty is associated with both comorbidity and disability, over one-quarter of the individuals identified as frail in the cohort (26.6%) had neither comorbidity nor disability. Both comorbidity and disability have been shown to be risk factors for becoming frail (Woods et al., 2005), and disability has been shown to be an adverse outcome of frailty. Indeed, frailty also significantly increases mortality risk, a focus of this chapter.

Although the paper of Fried and co-workers is well regarded, it did not establish a standard definition of frailty. During the years following its publication, many other definitions and descriptions of the frailty phenotype have been published. Significantly, most of these papers include weight loss, the other focus of this chapter, as an important component of the frailty phenotype. Indeed, a simple index (Ensrud et al., 2008) has been developed for determining frailty called the Osteoporotic Fracture Index; this index involves the measurement of weight loss, inability to rise from a chair five times without using the arms, and poor energy (based on the subject's answer to the question "Do you feel full of energy?" on the Geriatric Depression Scale). It has been found for both women (Ensrud et al., 2008) and men (Ensrud et al., 2009) that this simple index predicts mortality as effectively as the phenotype put forth by Fried and colleagues.

Whether weight loss has a causal role in frailty of aged people or is merely a marker of its occurrence is not known. However, loss of muscle mass is a significant component in the loss of body weight by the frail elderly (Roubenoff & Hughes, 2000); clearly, this loss of muscle mass has a causal role in the frail individual's difficulty in rising from a chair without the use of one's arms, a common and practical test of lower extremity strength used in the clinical setting.

It is believed that there is a specific physiological basis to the geriatric syndrome of frailty. While there is still much unknown regarding the physiological basis of frailty and whether there is variation among individuals (Cigolle et al., 2009), there seems to be some agreement and evidence to suggest that frailty is associated with derangements in multiple physiological systems (Walston et al., 2002, 2006).

In 2009, the following three hypotheses involving a complex of physiological processes were proposed as the basis of the occurrence of frailty in the elderly: (1) Kanapuru & Ershler (2009) point out that inflammatory and blood coagulation processes are commonly altered with advancing age. They hypothesize that interactions between these two altered processes lead to dysfunctions of the immune, neuroendocrine, metabolic, and vascular physiologies and that it is this complex of dysfunctions that leads to frailty. (2) Cappola and colleagues (2009) suggest that a generalized endocrine dysfunction resulting in anabolic hormone deficiencies underlies human frailty. This hypothesis is based on the evidence that women with low levels of three anabolic hormones (insulin-like growth factor-1, dehydroepiandrosterone, and testosterone) are more likely to be frail than women with none or one of these deficiencies. (3) Fried and colleagues (2009) have an even broader view; they hypothesize that frailty results from the aggregate loss of the complexity of physiological systems. This hypothesis is based on the evidence that frailty increases nonlinearly in relation to the number of physiological processes that are abnormal and that the number of abnormal systems is more predictive than a single abnormal system. The following abnormalities were assessed: anemia, insulin-like growth factor-1, inflammation, dehydroepiandrosterone sulfate, hemoglobin A_{1c}, micronutrients, adiposity, and motor speed. Further discussion of frailty is presented below in relation to the possible mechanisms underlying terminal weight loss.

UNINTENTIONAL TERMINAL WEIGHT LOSS

Older adults who are frail, as defined by Fried and colleagues (2001) or by the Osteoporotic Fracture Index, undergo weight loss, which often is also terminal weight loss (Morley, 2003). However, the relationship between weight loss and mortality should be viewed in a broader context than frailty. As early as 1992, a loss of body weight by men (mean age of 58 years) was found to be associated with a significant increase in mortality from all causes (Lee & Paffenbarger, 1992); clearly the association between unintentional weight loss and mortality is not limited to the frail elderly. In the same year, it was reported that loss of body weight by men and women with a maximum BMI of 26 (not thin or overweight) increased the risk of death (Panuk et al., 1992). Similar findings regarding weight loss and mortality were subsequently reported by several other studies for obese older adults (Zamboni et al., 2005); obese elderly community-dwelling women age 65 years and older living in Baltimore, Maryland (Reynolds et al., 1999); a Hong Kong Chinese population age 70 years and older (Woo et al., 2001); community-dwelling men and women living in southern California, mean age 71 years (Widlock et al., 2002); and primarily male older adult patients treated at a U.S. Veterans Affairs Hospital (Liu et al., 2002). A significant increase in mortality has even been observed in very markedly overweight people (baseline BMI of 31 or more) who lose 5% or more of their body weight over a 3-year period (Chapman, 2006).

It has been suggested that the association between weight loss and mortality is probably a reflection of illness (Losonczy et al., 1995). Although Wannamethee and colleagues (2002) too propose that preexisting disease underlies the association between weight loss and mortality, they also implicate disadvantageous lifestyles. However, the fact that the association between weight loss in the elderly is similar for all-cause mortality, cardiovascular mortality, and noncardiovascular mortality makes the causal basis somewhat enigmatic (Droyvold et al., 2005). Indeed, the importance of chronic disease as a predictor of death declines with increasing age after age 50 years (Covinsky, 2007). It has been pointed out that weight loss in the elderly is usually unintentional (Wallace & Schwartz, 1997). In about one-fourth of the deaths associated with unintentional weight loss in elderly people, there was no obvious medical cause (Alibhai et al., 2005).

Although unintentional weight loss is clearly associated with an increased mortality rate, there is a question as to whether intentional weight loss is also so associated. Williamson and colleagues (1995) reported that intentional weight loss increases longevity in middle-aged, obese white women with illnesses and that its effect is equivocal in those without illness. A more recent study on overweight and obese Americans over age 35 years found intentional weight loss to be associated with a decrease in mortality rate (Gregg et al., 2003). In older men, unintentional, but not intentional, weight loss was associated with a significant increase in all-cause mortality (Wannamethee et al., 2005). Moreover, a very recent study of repeated intentional weight loss among middle age and older women found it had no effect on all-cause or cardiovascular mortality (Field et al., 2009). Noteworthily, Coffey and colleagues (2005) concluded that the association of intentional weight loss and mortality rate is an inherently unobservable entity. They point out that the association between mortality rate and intentional weight loss is not the same as the association between mortality rate and weight loss among those intending to lose weight. Thus, based on currently available data, it is likely that an actual intentional weight loss is not associated with increased mortality.

The question arises as to what component of body mass accounts for the detrimental effects of weight loss in older people. Is it the fat component or the muscle component, or both, or are they merely markers with no causal role? The current data base does not provide a clear answer. Frailty is observed not only in those who are underweight; it also occurs in obese people (Blaum et al., 2005), and reduction of the BMI (in particular visceral adiposity) in these frail obese older adults would be expected to delay mortality both by its effects on the common age-associated disease processes and by retarding frailty. Moreover, does decreasing the BMI have positive or negative effects in those with a BMI in the normal range or in underweight people? A cohort studied by Allison and colleagues (1999) began to address this issue; they reported that weight loss increased the mortality rate and fat loss decreased it. However, recent studies have not provided a clear answer to this important issue. Wannamethee and colleagues (2007) reported the results of a study of men ages 60 to 79 years, which showed a positive association between visceral fat and mortality and an inverse relationship between muscle mass and mortality. In contrast, Bouillanne and colleagues (2009) found that fat mass protects hospitalized older people against morbidity and mortality. And Auyeung and colleagues (2010) reported that a 5-year study of 4000 older adults indicated that being slightly overweight and centrally obese benefits the survival of men and that this benefit relates more to adiposity than muscle mass.

A detailed study was made on terminal weight loss using male F334 rats fed ad libitum and those fed a life-prolonging caloric-restriction (CR) regime (Black et al., 2003). One hundred ad libitum-fed rats were kept specific-pathogen-free throughout life, and 82 of them exhibited terminal weight loss. Of 60 rats on a CR regime, 38 exhibited terminal weight loss. The rats that did not exhibit terminal weight loss died at younger ages than those that did. On average, the ad libitum-fed rats began to lose weight at 635 days of age and lost 28% of their body weight in the 146 days prior to death, while the rats on the CR regime began to lose body weight at 836 days of age and lost 18% of body in the 136 days prior to death. The rats in this study are discussed further under Factors other Than Decreased Food Intake in Terminal Weight Loss with regard to possible mechanisms underlying terminal weight loss. However, it should be pointed out here that the length of time during which terminal weight loss took place did not differ significantly between the ad libitum-fed rats and the rats on a CR regimen. This is surprising since the two groups had significantly different longevities and suffered from different diseases based on postmortem assessment of pathology (Maeda et al, 1985). It should also be noted that all the rats in the longest-lived decile of the ad libitum-fed group exhibited terminal weight loss, a finding in concurrence with the cross-sectional data on humans showing a decrease in body weight during the ninth and tenth decades of life. Thus, it appears that weight loss is an almost inevitable consequence of old age.

DECREASED FOOD INTAKE AND TERMINAL WEIGHT LOSS

It has been claimed that weight loss in older humans is mainly due to reduced food intake (Wilson & Morley, 2003). Roberts & Rosenberg (2006) further developed the view that a reduced ability of the elderly to respond to a negative energy balance by increasing food intake plays a major role in weight loss older adults. They believe this deficit results from a complex of interacting factors: a decrease in olfactory and gustatory functions, impaired functioning of the autonomic nervous system, slowing of gastric emptying, an increase in the levels of plasma glucose, insulin, glucagon, and free fatty acids; these factors, along with others, probably result in a decreased perception of hunger and/or increased satiation.

Clarkston and colleagues (1997) proposed that the slowing of gastric emptying may be a major contributor to reduced food intake in healthy older adults. It has also been suggested that reduced sensory perception within the gastrointestinal tract plays a role (Chapman et al., 2002). Involvements of increased levels of β−endorphin in the cerebrospinal fluid (Martinez et al., 1997) and cholecystokinin activity (MacIntosh & Morley, 2001) have also been viewed as factors in the food intake deficit of the elderly. Elevated serum levels of cholecystokinin and peptide YY levels in the healthy elderly are also believed to sustain satiety and inhibit hunger (Di Francisco et al., 2005). However, it is important to note that unintentional weight loss in older men was not associated with inappropriately elevated leptin levels (Yukawa et al., 2003). Depression, dementia, edentulism, dysphagia, and pharmacologic agents have also been cited as causes of reduced food intake by the elderly (Hays & Roberts, 2006). Clearly, multiple factors can underlie decreased food intake by older men and women.

Food intake during terminal weight loss has also been studied in the rat model. Blanton and colleagues (1998) reported that ad libitum-fed male F344 rats undergoing terminal weight loss eat the same number of meals per day as before weight loss but the meals are of a smaller size. They also determined whether there was a change in food preference during terminal weight loss and found it was the same as before weight loss. Based on the above findings, the investigators concluded that earlier satiation occurs in the rats undergoing weight loss. The results of this research also showed that the reduction in food intake did not result from an elevation of serum levels of leptin. Nor was it due to specific disease processes. However, there is evidence that elevated levels of leptin may play a role in the reduced intake of food by old Brown Norway rats (Wolden-Hanson, 2006).

Male F344 rats in the terminal weight-loss stage of life exhibit a reduced eating response to the intracerebroventricular administration of neuropeptide Y (Blanton et al., 2001). Neither a decrease in the expression of nor in the number of neuropeptide Y_1 and Y_5 receptors in the hypothalamic paraventricular nucleus underlies this reduced response to neuropeptide Y (Coppola et al., 2004). These investigators also found that alterations in the γ-aminobutyric acid pathway as well as the neuropeptide Y pathway probably play a role in the decreased food intake during terminal weight loss by male F344 rats (Coppola et al., 2005). Impairments in the neuropeptide Y pathway also appear to be involved in the age-associated reduction in food intake in senescent Brown Norway rats (Gruenwold et al., 1996).

There is also suggestive evidence linking other factors in the reduction in food intake by senescent rats. Alterations in the orexin A network appear to be so linked (Kotz et al., 2005; Takono et al., 2004). Age changes in the functioning of ghrelin 1 (Rigamonti et al., 2006) and the melanocortin system (Wolden-Hanson et al., 2004) may also be involved in terminal weight loss. However, further research is needed to establish the role of these factors firmly.

In clinical medicine, treatment of older adults for involuntary weight loss has primarily been aimed at increasing the intake of calories and protein. Although some mitigation of weight loss in older adults by nutritional means has been reported (Levinson et al., 2005), such therapy has not been highly successful. Only about half the subjects with unintentional weight loss in a residential health care facility responded to nutritional therapy by maintaining or gaining body weight (Splett et al., 2003). In a meta-analysis of older people (but not restricted to those undergoing weight loss), it was found that protein and caloric supplementation increased body weight by 2.51%

in patients in long-term care facilities and 2.25% in those living at home (Milne et al., 2006). A recently published 24-week, double-blind, placebo-controlled study of institutionalized older people receiving nutrient-dense drinks (250 kcal per day) was in accord with the meta-analysis findings (Manders et al., 2009); however, it was not reported whether frail people or those undergoing unintentional weight loss were part of the sample studied, and the enrollment criteria would probably have excluded the former.

In frail older adults, increasing caloric and protein intake was found to have only a small effect on body weight (Boockvar & Meier, 2006). Some success has been reported in treating weight loss in old adult residents of long-term facilities by diet modification, nutritional supplements, and flavor enhancers (Padala et al., 2007). In an analysis of the literature, Chapman (2007) found that treatments of older adults with weight loss that aimed at increasing food intake were not effective. In a report of the Working Group on Functional Outcome Measures for Clinical Trials (2009), the following was concluded about treatment of the older adult undergoing unintentional weight loss: the appetite stimulants that are currently available either are not very effective or may stimulate appetite but not the accretion of lean body mass. Further, these agents are often associated with adverse side effects, such as worsening of chronic illness, edema, and central nervous system effects; in the case of megestrol, which is commonly used as an appetite stimulant, there is an increased risk of deep venous thrombosis (Kropsky et al., 2003). Given these potential harmful side effects and the limited evidence of effectiveness of these agents, they are generally not recommended for use in older adults with weight loss.

FACTORS OTHER THAN DECREASED FOOD INTAKE IN TERMINAL WEIGHT LOSS

The generally held view is that a decrease in food intake is the major cause of terminal weight loss and that the limited success of nutritional therapies is primarily the result of those undergoing such loss not being able to engage fully in the therapy. The findings of the study of Black and associates (2003) on male F334 rats undergoing terminal weight (described above) question the validity of this view. Of the 82 ad libitum-fed rats that underwent terminal weight loss in that study, 41 exhibited an increase in food intake during terminal weight loss (Figure 14.2).

Clearly, reduction in food intake played no role in the loss of body weight in half of the ad libitum-fed rats undergoing terminal weight loss. Moreover, in many of the 41 rats that did exhibit a reduced intake of food during terminal weight loss, the reduction in food intake was small (Figure 14.2); thus, in many of these rats, it is likely that decreased food intake was not a major factor in the weight loss. In the CR group, 38 of the rats underwent terminal weight loss, and reduced food intake was associated with weight loss in this group (Figure 14.2). However, 8 of the 38 rats did not show a decrease in food intake (the experimental design precluded rats on the CR regimen from increasing their food intake). All of the rats in

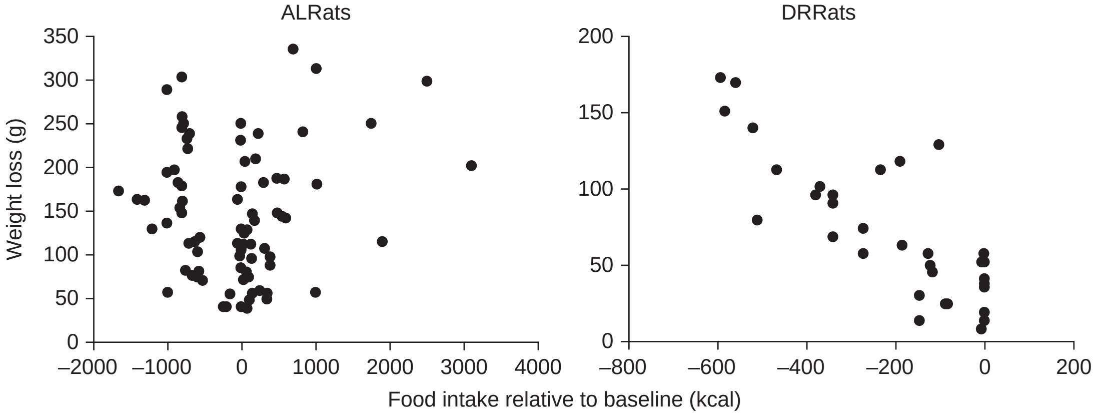

Figure 14.2 Terminal weight loss and food intake. Reprinted from Black et al., 2003, copyright American Physiological Society, reproduced by permission of the publisher. Terminal weight loss of AL (ad libitum-fed) and DR (restricted to 60% of the ad libitum-fed food intake) rats plotted against total food intake relative to baseline from the start of weight loss until death of the rat. Points to the right of 0 on the horizontal axis refer to rats that ate more than the basal intake during terminal weight loss; points to the left of 0 refer to rats that ate less than baseline during terminal weight loss.

the study underwent postmortem gross and histopathology analyses; specific disease processes were not found to explain the food intake findings.

Clearly in many of the male Fischer 344 rats, terminal weight loss was partly or fully related to factors other than a reduction in food intake. Those factors could be a loss of energy by excreting protein or fat or both in the feces and/or glucose or protein or both in the urine or by an increase in metabolic rate. Black and colleagues (2003) did not measure these possibilities. However, a lifelong longitudinal study of the metabolic rate of male F334 rats indicates that an increase in the metabolic rate may play a role (McCarter & Palmer, 1992). In that study, it was found that the metabolic rate of the ad libitum-fed rats increased from 18 to 24 months of age; by 24 months of age, many male F344 rats are undergoing terminal weight loss (Black et al., 2003).

A study in which male Brown Norway rats undergoing senescent weight loss were treated with ghrelin provides further support for the view that an elevated metabolic rate may play a major role (Yukawa et al., 2008). This research was based on the fact that ghrelin is known to promote appetite and increase food intake in humans. Starting at 33 months of age, the rats were administered subcutaneously either saline or ghrelin for 17 days, during which the rats receiving the saline lost a small but significant amount of body weight, while those receiving ghrelin maintained their body weight. However, the ghrelin administration did not increase the rats' food intake. That ghrelin is known to decrease sympathetic nervous system activity led Yukawa and colleagues to suggest that it may have blunted the loss of weight in these old Brown Norway rats by decreasing metabolic rate and cytokine-driven inflammation.

Weight loss in older adult humans has been viewed as being of three distinct types: wasting, cachexia, and sarcopenia (Roubenoff, 1999). Wasting results primarily from a decrease in food intake, cachexia is due to an increase in metabolic rate including an increased rate of protein degradation, and sarcopenia, or loss of muscle mass, is viewed as an inherent component of aging. If this classification of weight loss is correct, the frequent failure in humans of nutritional therapy in stemming weight loss is not surprising nor is the finding that half of the male F344 rats undergoing terminal weight loss exhibited an increase in food intake. Although there should be concern about their nutrition when older people lose weight (Hickson, 2006), it is likely that other factors may be involved even in the absence of overt disease. Yeh & Shuster (1999) pointed to the likely role of inflammatory cytokines in the occurrence of weight loss in the elderly. Indeed, sarcopenia in older adults has been associated with C-reactive protein and interleukin 6 (Bautmans et al., 2005).

WHAT IS KNOWN AND WHAT NEEDS TO BE DONE

About as many of the ad libitum-fed male F344 rats that undergo terminal weight loss have an increase in food intake as have a decrease. Male F344 rats on a life-extending CR diet also undergo terminal weight loss and a significant fraction of them exhibit either no or only a small decrease in food intake. Although terminal weight loss begins at an older age in the CR rats than in ad libitum-fed male F344 rats, the duration of the terminal weight loss is the same in the CR rat as in the ad libitum-fed rats. Postmortem pathological examination does not reveal a statistically significant relationship between a particular disease and food intake during terminal weight loss in either ad libitum-fed or CR rats.

What is not known is whether studies of female F334 rats and both genders of other rat strains would yield similar findings. Studies need to be done using female F344 rats and both genders of other rat strains such as the inbred Brown Norway, the outbred Sprague–Dawley, and F_1 hybrid rat models. Most importantly, studies using rat models are needed in which energy expenditure as well as the occurrence of energy loss via the gastrointestinal and urinary pathways is explored during terminal weight loss. Kirkland & Peterson (2009) point out the need to develop animal models of frailty in the quest to understand human frailty and ameliorate its effects; indeed, such models would also be useful tools for studying terminal weight loss. Walston and colleagues (2008) are in the process of developing a mouse model of frailty. Mouse models will be invaluable in investigating mechanisms underlying terminal weight loss since the tools for molecular genetic studies are so well developed in this species.

Surprisingly, there is almost no information on the extent to which alterations in metabolic rate underlie unintentional weight loss in humans. Also, the involvement of energy loss in humans via the urinary and gastrointestinal pathways has received little or no study. Therapeutic measures that are effective in preventing or slowing terminal weight loss may emerge from studies in these areas.

Ultimately, the physiological and pathophysiological bases of terminal weight loss need to be determined. Rodents in which terminal weight loss occurs, such as the male F344 rat, are good animal models for the initiation of such studies. Indeed, this approach has been initiated for the study of sarcopenia; findings from research using B6C3F1 mice suggest that an increase in muscle proteolysis underlies the loss of muscle mass (Reynolds et al., 2002). Also, a study of old male Wistar rats found that elevated levels of α_2 macroglobulin and fibrinogen are markers of loss of body weight and that very high levels of $\alpha_2$macroglobulin and fibrinogen are markers of impending mortality (Mayot et al., 2007).

Most importantly, human research on frailty and on factors predicting mortality may yield insight into basic mechanisms underling terminal weight loss. Some progress was made in this regard during the first decade of the 21st century. For example, a cluster of immunological changes and inflammation markers were found to be associated with impending death in humans (De Martinis et al., 2006). Also, IL-6 dysregulation has been implicated in the occurrence of frailty (Maggio et al., 2006); e.g., elevated levels of IL-6 occur in frailty (Leng et al., 2007). Elevated levels of neutrophils and monocytes are also associated with frailty and this association has been disassociated from the elevation of IL-6 levels (Leng et al., 2009). In addition, monocytes studied ex vivo from frail subjects have an enhanced expression of specific stress-response genes during a lipopolysaccharide challenge (Qu et al., 2009). High levels of oxidative stress have also been associated with frailty (Wu et al., 2009).

As mentioned under Frailty, above, three hypotheses have been proposed recently regarding the basis of frailty (Cappola et al., 2009; Fried et al., 2009; Kanapuru & Ershler, 2009); each of these hypotheses involves a complex of interacting physiological alterations. Testing these hypotheses will be extremely difficult because of the complexity of the several interacting processes proposed. However, it is likely that no single process underlies frailty or terminal weight loss. Indeed, the development of the concepts and the tools needed for the testing of these complex hypotheses is likely to yield rich dividends for the study and treatment of many geriatric conditions, including frailty and involuntary weight loss.

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Chapter | 15 |

Human Brain Myelination Trajectories Across the Life Span: Implications for CNS Function and Dysfunction

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CHAPTER CONTENTS

Introduction 333
Myelin in the Context of Human Brain Development and Degeneration 334
The "Myelin Model": Implications for Human Brain Function and Dysfunction 338
Conclusions 341
References 341

INTRODUCTION

The popular concept that postnatal human biology can be separated into three distinct stages (development → stability → degeneration) may be appropriate for features such as human height but is not helpful for understanding the human brain. The human brain is in a continuous state of flux defined by periods of relative development and periods of relative degeneration that together engender processes of growth, maturation, repair, and deterioration across the life span. The notion of an adult (defined as ages 18–55) human brain as fixed/stable or unchanging is therefore misleading and biologically invalid (Bartzokis et al., 2001; Kaes, 1907; Miller et al., 1980). The concept of an unchanging adult human brain *volume*, on the other hand, is valid (Bartzokis et al., 2001; Miller et al., 1980), but fails to capture ongoing changes in the underlying infrastructure of the human brain. One prime example in this regard is white matter (consisting largely of myelinated axons), which constitutes the focus of this chapter and accounts for the disproportionate enlargement of structures that mature latest in the human brain, such as the frontal and temporal lobes (Norton, 1981; Schoenemann et al., 2005; Semendeferi et al., 2002). Studies show that adult humans do not have disproportionately enlarged frontal lobes relative to overall brain size nor a disproportionate increase in gray matter (where neuromal cell bodies reside) compared to other primates; rather, human frontal lobes have disproportionately more (approximately 20%) white matter than nonhuman primate frontal lobes (Schoenemann et al., 2005; Semendeferi et al., 2002) (Figure 15.1).

The observations summarized above are consistent with the overall evolutionary trend that cognitive abilities do not correlate best with the number of neurons but rather with the number of glia (in particular oligodendrocytes, which produce myelin and astrocytes, which help regulate levels of energy, cholesterol, and neurotransmitters in the brain milieu). More specifically, nonneuronal cell numbers have increased disproportionately compared to neuronal cell numbers over evolutionary time, from 10–20% of all brain cells in the nematode to 25% in the fly to 65% in the rodent to 90% in the human (Pfrieger & Barres, 1995). Of additional interest, not only does the human brain have disproportionately more oligodendrocytes and more myelin content than other species, but the duration of active myelination is known to be extensive. On average the active myelination phase in human continues until approximately age 50–60 (Benes et al., 1994; Kaes, 1907; Kemper, 1994; Miller et al., 1980), which, from an evolutionary

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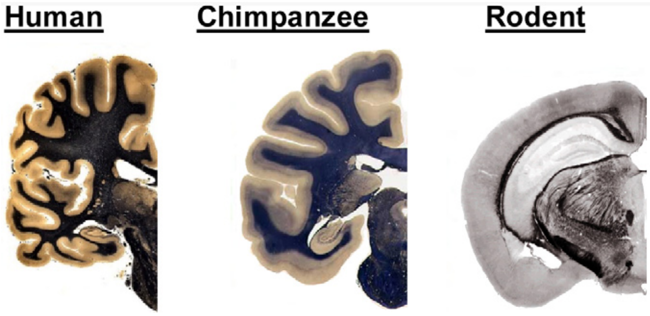

Figure 15.1 Coronal myelin stains of right hemisphere of human, chimpanzee, and rodent brains. Brains are not to scale. Chimpanzee and rodent brain are enlarged to approximate human brain to demonstrate more easily the striking differences in the *proportion* of myelin (stained black) in each brain. Human brain has approximately 20% more white matter than the chimpanzee (Schoenemann et al., 2005; Semendeferi et al., 2002).

perspective, may extend over the entire human life span, since most individuals died by age 50–60 until modern times (reviewed in Bartzokis, 2004a). In this chapter, changes in human brain myelination are discussed in the context of development and degeneration over the life span with a focus on implications for brain function as well as brain dysfunction.

MYELIN IN THE CONTEXT OF HUMAN BRAIN DEVELOPMENT AND DEGENERATION

The focus on myelin and myelination in this chapter is not intended to diminish the importance of other brain developmental or degenerative processes. The focus on myelin is especially not intended to dismiss the drastic regressive processes of neuronal, synaptic, and axonal as well as dendritic pruning that effectively reduce the "connectivity" of the developing brain by over one-third before adulthood and whose disruption can cause severe abnormalities that are usually evident in infancy (Rakic, 2002). However, it is important to note that these regressive processes, which occur in large part during puberty or midadolescence, occur in concert with the precisely regulated process of myelination (reviewed in Bartzokis, 2002, 2004b, 2005). In particular, both pruning of neural elements and myelin proliferation in human brain cortices occur in a sequential pattern that involves primary process areas first (motor, sensory), followed by association areas (parietal, temporal, and frontal lobes) (Huttenlocher & Dabholkar, 1997). More specifically, the pattern of sequentially regressive pruning (volume *reducing*) matches the pattern of sequentially boosted myelination (volume *increasing*) (Bartzokis et al., 2001; Benes et al., 1994; Kemper, 1994). As the human skull becomes rigid in late childhood, eliminating the possibility of further expansion of the brain, it might be argued that the regressive/pruning processes occur in part to provide the "room" (intracranial volume and possibly other resources) necessary to support the crucial process of myelination (reviewed in Bartzokis, 2004b, 2005).

Although regressive processes are not uncommon in biological systems, it is remarkable that such a profound (40%) loss of synapses and axons is compatible with the production of a functioning computational nervous organ. It could be in part that the dramatic gains in processing parameters offered by myelination (transmission speed, bandwidth, and "online" connectivity—see below) and perhaps other possible benefits (such as reduced energy consumption) compensate (functionally) for the regressive/pruning processes. Along these lines, the myelination process may markedly *increase* the computational potential (connectivity) of those neural networks and circuits that, because of the regressive/pruning process, have had their individual neuron computational capacity reduced (reviewed in Bartzokis, 2004a,b). Studies examining brain development in children and adolescents have demonstrated increasing white matter volume and decreasing gray matter volume in later childhood/adolescence (Giedd et al., 1999; Jernigan & Tallal, 1990; Pfefferbaum et al., 1994; Reiss et al., 1996; Shaw et al., 2006; Sowell et al., 2003). These findings were confirmed in prospective magnetic resonance imaging (MRI) studies of brain development that demonstrated that white matter volume increases linearly between ages 4 and 20 (Giedd et al., 1999; Shaw et al., 2006), a period characterized by robust myelination (Yakovlev & Lecours, 1967). But unlike the white matter changes within this age span (4–20), cortical gray matter trajectories were inverted-U shaped rather than linear and exhibited region-specific patterns of change. In particular, gray matter reached maximum volume at about age 12 in the frontal lobes and 16 in the temporal lobes (Giedd et al., 1999; Shaw et al., 2006), whereas maximal myelination was not reached until 30 years

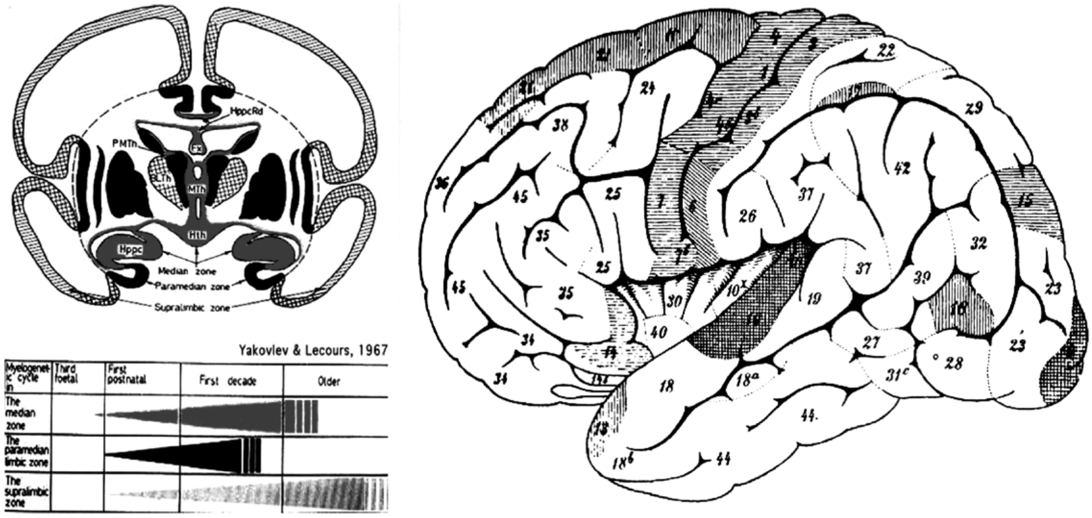

Figure 15.2 Developmental heterogeneity of brain myelination. On the left, the image shows three zones of cerebral myelination, which differ by age at myelination. Medial zone, light gray; paramedial zone, dark; dorsolateral zone, opercular and paralimbic areas, cross hatching; supralimbic association areas, diagonal hatching. The supralimbic association areas (diagonal hatching of the frontal and temporal cortices) continue myelinating into "older age." This is graphically depicted in the lower left graph, where the cortical supralimbic zones (lowest horizontal graph) are shown to continue myelinating well beyond the first decade. (Adapted from Yakovlev & Lecours, 1967). On the right, the myelogenetic fields numbered according to the time of myelination based on postmortem data (Meyer, 1981) (reproduced from Flechsig, 1920). This is one of the few works attempting to map the sequence of myelination. It illustrates the developmental heterogeneity of brain myelination with sensory and motor regions (shaded) myelinating first (in very early childhood) and temporal and frontal regions myelinating last—confirmed by Yakovlev & Lecours (1967). See Figure 15.4 for an example of how total myelinated white matter volumes and intracortical myelination can be measured on MRI.

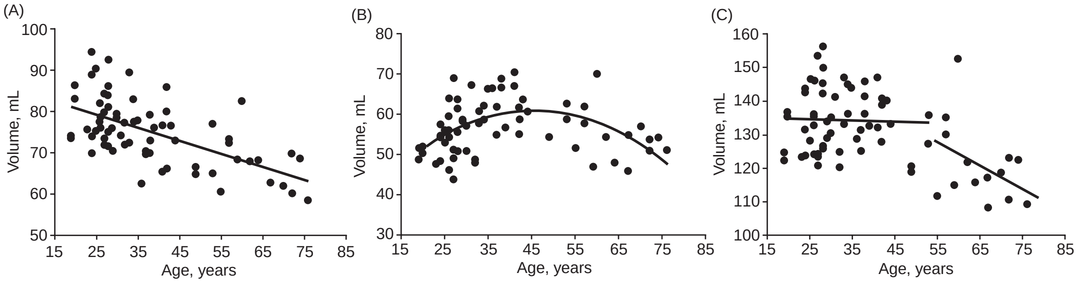

Figure 15.3 MRI volumes in frontal plus temporal lobes of healthy individuals across the life span. (A) Gray matter. Linear, $r = -0.62$, $P < 0.0001$; quadratic, not significant (NS), $P > 0.94$. Decrease in cortical gray matter volume does not necessarily reflect cell loss (see text). (B) White matter. Linear, NS, $P > 0.84$; quadratic, $t = 4.56$, $P < 0.0001$, peak = 45 years. (C) Gray plus white matter. Linear correlations for adults (18–54) and older age (55–76), confirming stable adult brain volume (gray plus white matter) observed in postmortem studies (see Miller et al., 1980). For details on MRI methods, see Bartzokis et al. (2001) and Figure 15.4.

later in both frontal and temporal lobes (Figures 15.2 and 15.3B).

The history of research on developmental changes in human brain structure has been immersed in confusion. Much of the confusion stemmed from the long-standing dogma that in normal "adulthood" (18–55 years of age) there were large reductions in cortical neuron numbers. This erroneous conclusion was based on data showing that neuronal *density* in young adults was higher than that of middle-aged and older adults. The age-related reductions in cortical neuronal density were subsequently shown to be caused by an age-related tissue fixation "shrinkage" artifact that caused cortices from younger individuals to contract more than those from older ones. Together with the assumption that neuronal *density* was equivalent to neuronal *number,* the doctrine that neuronal numbers were decreasing with age beginning in young adulthood took hold (reviewed by Haug, 1985). More recent postmortem data based on unbiased stereologic assessments, however, have demonstrated that there is minimal if any neuronal cell loss or cortical thickness decrement before the age of 55 (Haug, 1987; Pakkenberg & Gundersen, 1997; Peters et al., 1998; Terry et al., 1987). This confusion was also promoted by an underappreciation of the fact that, in primate brain, postnatal development is accounted for in large part by the protracted process of myelination. Unlike other brain cell lines, oligodendrocyte numbers increase into old age (O'Kusky & Colonnier, 1982; Peters & Sethares, 2004), presumably to support the lifelong process of subcortical and intracortical myelination (Kaes, 1907; Kemper, 1994). The excess cortical shrinkage of younger brains that occurred upon fixation was therefore most likely due to the lower levels of intracortical myelin at younger ages (Kaes, 1907; Kemper, 1994); intracortical myelin peaks in the middle to late fifth decade of life in association regions such as the frontal and temporal lobes (Bartzokis et al., 2001; Kemper, 1994), and lipid-rich myelin reduces the amount of dehydration-related contraction that occurs during tissue processing (Kretschmann et al., 1982; Ogata & Feigin, 1973). In other words, the lower the myelin content of any cortex the more shrinkage would be expected upon fixation and the higher the neuronal density (as opposed to numbers) (Bartzokis & Altshuler, 2005).

To complicate matters further, with the advent of MRI there was initial support for the hypotheses that intracortical neuronal numbers and gray matter thickness decrease beginning in the teens as part of healthy aging. This support emerged because the vast majority of imaging studies observed an apparent "decline" in gray matter volume in adulthood (ages 18–55). It is now known, however, that continued intracortical myelination into adulthood can produce the misleading appearance of cortical volume loss with age on MRI scans (Bartzokis et al., 2001; Kaes, 1907; Kemper, 1994) (Figure 15.2). Thus, as Figure 15.3 demonstrates, the apparent decrease in gray matter volume from age 18 to 55 (Figure 15.3A) can actually be accounted to by the increase in myelinated of the deep layers of the cortex (Figure 15.3B), to result in a stable brain volume in this age range (Figure 15.3C) as established by postmortem studies (Miller et al., 1980). As myelination increases information processing speed, the shift in the gray/white border that occurs as a result of intracortical myelination (Figure 15.4C) might also help explain why some studies report better performance associated with faster rates of cortical gray matter volume "decline" in healthy developing subjects (Shaw et al., 2006). Of note, the process of intracortical myelination may be altered by disease as well as specific pharmacologic intervention. Increases in intracortical myelination have been observed in patients with schizophrenia when medicated using atypical (second-generation)

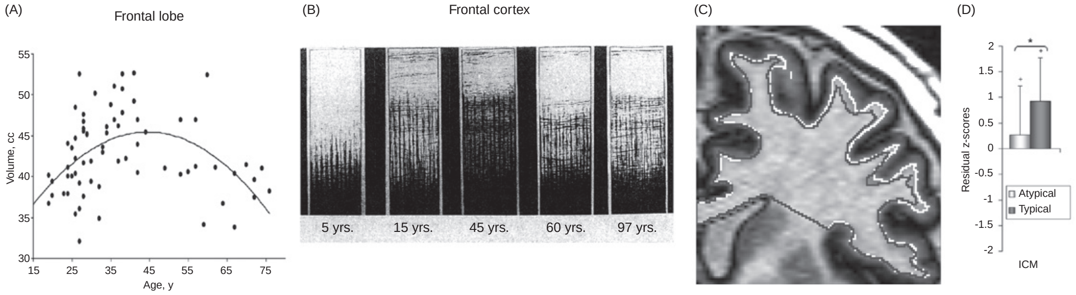

Figure 15.4 Myelination trajectories in human brain cortex. (A) In vivo MRI data of frontal lobe myelinated white matter volume (from Bartzokis et al., 2001). Data obtained using inversion recovery (IR) MR images that are most sensitive to the high cholesterol content in myelin and thus include heavily myelinated parts of the deeper portions of gray matter depicted in (B). (B) Postmortem intracortical myelin stain data (from Kaes, 1907; adapted and reproduced in Kemper, 1994; used with permission). For both (A) and (B) myelination (*y* axis) versus age (*x* axis) in frontal lobes of normal individuals. The data in (A) and (B) were acquired 100 years apart yet the two samples of normal individuals show remarkably similar life-span myelination trajectories of frontal lobes that peak at age 45 and even later in the temporal lobe (data not shown) (Bartzokis et al., 2001). (C) IR image of one frontal lobe hemisphere depicting an in vivo measure of intracortical myelin as the volume between the white and the gray lines. The same slice of brain is imaged twice, using different contrasts, with both images obtained sequentially in the same imaging session. The IR image optimally detects the high cholesterol in myelin (the white region of interest (ROI)) and is used to obtain the "myelinated WM volume" (depicted in (A)) that includes heavily myelinated parts of the deeper portions of gray matter (depicted in (B)). Thus, the white ROI line separates the myelinated WM and the unmyelinated portion of gray matter. Proton density image (not shown) is *not* sensitive to the cholesterol in myelin and is used to determine the border between the gray and the white matter and is depicted as the gray ROI line. The difference between the gray and the white ROI lines is the measure of intracortical myelin content (ICM). (D) Comparison of atypical and typical antipsychotic medications for ICM (same as the volume between the white and the gray lines in (C)). Residual scores are depicted ($*P < 0.05$) (adapted from Bartzokis et al., 2009).

antipsychotic medications to a significantly larger extent than when using typical (first-generation) antipsychotics (Figure 15.4D) (Bartzokis et al., 2009).

Thus, during postadolescent maturation of prefrontal and association areas, apparent gray matter volume reduction observed on imaging studies occurs in concert with an expansion in "myelinated white matter volume" (this phrase used to denote the volume of white matter that, in addition to axons and myelin that form white matter, includes highly myelinated lower layers of cortical gray matter) that continues into middle age (Bartzokis et al., 2001; Kaes, 1907; Kemper, 1994; Yakovlev & Lecours, 1967), when peak myelin brain levels are reached (Figure 15.4B). This white matter expansion volumetrically cancels out the apparent gray matter reduction such that both imaging and postmortem studies show minimal, if any, changes in total brain volume (gray plus white matter) occurring in normal individuals during adulthood (18–55 years of age) (Miller et al., 1980) (Figure 15.3C). At older ages the cortex undergoes additional changes consisting primarily of shrinkage of large neurons and an increase in the proportion of small neurons (Haug, 1987; Peters et al., 1998; Terry et al., 1987). It remains to be fully elucidated what impact such changes, combined with an increased number of oligodendrocytes (O'Kusky & Colonnier, 1982; Peters & Sethares, 2004) and intracortical myelin growth and eventual loss (Bartzokis et al., 2001; Kaes, 1907; Lintl & Braak, 1983; Miller et al., 1980), have on imaging results (see next section). Regardless of the precise proportions of these contributions, the biology of brain myelination across the life span can be appropriately conceptualized as following dynamic, roughly inverted-U-shaped trajectories (Allen et al., 2005; Bartzokis et al., 2001, 2003, 2004; Benes et al., 1994; Ge et al., 2002; Ho et al., 2003; Jernigan & Fennema-Notestine, 2004; Jernigan & Gamst, 2005; Kemper, 1994; Miller et al., 1980; Sowell et al., 2003; Walhovd et al., 2005) that vary temporally according to brain region (Bartzokis et al., 2001, 2004; Kemper, 1994) (Figure 15.2) and function (Bedard et al., 2002; Williams et al., 1999). The inverted-U-shaped trajectory of myelination across the life span can help explain MRI measures of white matter volume as well as structural integrity (Figure 15.4A) and relate these measures of brain to measures of function (Bartzokis et al., 2008).

In contrast to the expansion of myelin volume during developmental phases, there is eventually a loss of myelin integrity that emerges at older ages that has a profound effect on brain function in human and nonhuman primates (Bartzokis et al., 2007a, 2008; Berlet & Volk, 1980; Meier-Ruge et al., 1992; Peters et al., 1996). The breakdown in the structural integrity of myelin sheaths can be indirectly measured in vivo with MRI using multiple methods (Blezer et al., 2007; Kochunov et al., 2009; reviewed in Wozniak & Lim, 2006). One of the first sensitive methods employed used transverse relaxation rate measures (R_2; R_2 is directly associated with transverse relaxation time (T_2) by the formula $R_2 = 1/T_2 \times 1000$) to detect small changes in the amount of tissue water (Oldendorf & Oldendorf, 1988) (Figure 15.5); ultrastructural electron microscopy studies demonstrate that age-related myelin breakdown results in microvacuolations consisting of splits in myelin sheath layers that create microscopic fluid-filled spaces and increase MRI "visible" water, thus decreasing R_2 (Bartzokis et al., 2004; Peters et al., 1996). These microvacuolations are ultrastructurally very similar to reversible myelinopathies produced by certain toxins (Peters et al., 1996). Animal studies have confirmed that this type of myelin breakdown can be detected with MRI in circumscribed susceptible regions and that the histopathologic changes produced by toxins as well as the recovery process can be tracked by changes in MRI relaxation rates (reviewed in Cohen et al., 2000). In asymptomatic individuals focal white matter regions of R_2 decrease (T_2 increase) have also been associated with myelin pallor on histological stains (Blezer et al., 2007; Fazekas et al., 1998; Takao et al., 1999) (Figure 15.5). Although R_2 has not been directly correlated with myelin breakdown due to normal aging (as opposed to the reversible toxin-induced myelin breakdown in focal regions described above), healthy aging in humans and primates is not associated with neuronal loss (reviewed in Peters, 2002; Peters et al., 1998), whereas the process of myelin breakdown and loss has been repeatedly

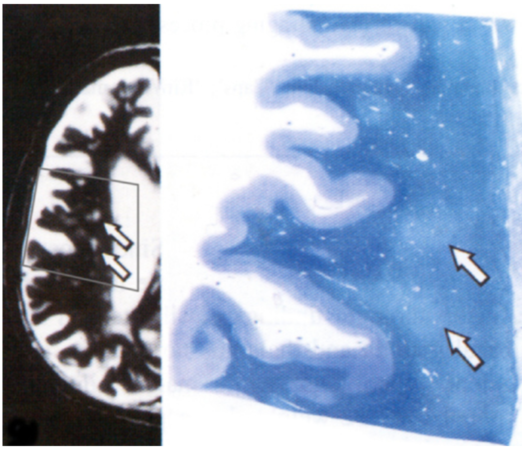

Figure 15.5 White matter "hyperintensities" on MRI are often the center of much larger areas of diffuse myelin breakdown. Small patchy hyperintense white matter lesions on T_2-weighted MRI (left) are often the very center of much more widespread and diffuse regions of myelin breakdown as shown in the postmortem myelin stain on the right (adapted from Takao et al., 1999). The left is a T_2-weighted MRI and the right is a histologic section (luxol fast blue staining for myelin) through approximately the same slice of the same brain (showing the boxed region on the left).

demonstrated (Kemper, 1994; Marner et al., 2003; Peters et al., 1996, 2001; Peters & Sethares, 2003, 2004; Sloane et al., 2003).

In addition to the geographically diverse temporal sequence of brain myelination described earlier, there is also a geographic diversity to the structure of myelin. For example, fiber tracts that connect early-myelinating regions such as the primary sensory and motor cortices (e.g., the splenium) contain large axons that are very heavily myelinated by one oligodendrocyte per myelin segment (Hildebrand et al., 1993) (Figure 15.6B). On the other hand, fiber tracts that connect late-myelinating regions such as frontal or temporal lobes (e.g., anterior parts of the corpus callosum such as the genu) contain much smaller axons. In these areas, one oligodendrocyte can be seen to myelinate *multiple* axons (as many as 50–60 in the latest myelinating association cortices; Hildebrand et al., 1993) (Figure 15.6A). It is in these areas that postmortem data from human brain show a 45% loss of myelinated fiber length during aging (Marner et al., 2003; Tang et al., 1997). This decline in myelin integrity is accompanied by age-related decrements in ferritin iron, which are concentrated in oligodendrocytes (reviewed in Todorich et al., 2009). It is postulated that age-related myelin breakdown and oligodendrocyte loss result in the release of considerable iron stores, which are redistributed and contribute to the age-related increase in brain iron levels observed in heavily myelinated subcortical gray matter regions (reviewed in Bartzokis, 2009; Bartzokis et al., 2007c).

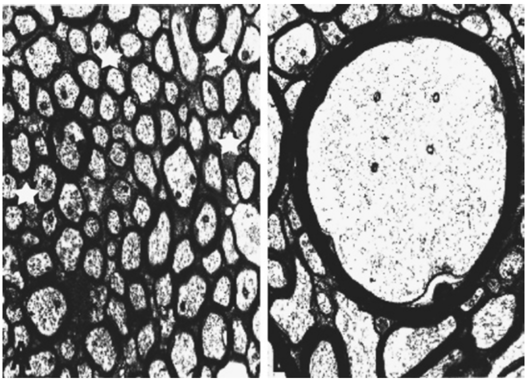

Figure 15.6 Corpus callosum regions differ in axon size, myelin content, and timing of myelination. Reproduced from Lamantia & Rakic (1990) with permission from John Wiley & Sons, Inc. (A) Genu (connecting late-myelinating frontal regions)—many small thinly myelinated axons and clusters of unmyelinated axons (20% identified by white stars) that may still be myelinating in adulthood. (B) Larger, more heavily myelinated axons and much fewer unmyelinated axons (<7%; none in this image) are observed in regions such as ventral splenium, which connect primary sensory (visual) early-myelinating regions.

THE "MYELIN MODEL": IMPLICATIONS FOR HUMAN BRAIN FUNCTION AND DYSFUNCTION

Lay comparisons of human brain function to that of a computer are outdated, as are perceptions that brain function depends solely on neurons. More useful and accurate would be a comparison of brain function to that of a laptop computer with Web-compatible software and a high-speed wireless Internet connection. Such a model is more useful and accurate because it incorporates an understanding of the collaboration between neurons, which provide "hardware"; synapses, which provide "software"; and myelin, which provides Internet processing characteristics: speed, bandwidth, and being "online." In the context of this analogy, it is myelin that increases signal transmission speed (>100-fold; Waxman, 1977) enough to integrate information across the spatially distributed neural networks that underlie our mental functions (Bartzokis, 2004b, 2005, 2009; Bartzokis et al., 2001; Fuster, 1999; Mesulam, 2000) and it is myelin that decreases the refractory time (by as much as 34-fold) (Felts et al., 1997) enough to produce a sizable bandwidth for information-rich coding (Bartzokis, 2004b, 2005). Thus, the extensive process of myelination markedly increases the quality and quantity of information that can be processed in the human brain, which may well underlie many unique human capabilities, including consciousness, language, memory, inhibitory control, and other higher cognitive functions (Salthouse & Kail, 1983; Verhaeghen & Salthouse, 1997; reviewed in Bartzokis, 2004b, 2005). As a corollary, damage to oligodendrocytes and the myelin they produce (because of aging or endogenous or exogenous insults) may also underlie unique human disorders ranging from autism to schizophrenia to Alzheimer disease (reviewed in Bartzokis, 2002, 2004b, 2005, 2009; Dwork et al., 2007).

The view of development and aging as a continuum of interactive structural and functional dynamic trajectories put forth in this chapter begs for scrutiny of the multiple factors that promote or inhibit myelination throughout the protracted process of human brain development. The inverted-U shape to patterns of change over the life span for myelin has also been demonstrated in neurophysiologic and cognitive models of brain function (Bedard et al., 2002; Dustman et al., 1996; Salthouse, 2000; Salthouse & Kail, 1983; Williams et al., 1999) as well as clinical symptomatology in a variety of neuropsychiatric disorders (Cjte et al., 2002; Cote et al., 2002; Grant, 1997; Vega et al., 2002; Warner et al., 1995; reviewed in Bartzokis, 2004b, 2005). For example, there is an inverted-U shape to the age-related changes in cognitive speed; cognitive speed increases throughout childhood and early adulthood and then decreases at an accelerating rate in old age (Salthouse & Kail, 1983; Schaie, 2005; Singer et al., 2003; Verhaeghen & Salthouse, 1997). Salthouse (1996) delineated the hypothesis that a basic parameter such as speed is directly related to biological factors and is essential for higher-order cognitive processing. Findings from our lab support this phenomenon using a finger-tapping measure of fine motor speed for comparison with an imaging biomarker of myelin integrity in a highly vulnerable late-myelinating region (Bartzokis et al., 2008) (Figure 15.7). Similar inverted-U-shaped trajectories are also apparent in paradigms that test inhibitory control (Bedard et al., 2002; Williams et al., 1999) as well as in certain neurophysiologic parameters (electroencephalogram) (Dustman et al., 1996) that may well relate to the process of intracortical myelination (Bartzokis, 2005; Kaes, 1907; Kemper, 1994; Miller et al., 1980; Peters & Sethares, 2002).

In the context of the model proposed herein, myelination may be one of the central biologic processes underlying changes in human brain function and dysfunction across the life span. It is important to point out that age-related cognitive improvements in processing speed and inhibitory control peak in adulthood (Bedard et al., 2002; Dustman et al., 1996; Salthouse, 2000; Schaie, 2005; Singer et al., 2003; Verhaeghen & Salthouse, 1997) and do not temporally "fit" (by more than a decade) the regressive processes of neuronal, synaptic, axonal, and dendritic pruning that occur primarily before midadolescence (see below) (Huttenlocher & Dabholkar, 1997; Rakic, 2002). Neuroimaging and postmortem data have demonstrated that the process of myelination peaks in middle age, followed by myelin breakdown and loss (Bartzokis et al., 2001; Kemper, 1994; Miller et al., 1980) (Figures 15.2, 15.5, and 15.7B). Age-related slowing in the speed of information processing, particularly after middle age, has been repeatedly documented in the neuropsychological literature and is a well-accepted clinical phenomenon (Cerella, 1990; Gottsdanker, 1982; Salthouse, 2000; Swihart & Pirozzolo, 1988; Tombaugh, 2004; van Gorp et al., 1990; Wilkinson & Allison, 1989). Salthouse and others (Hedden et al., 2005; Salthouse, 1996, 2000; Schaie et al., 2004) have demonstrated that the slowing in cognitive processing speed with age probably underlies the age-related decline observed in most if not all human cognitive functions. Investigations of cognitive processing speed across the life span reveal a trajectory that parallels myelination and subsequent myelin breakdown. The similarity in this trajectory supports the hypothesis that in older age myelin breakdown may be one of the main cellular mechanisms underlying the age-related decline in cognitive speed (Bartzokis et al., 2006, 2007a). As the uniquely extensive myelination of the human brain makes

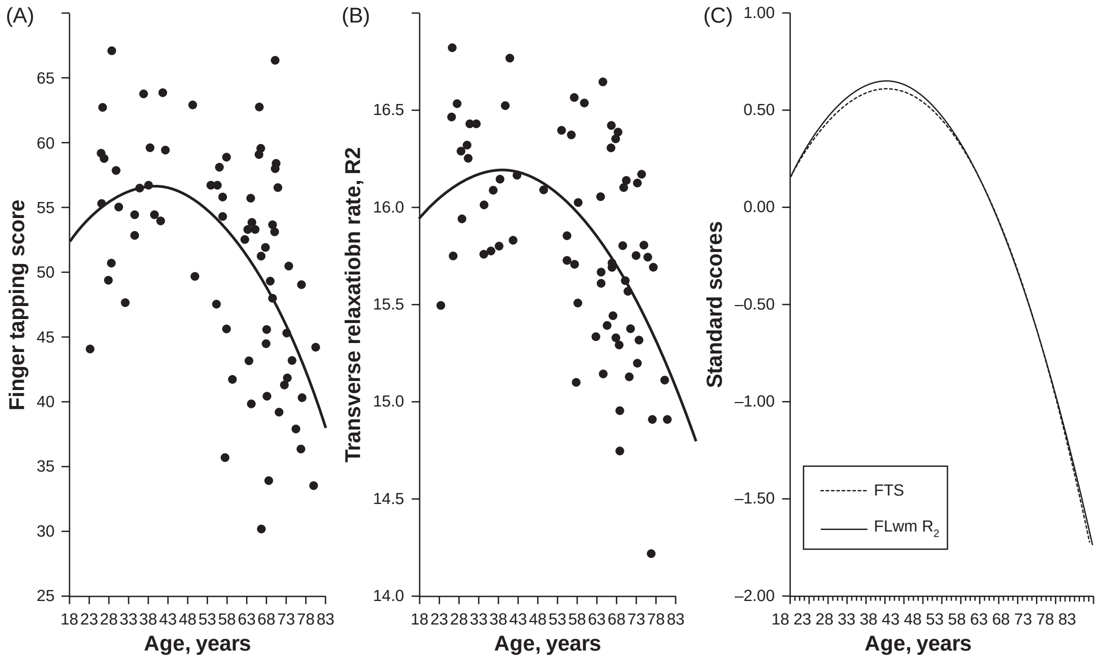

Figure 15.7 Age trajectories for finger tapping speed (FTS) and white matter transverse relaxation rate (R_2) in frontal lobe white matter (FLwm). The relationships of (A) FTS performance and (B) a measure of myelin integrity (FLwm R_2) with age are depicted. (C) The trajectories of FTS and FLwm R_2 across the age range of 23–80 based on mixed-effects regression models to show the remarkable similarity of the trajectories (adapted from Bartzokis et al., 2008).

myelin maintenance and repair especially critical for sustaining high cognitive processing speed (Bartzokis, 2004a, 2005), net myelin breakdown may result in a progressive "disconnection" of widely distributed neural networks that occurs in Alzheimer disease (AD) (Bartzokis, 2004a, 2009).

The rate of decline can be modified by multiple environmental risk factors such as brain trauma, hypertension, cholesterol levels, etc. (see below), as well as well-known and powerful genetic factors. The most powerful genetic factor is the apolipoprotein E (ApoE) 4 genotype, which explains as much as 50% of all genetic risk for late-onset AD and is second only to advancing age in importance as a risk factor for developing AD. Epidemiological studies have demonstrated that ApoE4 genotype shifts the age of onset for AD by more than a decade, thus accounting for the vast majority of observed cases of AD that occur before age 80 (Poirier, 1994; Raber et al., 2004). Conversely, age of onset is delayed in carriers of the ApoE2 allele (Corder et al., 1994; Khachaturian et al., 2004; Raber et al., 2004). The physiologic role of ApoE suggests that myelin may be a central biological link between the presence of this gene and AD. ApoE is the primary transporter of endogenously produced lipids such as cholesterol and sulfatide (Dietschy & Turley, 2004; Poirier, 2003; Vos et al., 1994), which are produced in large part by oligodendrocytes and essential for myelin production and function (Dietschy & Turley, 2004; Marcus et al., 2006; Saher et al., 2005; Vos et al., 1994). ApoE coordinates mobilization and transport of such lipids for uptake and use in repair, growth, and maintenance of myelin as well as other membranes, including synapses and dendrites essential for brain plasticity and learning (Bartzokis et al., 2006; Poirier, 2003). Boyles et al. (1989) demonstrated that ApoE is essential to the extensive process of "recycling" lipids such as cholesterol (Ando et al., 2003) released when damaged myelin is degraded and supplying these lipids for rapid membrane repair and biogenesis.

This process of remyelination has been demonstrated in the nonhuman primate model (Peters & Sethares, 2003, 2004; Peters et al., 2001; Sloane et al., 2003). Poirier (2005) demonstrated that the quantity of ApoE molecules, which are necessary for this recycling process, is lowest in ApoE4 carriers and highest in individuals with the ApoE2 allele. Thus, it has been hypothesized, and imaging data have supported, that the disruption of myelin repair secondary to insufficient ApoE molecules results in greater age-related myelin breakdown and contributes to the association between ApoE4 and AD as well as the associated influence of ApoE genotype on age-related cognitive decline (Bartzokis et al., 2007a). Healthy older individuals possessing the ApoE4 genotype show more myelin breakdown with age than matched controls; this relationship was absent for participants with the ApoE2 genotype, while ApoE3 individuals displayed an intermediate slope of decline. This genetic effect was specific to late-myelinating regions and not observed in early-myelinating regions such as the splenium of the corpus callosum (Bartzokis et al., 2007a). Furthermore, cognitive processing speed performance (as assessed by Trails A and Digit Symbol neurocognitive tasks) was significantly associated with myelin integrity in late-myelinating regions (frontal lobe white matter and genu) but not in the early myelinating splenium region (Figure 15.8). Together with postmortem data that show AD pathology to recapitulate the myelination process in reverse (Braak & Braak, 1996) (beginning in late myelinating regions such as the medial temporal lobe and inferior frontal lobe and spreading to earlier myelinating regions), these data suggest that myelin breakdown in healthy older individuals underlies the age-related cognitive decline that ultimately progresses to AD (reviewed in Bartzokis, 2009).

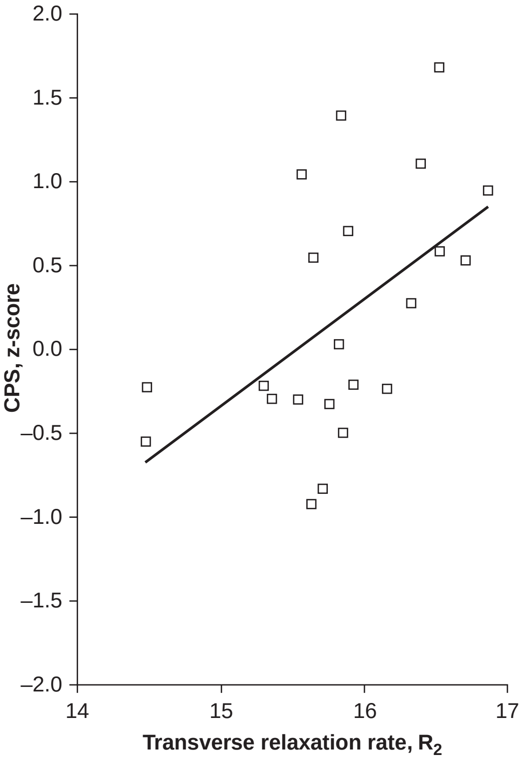

Figure 15.8 Correlation of cognitive processing speed (CPS; composite measure of Trails A and Digit Symbol scores; higher score represents faster performance) versus R_2 in late-myelinating white matter (average R_2 of frontal lobe and genu of corpus callosum white matter) for ApoE4$^+$ individuals (adapted from Bartzokis et al., 2007b).

A review of the fast-developing MRI techniques that are sensitive to changes in white matter and myelin, in particular, such as diffusion tensor imaging, magnetization transfer imaging, and myelin water imaging, is beyond the scope of this chapter and the reader is referred to a comprehensive review of the subject (Wozniak & Lim, 2006). Using these newer methods, several studies have provided additional converging evidence for the concept of myelin degradation in vulnerable late-myelinating regions being a very early event in aging and age-related cognitive declines (Davis et al., 2009; Kennedy & Raz, 2009), as well as evidence that common age-related disorders present in the general population, such as hypertension, diabetes, etc., can exacerbate the process (Kennedy & Raz, 2009; Vernooij et al., 2009). As described in earlier sections, in addition to metabolic/health factors many of the influences on myelination trajectories and cognition may ultimately be rooted in genetic influences such as ApoE, presenilin, neuregulin 1–ErbB4 receptor signaling, and neurotropins (Bartzokis et al., 2006; Kennedy et al., 2009; Konrad et al., 2008; Ringman et al., 2007). It is, however, argued that the unique vulnerability of myelin can be conceptualized as "the weakest link" in the chain of coordinated action of all brain cells and can most frequently undermine the connectivity of brain circuitry. Myelin may therefore represent a common underlying substrate for genetic, environmental, and age-related risk factors that directly contribute to behavioral and cognitive deficits of both developmental and degenerative diseases (reviewed in Bartzokis, 2002, 2004b, 2005, 2009; Dwork et al., 2007).

CONCLUSIONS

Postmortem data and in vivo imaging evidence suggest that the process of myelin production and subsequent breakdown may be directly relevant to deepening our understanding of the biology and function of the human brain. The inverted-U-shaped trajectories of myelination that occur over the life span may help explain the patterns of cognitive, behavioral, and emotional changes that define our everyday lives from the impulsiveness of youth to the wisdom of older ages. More importantly the focus on myelination trajectories of the human brain may help explain many of the unique but highly prevalent and devastating diseases that plague our species at various phases of life (Bartzokis, 2004b, 2005, 2009). The myelin model of human brain function posits that the uniquely pervasive and protracted myelination process renders a continuum of vulnerability that culminates in the later-developing oligodendrocytes. Genetic and/or environmental effects that impact myelin development and breakdown will manifest as risk factors (or protective factors) for developmental disorders such as autism and schizophrenia, as well as aging-related disorders such as AD and Parkinson disease (Bartzokis, 2002, 2004b, 2005, 2009; Dwork et al., 2007).

The creation and maintenance of myelin are at the core of postnatal brain development and, in older age, the aging process itself and perhaps the emergence of degenerative disorders such as AD (Bartzokis, 2005, 2009). Myelin deficits disrupt brain functions that depend on highly synchronized timing of high-frequency bursts of action potentials and eventually result in functional "disconnections" of associated cortical regions. This model suggests that pathological states (e.g., genetic, hormonal, head trauma, hypertension, hypercholesterolemia, substance abuse) that affect myelin development and breakdown may ultimately manifest as neuropsychiatric disorders. The life-span model of brain development and its dynamic interaction with degenerative processes can be directly tested using prospective in vivo imaging studies (Jack et al., 2009; Raz et al., 2010). The focus on myelination provides a conceptual framework for considering the development of novel myelin-centered treatments with potentially wide spectra of efficacy that could encompass many disorders that share derangements in myelin health. The model suggests that the medical field's current focus on *neuronal* neurotransmitter "imbalances" may be too narrow (Bartzokis, 2007). Expanding the research focus to include interventions that influence the vulnerable developmental processes of myelination may provide opportunities for novel and powerful treatment and prevention interventions (Amminger et al., 2010; Fotuhi et al., 2009).

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Chapter 16

Aging and the Cerebral Microvasculature: Clinical Implications and Potential Therapeutic Interventions

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CHAPTER CONTENTS

Introduction 347
Aging, Endothelium, and Vascular Dysfunction 349
Cerebrovascular Hemodynamics 349
Neurovascular Coupling 349
Brain Structural Changes may Result from Chronic Cerebral Hypoperfusion 351
Cerebrovascular Endothelial Dysfunction and Microvascular Disease 352
Clinical Implications of Cerebral Microvascular Disease in Aging 352
  Gait Disorders and Falls 353
    Vascular Disease is Associated with Impaired Gait and Falls 353
    Impaired Gait and Falls are Associated with Structural Changes in the Brain 353
    Higher Reserve may Attenuate the Impact of Cerebral Microvascular Disease on Gait and Falls 355
  Cognitive Dysfunction 356
    Vascular Pathology is Often the Cause of Cognitive Impairment and Dementia 356
    Alzheimer Disease is More Than Plaques and Tangles 356
    Brain Blood Flow, Structural Changes, and Cognition 357
Potential Therapeutic Interventions 358
  Hypoxia-Inducible Transcription Factor-1 358
    Activating HIF-1 with Desferrioxamine 358
    HIF-1 and Quercetin 359
  Flavonoids and Vascular Health 359
  Inhibitors of The Renin–Angiotensin System 360
Conclusions 361
Acknowledgments 362
References 362

INTRODUCTION

As of 2010, there were 35 million people over the age of 65 in the United States. This number is estimated to double by the year 2030 (Lakatta & Levy, 2003). As the elderly population rapidly expands over the next 2 decades, so will the prevalence of disease and disability related to vascular dysfunction. As a result of biologic aging, as well as the accumulation of cardiovascular risk factors over time, abnormalities in vascular function will continue to increase in prevalence (Burt et al., 1995; Kannel, 2002; Wolf, 1993). These abnormalities may become clinically manifest as both macro- (large) and micro- (small) vascular disease. Although the consequences of large vessel atherosclerosis, such as coronary artery disease, stroke, and renal failure, have received the greatest attention over the past 50 years, it is now becoming clear that alterations in the microvasculature, particularly small vessels of the brain, can have equally important clinical

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consequences. Advanced imaging techniques are now identifying abnormalities in the brain that were initially thought to be "covert" strokes (not associated with acute stroke symptoms) (Thompson & Hakim, 2009) or "incidental" hyperintensities (bright areas on magnetic resonance imaging), "consistent with age," and of no major significance. However, these findings are rapidly gaining importance as significant clinical entities, with profound effects on cognition, mobility, affect, and continence.

It is estimated that 11 million Americans have covert strokes annually, and by the time people reach their 70 s, one in three suffers a covert stroke every year. Moreover, nearly 18% of people over age 70 who undergo brain magnetic resonance imaging (MRI) scans are found to have periventricular or subcortical (deep) white matter hyperintensities (WMH). These WMH, which probably represent ischemic damage due to cerebral microvascular disease (Morris et al., 2009; Vermeer et al., 2007), are areas of high intensity (brightness) on MRI (Figure 16.1).

Figure 16.2 illustrates the cascade of conditions associated with aging that are currently thought to lead to cerebral microvascular disease and its clinical manifestations. Aging and the accumulation of vascular risk factors are associated with cerebral endothelial

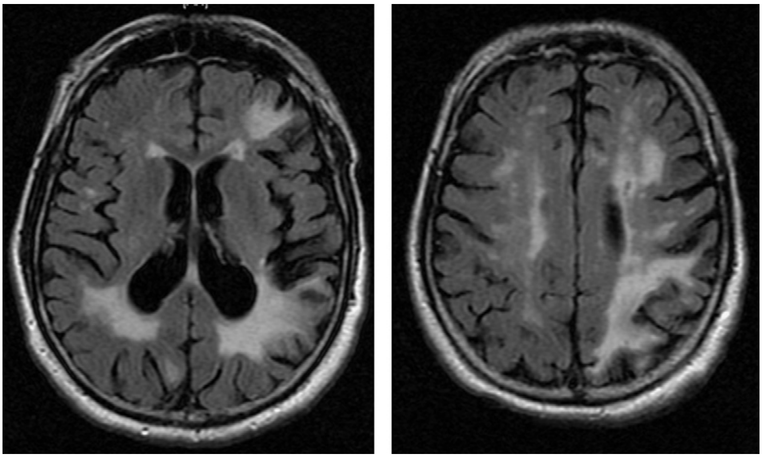

Figure 16.1 MRI scans showing typical white matter hyperintensities in the periventricular and deep white matter regions of the brain.

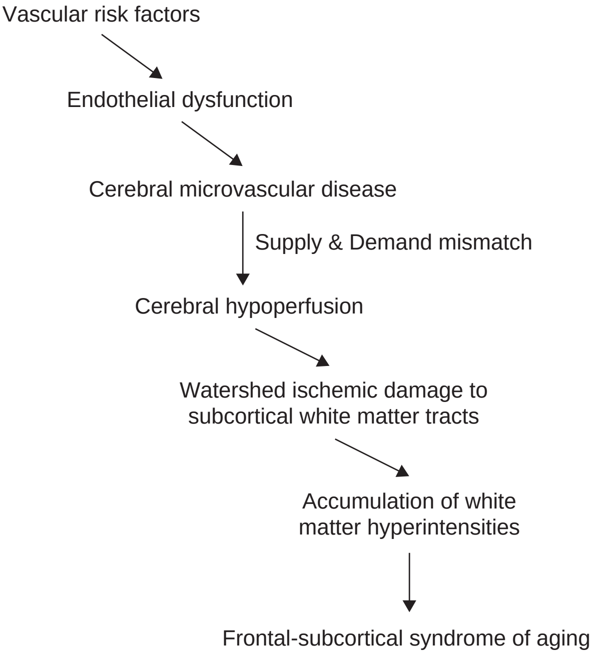
Vascular risk factors
Endothelial dysfunction
Cerebral microvascular disease
Supply & Demand mismatch
Cerebral hypoperfusion
Watershed ischemic damage to subcortical white matter tracts
Accumulation of white matter hyperintensities
Frontal-subcortical syndrome of aging

Figure 16.2 The hypothesized relationship between vascular risk factors and cerebral microvascular aging.

dysfunction that may impair the ability of cerebral microvessels to meet the metabolic demands of various neuronal networks in the brain. The mismatch between blood supply and metabolic demand may lead to the accumulation of ischemic damage in the watershed regions of the brain. Watershed regions are areas of the brain that receive a dual blood supply from the most distal branches of two large cerebral arteries and are particularly vulnerable to ischemia during hypoperfusion. They are particularly abundant around the deep subcortical white matter tracts, particularly those close to the ventricles (periventricular white matter). With chronic cerebral hypoperfusion, there is repeated damage to these watershed regions leading to progressive accumulation of WMH. Over time, these accumulated WMH become clinically manifest as a gradual slowing of gait, development of executive dysfunction, depressed affect, and other symptoms of frontal subcortical disease. We have previously called this constellation of symptoms the "microvascular frontal–subcortical syndrome of aging" (Pugh & Lipsitz, 2002) and have recently confirmed the coexistence of slow gait, executive dysfunction, and depressed affect, as well as their association with vascular risk factors, in 17% of a community-dwelling elderly population (Hajjar et al., 2009).

In this chapter we review age-related changes in the cerebral microvasculature, their clinical consequences, and potential preventive and therapeutic approaches that are under investigation.

AGING, ENDOTHELIUM, AND VASCULAR DYSFUNCTION

Aging is associated with a number of alterations in the structure and function of blood vessels. Age-associated structural changes include luminal enlargement with intimal and medial thickening, loss or thinning of the endothelium, endothelial dysfunction, and a reduction in the regenerative capacity of the endothelium (Hajdu et al., 1990; Lakatta & Levy, 2003). Resulting functional changes include arterial stiffness and reduced endothelial function (Brandes et al., 2005; Hajdu et al., 1990). Endothelial cells regulate several essential arterial properties, including vasomotor tone, permeability, angiogenesis, and inflammatory responses (Al-Shaer et al., 2005; Mombouli & Vanhoutte, 1999). Endothelial function is a nitric oxide (NO)-dependent process and usually is clinically assessed in the peripheral vessels by determining changes in blood flow or arterial diameter in response to endothelial vasodilators such as acetylcholine. Several clinical studies have shown that endothelium-dependent vasodilation progressively declines with age (Brandes et al., 2005; Drexler et al., 1991; Mayhan et al., 1990, 2008; Quyyumi et al., 1995; Reddy et al., 1994; Zeiher et al., 1991). Impaired endothelium-dependent vasodilation, referred to as endothelial dysfunction, is associated with all of the major cardiovascular diseases (Drexler & Hornig, 1999).

CEREBROVASCULAR HEMODYNAMICS

Cerebrovascular hemodynamics also change with aging (Bakker et al., 2004). Normal human aging is associated with impaired cerebral vasomotor reactivity (VR) or attenuated vascular dilation or constriction in response to chemical stimuli such as carbon dioxide (CO_2) (Bakker et al., 2004; Lipsitz et al., 2000, 2005; Serrador et al., 2005). Impaired cerebral VR, which is also NO dependent (Lavi et al., 2003, 2006), has been interpreted as a marker of endothelial dysfunction in the cerebral arteries (Akopov et al., 1996; Appenzeller et al., 2004; Hilz et al., 2004; Zimmermann et al., 2004; Zvan et al., 2002). In the population-based Rotterdam study (Bakker et al., 2004), VR was assessed using transcranial Doppler ultrasound (TCD) in 1720 participants. VR declined significantly with increasing age up to 90 years at a rate of −0.6%/kPa per year. In our studies of healthy elderly participants, we have also shown that cerebral VR is reduced in healthy aging (Lipsitz et al., 2000, 2005; Serrador et al., 2005). Other studies have shown that impaired cerebral VR is associated with an increased risk of stroke (Zimmermann et al., 2004), impaired cognition, cerebral hypoperfusion, and white matter changes on MRI (Adak et al., 2004; Bakker et al., 1999, 2000, 2004; DeCarli et al., 1995, 2005; DeStefano et al., 2006; Fazekas et al., 1987; George et al., 1986; Salat et al., 2002; Tullberg et al., 2004).

NEUROVASCULAR COUPLING

There is a close spatial and temporal relationship between neuronal activity and cerebral blood flow (CBF), termed neurovascular coupling, which ensures that the blood supply to the brain matches the energy needs of its cellular components. CBF is higher when participants engage in cognitive activities, compared to when they are resting (Stroobant & Vingerhoets, 2000, 2001). Functional TCD ultrasound is a noninvasive method used to measure neurovascular coupling by recording CBF velocity changes during the performance of cognitive tasks. Studies utilizing functional TCD have substantially contributed to the field of functional neuroimaging (for detailed review see Deppe et al., 2004; Stroobant & Vingerhoets, 2000, 2001).

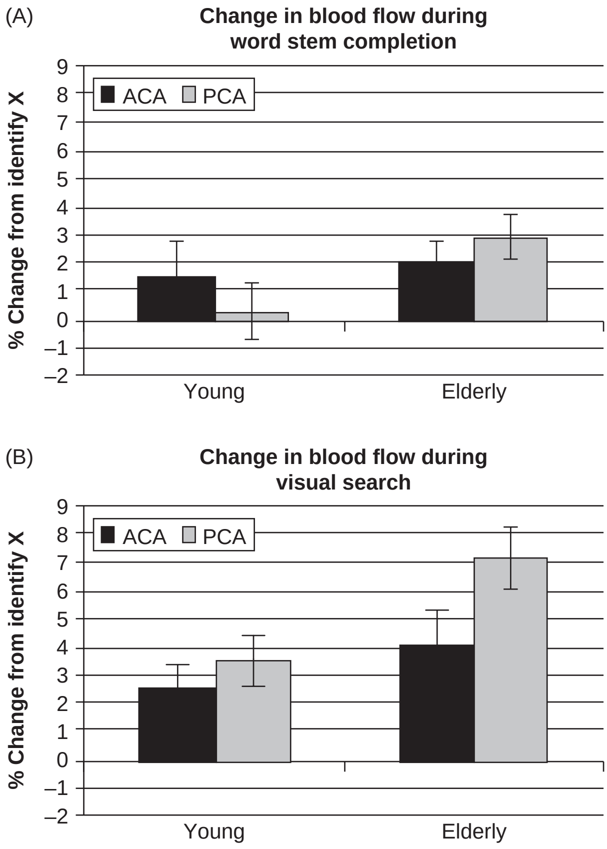

Figure 16.3 Percentage changes in cerebral blood flow during (A) an executive and (B) a visual task in healthy young and old subjects. The bars reflect the mean percentage change in blood flow velocity relative to an "identify X" control task in ACA (anterior cerebral artery) and PCA (posterior cerebral artery) territories in young and elderly participants. Error bars represent the standard error of the mean. (A) Changes during a word-stem completion task (an executive function task used to activate the frontal lobes) in which participants are asked to generate as many completions to a given three-letter stem as possible and indicate with a button press whether a completion came to mind. There was no main effect of group or location, but there was a significant interaction, such that the elderly had greater activation than the young in both territories. (B) Changes during a visual search task in which participants were asked to indicate with a button press the presence of a line bisecting a circle among a random array of circles. There was a trend toward a main effect of group, but no effect of location or interaction.

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Neurovascular coupling is disrupted in a number of age-related conditions, such as hypertension and Alzheimer disease. Consequently, CBF is no longer matched to the metabolic requirements of the tissue, resulting in relative hypoperfusion and brain dysfunction. We have used functional TCD to assess neurovascular coupling in the anterior and posterior cerebral artery territories in healthy aging (Figure 16.3). We measured simultaneous blood pressure and CBF velocity responses in these two arteries during cognitive tasks designed to activate the frontal and occipital lobes, respectively. An executive function task, word-stem completion (a verbal test of memory in which participants are presented with the first few letters of a word and asked to complete the word stem with the first word that they can think of), was used to activate the frontal lobes, and a visual task (find an object on a computer screen) was used to activate the occipital lobes. While healthy young subjects showed task-specific patterns of flow activation, healthy elderly subjects showed a general increase in blood flow activation in both territories in response to both tasks. This finding suggests a possible generalization of cerebral activity to compensate for age-related loss of region-specific functions. This generalization of cerebral activity has been seen with other cognitive tasks in functional MRI studies of elderly people (Cabeza et al., 2001).

BRAIN STRUCTURAL CHANGES MAY RESULT FROM CHRONIC CEREBRAL HYPOPERFUSION

The pathogenesis of WMH, also referred to as leukoaraiosis, white matter signal abnormalities, and white matter lesions, is poorly understood. A better understanding of the mechanistic pathways leading to structural damage in cerebral white matter regions is essential to the prevention of several geriatric conditions, including cognitive impairment, gait disorders, and falls. The most commonly reported pathologic abnormality in the cerebral small vessels associated with WMH is a diffuse arteriopathy with hyaline deposition, an appearance described as lipohyalanosis (Fisher, 1968). The neuropathological appearance corresponding to WMH in postmortem brains is neuronal loss, ischemic demyelination, and gliosis (Pantoni & Garcia, 1997). Both clinical and pathological studies support the hypothesis that lacunar infarction and WMH represent different forms of small vessel disease. Pathophysiologically, it is thought that a diffuse arteriopathy of the cerebral small vessels results in impaired cerebral autoregulation, hypoperfusion, and subsequent ischemia (Bakker et al., 1999; Pantoni & Garcia, 1997; Terborg et al., 2000) (see Figure 16.2). If acute, this causes small focal regions of damage in perforating arteriole territories (lacunar infarction), while if it is more chronic it results in diffuse ischemic injury (WMH). The perforating arteries supplying the deep white matter regions are end arteries resulting in internal watershed regions. Therefore, perfusion pressures will be lowest in these areas. The low perfusion pressures, exacerbated by any diffuse arteriopathy or orthostatic hypotension—so common in the elderly (Lipsitz, 1989), makes these areas most susceptible to chronic ischemic damage and the development of WMH.

The association of WMH and hypoperfusion is supported by several studies. In a mouse model, WMH were induced after chronic cerebral hypoperfusion (Shibata et al., 2004; Tomimoto et al., 2003). In humans, low regional CBF has also been associated cross-sectionally with the presence of WMH (Figure 16.4) (Enzinger et al., 2007; Hatazawa et al., 1997; Herholz et al., 1990; Isaka et al., 1994, 1997; Kawamura et al., 1991; Markus et al., 2000; Marstrand et al., 2002; Meguro et al., 1990; Schmidt et al., 2007; Waldemar et al., 1994; Yamauchi et al., 1991, 1999). These studies, which used positron emission tomography (PET), single-photon emission tomography (SPECT), or perfusion-weighted MRI, demonstrated an association between WMH and regional cerebral hypoperfusion (Bisschops et al., 2004). More recently a longitudinal study of 390 elderly individuals also showed that during a 33-month follow-up, the decline

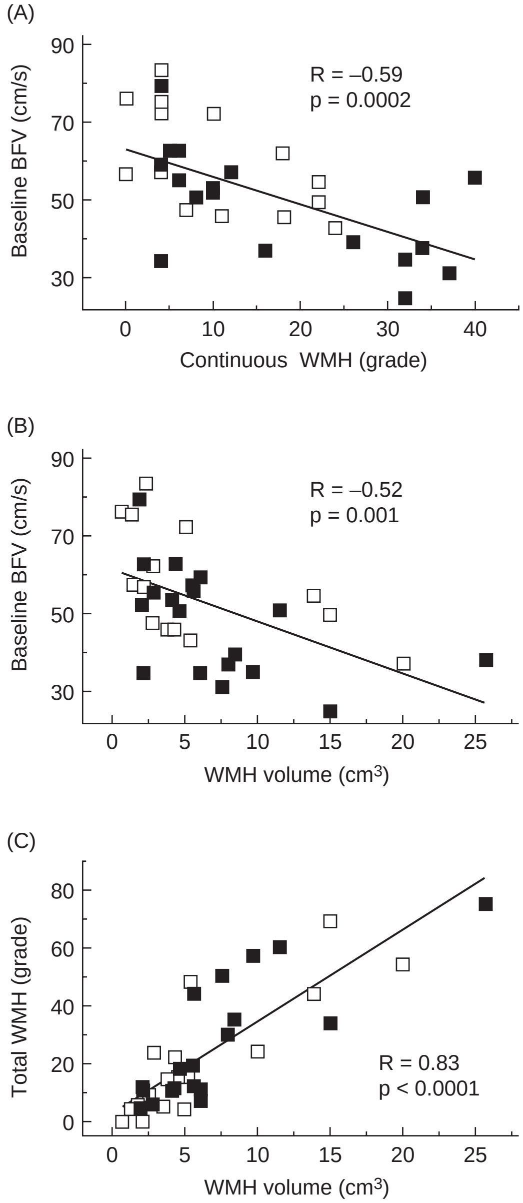

Figure 16.4 Relationship between baseline mean blood flow velocities (BFVs) and (A) sum grade of continuous WMH on a visual rating scale and (B) WMH volume on MRI. Regression analysis revealed that BFV significantly declined with increased WMH grade and volume. (C) Regression analysis between the WMH volume on MRI and the sum of continuous and punctate WMH (total WMH grade) on the visual rating scale for control (□) and diabetic (■) subjects.

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in total CBF was linked to an increase in WMH (ten Dam et al., 2007).

WMH have also been associated with low blood-flow velocity in the middle cerebral artery (Tzourio et al., 2001). This study reported a fourfold increase in risk of severe WMH in patients with low CBF velocities compared with patients with high CBF velocity in the middle cerebral artery. In another study of an unselected cohort of elderly people, molecular analysis of donated brains showed that WMH were also associated with increased levels of protein markers of hypoxia, again pointing to chronic hypoperfusion as an underlying etiology for WMH accumulation (Fernando et al., 2006).

More recently, Guttmann and colleagues compared the location of WMH to established areas of perfusion documented in perfusion maps of the brain in three cohorts of elderly people, those with Alzheimer disease, with cerebral amyloid angiopathy, and with healthy aging. They showed that in all three groups, WMH are much more frequent in known deep watershed areas and that regions that normally exhibit lower perfusion levels are increasingly affected by white matter damage (Holland et al., 2008). In summary, cross-sectional animal and human studies suggest that chronic hypoperfusion may play a role in the pathogenesis of cerebral microvascular disease.

CEREBROVASCULAR ENDOTHELIAL DYSFUNCTION AND MICROVASCULAR DISEASE

A number of lines of evidence suggest that chronic endothelial dysfunction plays a pivotal role in the pathogenesis of WMH (Hassan et al., 2003, 2004; Markus, 2008). Under stimulation by numerous agents, the endothelium undergoes changes that allow it to participate in the inflammatory response; this is known as endothelial cell activation (Hunt & Jurd, 1998). In WMH, there is histopathological evidence of endothelial cell activation, with subsequent breakdown of the blood–brain barrier (Lin et al., 2000). One of the consequences of endothelial cell activation is increased vascular permeability, which may have toxic effects by promoting the entry of serum proteins into the vascular wall and perivascular neural parenchyma (Tomimoto et al., 1996).

Previous studies have also shown that cerebral endothelial function, as measured by the cerebral vascular response to CO_2 (VR) (Akopov et al., 1996; Appenzeller et al., 2004; Hilz et al., 2004; Zimmermann et al., 2004; Zvan et al., 2002), is impaired in WMH, particularly in periventricular regions. In 73 individuals from the Rotterdam Study VR was inversely associated with deep subcortical and total periventricular WMH (Bakker et al., 1999). Furthermore, a strong correlation was found between impaired VR and periventricular WMH adjacent to the lateral ventricular wall. While this is the only small-population-based study establishing this link, other smaller case–control studies have also shown that in patients with WMH, VR is lower compared with controls (Bisschops et al., 2003; Bonoczk et al., 2004; Fu et al., 2006; Rossini et al., 2004). Systemic endothelial function has also been associated with WMH. Hoth and colleagues reported that in older adults with cardiovascular disease, endothelial-dependent vasodilation in the brachial artery was significantly and inversely associated with WMH volume (Hoth et al., 2007).

CLINICAL IMPLICATIONS OF CEREBRAL MICROVASCULAR DISEASE IN AGING

The process of human aging is associated with changes within motor and cognitive systems that lead to cognitive impairment, urinary incontinence, falls, and depression. Several features of this geriatric syndrome (see Table 16.1), such as slow gait, skeletal muscle disinhibition, uninhibited bladder contractions, and executive cognitive impairment, are suggestive of dysfunction in both frontal and subcortical regions of the brain, which we have called the microvascular frontal–subcortical syndrome of aging (Pugh & Lipsitz, 2002). MRI findings of periventricular and subcortical WMH in the aging brain are associated with executive impairment and slow gait. Since these findings are also associated with the presence of cardiovascular risk factors, especially hypertension and

Table 16.1 Geriatric syndromes related to cerebral microvascular disease

CLINICAL CONDITION	FEATURES
Cognition	Executive dysfunction, psychomotor slowing, poor retrieval
Gait	Disequilibrium, postural instability, slow gait speed, falls
Mood	Depression, apathy, disinhibition
Urinary function	Detrusor hyperactivity, urgency, incontinence

From Pugh & Lipsitz (2002).

diabetes, they may be due to the presence of cerebral microvascular disease rather than normal aging per se. We focus here on the evidence linking gait disorders and cognitive dysfunction to cerebral microvascular disease.

Gait Disorders and Falls

Slowing of gait is particularly common in elderly people and is associated with the development of falls (Guralnik et al., 1995, 2000; Robbins et al., 1998; Verghese et al., 2002a,b). Between the ages of 60 and 85 years there is a significant decline in the proportion of people who have normal gait. More than 80% of those 85 years or older have abnormal gait as defined by slow speed, increased step variability, impaired gait initiation, and instability when turning (Bloem et al., 1992; Snijders et al., 2007; Sudarsky, 2001).

Falls are a common, morbid problem that occur annually in approximately 30–40% of community-dwelling elders and often result in fractures, intracranial hemorrhage, soft-tissue injury, loss of independent function, nursing home placement, and death (Centers for Disease Control and Prevention, 2008). Among many reported risk factors, abnormal gait is strongly correlated with the occurrence of falls (Verghese et al., 2002a), as well as functional disability (Guralnik et al., 1995, 2000), hospitalizations (Robbins et al., 1998), and dementia (Verghese et al., 2002b). For such a common abnormality in older age there is limited information about mechanisms underlying acquired abnormalities in gait and their progression to falls and functional decline.

Prior cross-sectional studies have demonstrated a significant association between gait and balance and WMH (Baloh et al., 1995; Bennett et al., 1996; Briley et al., 1997, 2000; Guralnik et al., 1995; Guttmann et al., 2000; Kerber et al., 1998). A study by Srikanth and colleagues (2009) showed that abnormal gait and the occurrence of falls are associated with WMH. However, this relationship was not adjusted for potential confounders such as stroke and hypertension. The bivariate associations between vascular risk factors and CBF dysregulation, vascular risk factors and WMH, WMH and slow gait speed, and WMH and falls argue for alterations in cerebral vasoregulation and resultant ischemic microvascular disease as a potentially preventable mechanism underlying the development of falls in elderly people. However, direct data from longitudinal population-based studies linking cerebral vasoregulation to WMH and the subsequent development of slow gait and falls are lacking.

Vascular Disease is Associated with Impaired Gait and Falls

A number of recent studies have shown that gait disorders in the elderly may be a marker for underlying subclinical vascular disease, particularly cerebrovascular disease. In a study of hypertension and gait in the elderly, increased blood pressure was associated with worse performance on measures of gait and balance (Hausdorff et al., 2003). In a population-based longitudinal study of gait disorders and survival, Bloem and colleagues showed that all-cause mortality risk was increased in subjects with senile gait disorders compared with subjects with a normal gait (RR = 2.8; 95% CI 1.1–7.3, P = 0.03) and that the risk of cardiovascular death in subjects with senile gait disorders was twofold greater than in subjects with a normal gait (RR = 2.1; 95% CI 0.4–10.3) (Bloem et al., 1992). For the 455 individuals who suffered a stroke in the Cardiovascular Health Study, one independent predictor of death after any stroke was poor performance on a timed walk (slow gait) measured before the incident stroke (Longstreth et al., 2001). More recently, among more than 13,000 postmenopausal women in the Women's Health Initiative, slower walking speeds at baseline were also associated with higher risk of incident ischemic stroke (McGinn et al., 2008). Compared to women in the fastest tertile of walking speed (>1.24 m/s), those with walking speeds in the second tertile (1.06 to 1.24 m/s) had a 29% increase in incident ischemic stroke risk (95% CI 0.92 to 1.82), and those in the slowest tertile (<1.06 m/s) had a 69% increased incidence of ischemic stroke risk (95% CI 1.21 to 2.36). The strength of the association of walking speed with incident ischemic stroke in this group of women was independent of and comparable to, if not stronger than, established risk factors for stroke, including hypertension and diabetes. Therefore, slowing of gait among elderly people may be related to subclinical cerebrovascular disease and may be potentially preventable with a reduction in cerebrovascular risk factors.

Impaired Gait and Falls are Associated with Structural Changes in the Brain

White Matter Hyperintensities

One possible mechanism linking vascular disease to slow gait is cerebrovascular ischemia. The overwhelming body of literature, which shows that cerebral microangiopathy or subcortical WMH is associated with both vascular disease and slow gait, supports this mechanism. Several studies have reported that subclinical white matter alterations, brain infarcts, and measures of ventricular enlargement detected on brain MRI are associated cross-sectionally with measures of lower extremity performance, including walking speed (Longstreth et al., 1996, 1998; McGinn et al., 2008; Starr et al., 2003) (see Figure 16.5, adapted with permission from Dr. Charles Guttmann). Other studies have also shown that balance and gait dysfunction are associated with gradual onset of white

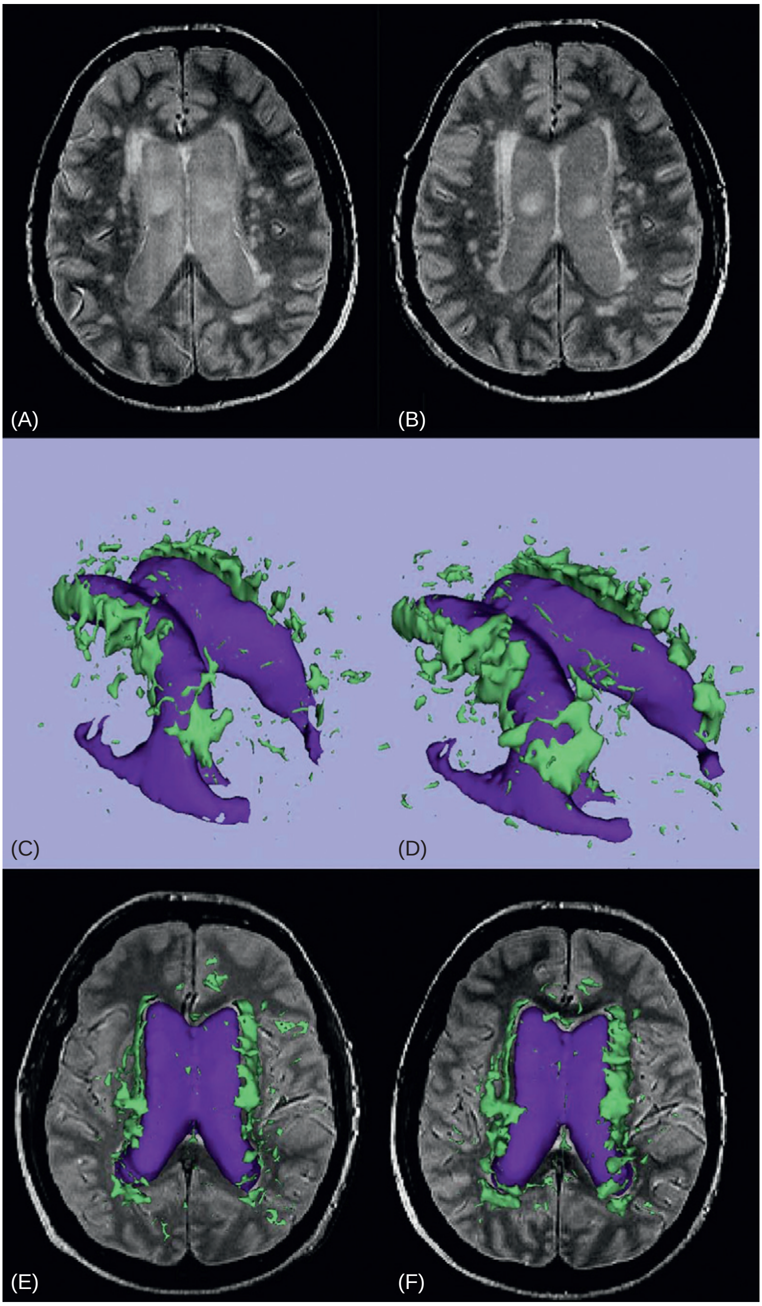

Figure 16.5 An 81-year-old man with gait impairment. Left column shows baseline MRIs and right column is 592 days later. (A, B) Sample cross-sectional proton-density-weighted image at the level of the lateral ventricles. (C, D) Three-dimensional rendering of the segmented WMH surrounding the ventricles (dark gray). (E, F) Cranial view of the three-dimensional renderings of WMH and ventricles in the context of a sample cross-sectional proton-density-weighted image. The measured WMH increased 30.7% from 20.1 to 26.3 cm^3.

(reproduced from Guttmann et al., 2000; copyright American Academy of Neurology Press; used by permission of the publisher).

matter disease (Guttmann et al., 1999, 2000; Rosano et al., 2007; Whitman et al., 2001). More recent support for the hypothesis that abnormal gait is associated with WMH comes from the Leukoaraiosis and Disability Study, in which WMH were shown to be an independent determinant of the transition to disability in the elderly (65 to 84 years). In cross-sectional analysis, deficiencies in gait and balance performance were inversely correlated with the severity of WMH. Walking speed correlated inversely with the severity of WMH (1.24 ± 0.28 m/s in the mild, 1.18 ± 0.32 m/s in the moderate, and 1.09 ± 0.31 m/s in the severe group; $P < 0.001$) (Baezner et al., 2008). Srikanth and colleagues (2009) showed that white matter lesion volume was also associated with gait characteristics and variability. Individuals with a poor gait score, which was a composite score of a number of gait variables, had a greater volume of WMH. Similarly, those with a greater gait variability score, a composite measure of stride length, stride time, and step width, also had a greater volume of WMH. Moreover, WMH was prospectively associated with the occurrence of falls (Srikanth et al., 2009).

Reduced Gray Matter Volume and Cerebral Atrophy

A variety of other gait characteristics have also been associated with atrophy in specific areas of the brain. However, these data are conflicting. First, in 321 high-functioning older adults participating in the Cardiovascular Health Study, Rosano and colleagues showed that slower gait speed, shorter stride, and longer double-support time were associated with higher WMH burden and subclinical strokes (Rosano et al., 2006). Next, in a sample of 327 participants of the same Cardiovascular Health Study, Rosano and colleagues examined the association between gray matter volumes in regions related to motor control, gait, and balance and showed that smaller gray matter volumes in the cerebellum, putamen, prefrontal, and parietal regions were also associated with slower gait and poorer balance. However, the investigators reported that this association was independent of WMH (Rosano et al., 2007). Most recently, Rosano and colleagues demonstrated that shorter steps and longer double-support times were associated with smaller volumes of gray matter in sensorimotor and frontoparietal regions of the brain (Rosano et al., 2008a). Finally, the same group showed that in 795 participants of the AGES–Reykjavik Study cohort, brain atrophy was also significantly associated with longer time to walk in the elderly (Rosano et al., 2008b).

These seemingly disparate findings may be due to the fact that gray and white matter structural changes are not independent. Since normal gait requires input from motor, visuospatial, attentional, and other centers of the brain, which all send myelinated association fibers through subcortical regions affected by WMH, it would be expected that lesions in both gray and white matter should be associated with impairments in gait. In fact, atrophy in gray matter regions could result from Wallerian degeneration of myelinated neurons that suffer ischemic damage in subcortical watershed areas. This notion is supported by data from patients with multiple sclerosis in which retrograde damage of the perikarya from axonal injury is one of the significant factors leading to gray matter atrophy (Sepulcre et al., 2009).

More recently Varga and colleagues showed that CBF is decreased in the normal-appearing white matter of early and advanced multiple sclerosis but only in the subcortical normal-appearing gray matter of patients with advanced disease (Varga et al., 2009). This finding suggests a gradient in brain tissue perfusion during disease progression such that there is an initial decrease in flow in the white matter, which gradually extends to involve the gray matter. This notion is supported by data from Wen and colleagues, who showed that in the elderly, WMH are associated with decreased cortical blood flow after adjusting for age, stroke, and atrophy (Wen et al., 2004). However, in the absence of longitudinal studies to provide a chronological record of accumulating brain lesions, we do not know the sequence of white and gray matter structural changes.

Higher Reserve may Attenuate the Impact of Cerebral Microvascular Disease on Gait and Falls

Research into the causes of gait disorders and falls have shown them to be multifactorial and rarely due to single risk factors or pathophysiologic processes. Moreover, there is very large variability in gait characteristics and the development of falls between individuals, even in the presence of risk factors that appear similar in nature and severity. Similarly, there is rarely a direct linear relationship between the degree of brain pathology and the clinical manifestation of that pathology. One of the fundamental principles of geriatric medicine states that the relationship between any given risk factor and an adverse outcome, such as falls, is modified by an individual's underlying "homeostatic reserve" or ability to adapt to a given perturbation or stress. In the cognitive literature these observations have led to the development of the cerebral reserve hypothesis to explain the apparent protection from cognitive decline in some older individuals with brain pathology.

The concept of reserve may also be a critical factor that protects some elderly people from declining mobility. Gait and mobility are under not only motor control, but also visuospatial and cognitive control. A number of investigators have reported that measures of cognitive function, in particular those in the executive domain, are associated with gait disorders and falls (Rosano et al., 2008b; Srikanth et al., 2009). Rosano and colleagues showed that higher levels of executive function substantially attenuated the association between brain structural changes as detected by MRI and slow gait speed (Rosano et al., 2008b). Muscle strength, which can be a measure of peripheral physiologic reserve, has also been shown to attenuate the relationship between brain structural lesions and gait disorders and falls. Srikanth and colleagues recently reported that, while more WMH were associated with worse gait, the effect of these lesions on falls was magnified in those with weaker quadriceps muscle strength (Srikanth et al., 2009). Similarly, Rosano and colleagues showed that lower extremity muscle strength attenuated the association between brain structural lesions and slow gait speed (Rosano et al., 2008b).

Cognitive Dysfunction

Cognitive decline is among the most common and devastating consequences of growing older. Evidence is accumulating that vascular disease may play a role not only in vascular cognitive impairment, but also in neurodegenerative processes such as Alzheimer disease. A great deal of interest has focused on the mechanisms by which vascular disease might lead to neurodegeneration and cognitive impairment. There is indirect evidence that impaired cerebrovascular hemodynamics and cerebral hypoperfusion may play an important role in this neurodegenerative process. The coexistence of vascular and Alzheimer pathology in elderly people with dementia and the close link between cardiovascular disease (CVD) and dementia point to disrupted CBF as a potential pathophysiologic mechanism in dementia.

Vascular Pathology is Often the Cause of Cognitive Impairment and Dementia

Clinicians have known for more than a century that CVD can lead to dementia. Around 25% of patients with CVD meet criteria for dementia 3 months after a stroke (Pohjasvaara et al., 1997) and a greater number have cognitive impairment short of dementia (Tatemichi et al., 1994a). Compared with individuals without ischemic brain disease, patients who are cognitively intact 3 months after a stroke have a six- to ninefold greater risk of developing dementia in the following year (Kokmen et al., 1996; Tatemichi et al., 1994b). Whereas the increased risk is greatest in the first 12 months (Kokmen et al., 1996), it is still present several years later. Large cortical infarcts, single strategic infarcts, multiple small infarcts, and cerebral hemorrhage, as well as a number of vasculopathies including cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL) and amyloid angiopathy, are all associated with cognitive impairment (Hachinski & Bowler, 1993; Kalaria et al., 2004). In addition to CVD, traditional cardiovascular risk factors, including arterial hypertension, history of high cholesterol, diabetes, or forms of heart disease, are also independently associated with an increased risk of cognitive impairment and dementia.

Why do patients with stroke and CVD risk factors have such a high risk of developing dementia? Traditionally, cognitive decline in patients with CVD was attributed to large volumes of brain affected by infarcts or to strategic lesions within cortical or subcortical areas important for cognition. However, recent epidemiological and neuropathological studies suggest that microvascular white matter lesions and covert infarcts are also associated with cognitive impairment and dementia (Frisoni et al., 2007; Vermeer et al., 2007). Given the high prevalence of CVD and Alzheimer disease pathology in the elderly, it is likely that most of these patients have overlapping pathological processes (Medical Research Council Cognitive Function and Ageing Study, 2001). Also, patients who at autopsy have coexistent Alzheimer-type changes and cerebral infarcts had more severe cognitive impairment (and a higher prevalence of dementia) during life than patients with isolated senile plaques and neurofibrillary tangles (Snowdon et al., 1997). However, our understanding of this process is limited. A broader understanding of the interplay between vascular disease and dementia is necessary for early diagnosis and the realization of successful therapeutic interventions.

Alzheimer Disease is More Than Plaques and Tangles

Alzheimer disease (AD) is a complex, multifactorial disease with a long preclinical phase, suitable for therapeutic interventions. While the prevailing hypothesis maintains that AD is due to abnormal amyloid β-peptide (Aβ) deposits (plaques) in the brain (Hardy & Selkoe, 2002), more recent data support the notion that the neuropathology of Alzheimer-type dementia comprises more than amyloid plaques and neurofibrillary tangles. First, as described above, there is an interaction between cerebral infarcts and Alzheimer pathology. Patients with infarcts and Alzheimer-type changes have a greater degree of cognitive impairment than those with similar severity of either pathology (Snowdon et al., 1997). Second, epidemiological studies suggest that the traditional CVD risk factors are also risk factors for AD (Luchsinger & Mayeux, 2004). Common risk factors could imply common pathological mechanisms. Finally, data from animal studies suggest that cerebral hypoperfusion may be a preclinical event in AD (de la Torre, 2002; Ruitenberg et al., 2005). These findings support a role for CVD in AD, but how this role relates to the neurodegenerative process underlying AD is unclear.

Alterations in cerebrovascular structure, which have frequently been reported in patients with AD, may be a possible underlying mechanism (Buee et al., 1997; Claudio, 1996; Kalaria, 1996). AD brains consistently show capillary basement membrane thickening and collagen type IV accumulation, also known as fibrosis. Similar ultrastructural alterations of cerebral microvessel walls are also seen in aging and experimental cerebral hypoperfusion in rats (de Jong et al., 1990, 1999). There is now evidence to suggest that the abnormal capillary structure and function seen in AD is related to the β-amyloid level in these capillaries (Salloway et al., 2002). Investigations have also shown increases in luminal diameter and wall thickness of the large cerebral arteries of patients with AD at autopsy (Trembath et al., 2007). Moreover, deposition of β-amyloid in arterial walls is associated with decreased smooth muscle action in AD (Ervin et al., 2004). These findings suggest that cerebral microvascular morphology and function are compromised in AD.

Even more intriguing commonality between AD and CVD emerges when one considers apolipoprotein E (apoE), the main apoprotein of the chylomicron, which is essential for the normal catabolism of triglyceride-rich lipoprotein constituents. The apoE4 allele is known to be strongly associated with AD risk, pointing to a genetic basis for this disorder (Han et al., 1994; Riekse et al., 2004; Saunders et al., 1993; Xu et al., 1999; Yip et al., 2005). Inheritance of the apoE4 allele increases the risk of development of AD and reduces the age of onset (Saunders et al., 1993). Yet, at the cellular level, apoE plays a major role in lipid function and specifically the metabolism of cholesterol (Lane & Farlow, 2005). Given the well-recognized role of lipids, including cholesterol, in CVD, it may be that variations in metabolic function related to apoE share common influences in AD as well (Grammas et al., 2000). However, relatively few data exist on the interrelationship among apoE4 allele, cerebrovascular pathology, and specific AD factors.

Brain Blood Flow, Structural Changes, and Cognition

The vascular pathogenesis of AD was initially based on the finding that reduced CBF in aging rats resulted in metabolic, memory, and neurodegenerative abnormalities similar to those seen in AD pathology (de la Torre et al., 1992). It was proposed that excess Aβ production and cholinergic neuronal loss are products of a dysfunctional cerebrovasculature that begins with chronic hypoperfusion of the brain (de la Torre, 2004). This concept has been further advanced by a study that showed that the homeobox gene MEOX2 plays a role in angiogenesis and vascular smooth muscle cell migration (Witzenbichler et al., 1999), which is linked to the dysfunctional brain vasculature in patients with AD (Wu et al., 2005). MEOX2 is a transcription factor expressed in vascular endothelial cells (Patel et al., 2005). Wu and colleagues have shown that in response to hypoxia, MEOX2-deficient mice had a 50% lower cortical CBF response and lower angiogenic ability, independent of their vascular reactivity, compared to control mice (Wu et al., 2005). Given that isolated brain endothelial cells at autopsy from severely affected AD patients also show low expression of MEOX2, it may be that preclinical hypoperfusion detected in AD patients results from an endotheliopathy, which further diminishes CBF by limiting the vasodilatory capacity of the involved vessels (de la Torre & Stefano, 2000).

Studies of regional CBF, measured by SPECT and PET studies, show a reduction in CBF in AD patients (DeKosky et al., 1990; Duara et al., 1986; Eberling et al., 1992; Johnson et al., 1987; O'Brien et al., 1992; Ohnishi et al., 1995). However, the low CBF in AD may be a consequence of reduced neuronal metabolic demand (Friedland et al., 1983). Several studies have attempted to address the perfusion versus demand debate in AD. Ruitenberg and colleagues examined CBF velocity using TCD ultrasound in 1730 participants of the Rotterdam Study and showed that greater CBF was related to a lower prevalence of dementia and cognitive decline and less hippocampal and amygdalar atrophy on MRI (Ruitenberg et al., 2005). While their study could not exclude that this was caused by preclinical neurodegeneration (low demand) leading to hypoperfusion, it is supportive of the interplay between CBF and cognition. More recently, the relationship between mild cognitive impairment, apoE4 allele, and CBF velocity, as measured by TCD ultrasound, was studied in patients with mild cognitive impairment and controls (Sun et al., 2007). This study showed that, compared with controls, patients with mild cognitive impairment had significantly lower blood flow velocities in the middle and anterior cerebral arteries. Moreover, in those with mild cognitive impairment, the presence of the apoE4 allele was associated with even lower CBF velocities. While findings from this study are supportive of a role for perfusion as a causal factor, they illustrate the need for longitudinal studies of CBF regulation and cognition. Such studies would not only help better define the causal relationship between flow and cognition, but they would also have important implications as diagnostic tools for the early diagnosis of dementia.

Finally, there is evidence from both animal and human studies to suggest that Aβ can impair cerebral vasoreactivity, leading to vasoconstriction and a decrease in CBF (Crawford et al., 1997; Paris et al., 2000; Suo et al., 2000). In the longitudinal population-based Rotterdam Study, plasma Aβ levels and cerebral vasoreactivity to carbon dioxide were measured using TCD in 441 people ages 60–90 years. Using age- and sex-adjusted logistic regression analyses, plasma Aβ levels assessed on average 6.5 years before TCD studies were linearly associated with impaired cerebral vasoreactivity (van Dijk et al., 2007). Therefore, at the clinical level, it is possible that Aβ reduces perfusion and perhaps causes ischemia in AD brains, thereby amplifying the pathological neurodegenerative process.

POTENTIAL THERAPEUTIC INTERVENTIONS

If, as suggested above, cerebral hypoperfusion is one of the mechanisms underlying cerebral microvascular damage and its clinical effects on gait and cognition, therapeutic efforts aimed toward preserving brain perfusion may be able to prevent the disorders of gait and cognition associated with aging. Unfortunately, in early trials, vasodilators such as ergot alkaloids (hydergine), papaverine, or cyclandelate (cyclospasmol) (Sokoloff, 1959) were not shown to have therapeutic value. These results suggest that nonselective systemic vasodilation without improving vascular function may not be the solution. There are several promising molecular targets for improving CBF and vascular function in advanced age that may ultimately prove useful in preventing cerebral microvascular disease. These include hypoxia-inducible transcription factor 1 (HIF-1), flavonoids, and inhibitors of the renin–angiotensin system. More recently, there has been significant supportive evidence that exercise and statin therapy may also promote cerebrovascular health and endothelial function (for a detailed review of these mechanisms see Moreno et al., 2009; Di Francescomarino et al., 2009).

Hypoxia-Inducible Transcription Factor-1

Investigations directed at deciphering the molecular mechanisms underpinning vascular aging have implicated a regulatory role for HIF-1(Di Giulio et al., 2003, 2005; Frenkel-Denkberg et al., 1999; Rivard et al., 1999, 2000). HIF-1, which is a heterodimeric transcription factor composed of α and β subunits, is expressed in all tissues in response to hypoxia. HIF-1 is responsible for a coordinated genetic program mediating such diverse but related functions as increased respiratory rate, erythropoiesis, glycolysis, and angiogenesis, all in response to hypoxic stress (Semenza, 1998) and directed at minimizing the mismatch between substrate supply and metabolic demand. HIF-1 targeted genes are also responsible for cellular processes related to vascular remodeling, such as proliferation, migration, differentiation, extracellular matrix metabolism, pH adjustment, and regulation of enzymes, ligands, receptors, and ion channels, which mediate either vasoconstrictor or vasodilator effects (Hanze et al., 2007). The specific gene products involved in these processes include direct and indirect HIF-1-dependent target genes, such as vascular endothelial growth factor (VEGF; Forsythe et al., 1996), endothelial nitric oxide synthase (eNOS; Palmer et al., 1998), matrix metalloproteinases (Ben-Yosef et al., 2002), angiopoietin 2 (Pichiule et al., 2004), and potassium channel Kv 1.5 (Bonnet et al., 2006), to name a few (for a detailed review see Hanze et al., 2007).

Animal studies have shown that aging is associated with decreased hypoxic–ischemic HIF-1 activation and impaired physiological responses mediated by HIF-1 (Bianchi et al., 2006; Chavez & LaManna, 2003; Di Giulio et al., 2005; Frenkel-Denkberg et al., 1999; Kang et al., 2005; Rivard et al., 2000; Zarember & Malech, 2005). In senescent animals, the ability of HIF-1 to bind to the hypoxia-response element present on HIF-1-regulated genes is significantly reduced (Frenkel-Denkberg et al., 1999), hence decreasing the transcriptional rate of HIF-1-regulated genes. Studies have shown that aging is associated with a marked impairment in hypoxia-induced angiogenesis and anemia-induced erythropoiesis due to reduced production of VEGF (Rivard et al., 2000) and erythropoietin (Wang et al., 1996, 1998), respectively. Several studies also report that eNOS expression and NO production decline with age (Aicher et al., 2003; Barton et al., 1997; Cooke & Losordo, 2002; Csiszar et al., 2003; Hoffmann et al., 2001; Iemitsu et al., 2004; Tschudi et al., 1996). Although the exact mechanisms underlying an age-dependent reduction in hypoxic–ischemic HIF-1 activation remain unknown, pharmacological activation of HIF-1 with cobalt chloride, a well-established HIF-1 activator, has been shown to reverse age-related decline in HIF-1 activation in aged mice (LaManna, 2007). Age-related changes in HIF-1 have not been investigated in humans.

Activating HIF-1 with desferrioxamine

HIF-1 activation can be achieved by means other than ischemia or hypoxia. Systemic administration of iron chelators such as desferrioxamine (DFO) to 7-day-old rats was associated with an increase in brain HIF-1 protein levels 1–3 h after injection (Bergeron et al., 2000). We have also shown that DFO administration and oxygen–glucose deprivation resulted in HIF-1 activation in primary mature cortical neuronal cultures (Sorond et al., 2001). There is also indirect evidence that iron chelators can modulate vascular function, possibly via HIF-1 activation (Duffy et al., 2001). We have shown that DFO infusion in healthy human volunteers was associated with a significant cerebral vasodilatation, which was temporally correlated with increased hypoxia-inducible transcription factor-1 protein concentration (Sorond et al., 2008) (see Figure 16.6). Therefore, acute DFO infusion and HIF-1 activation may be associated with improved vascular function.

Although all the above studies describe the responses to acute DFO administration, only a few studies have used DFO for longer periods and

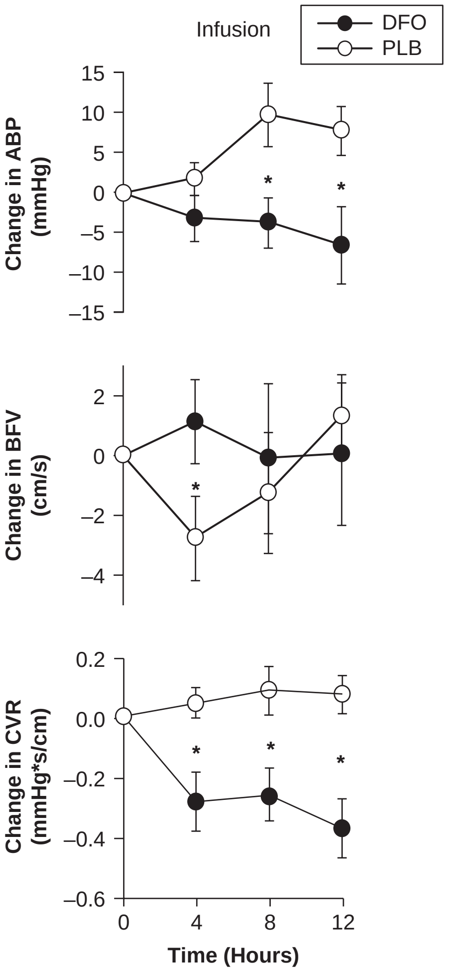

Figure 16.6 Cerebrovascular hemodynamic changes in response to 8 h of desferrioxamine (DFO) versus placebo (PLB) infusion. ABP, mean arterial blood pressure, expressed as percentage change from baseline; BFV, blood flow velocity, expressed as percentage change in mean flow velocity; CVR, cerebrovascular resistance (ABP/BFV) expressed as percentage change from baseline. *(reproduced from Sorond et al., 2009; copyright The Biochemical Society (http://www.clinsci.org); used by permission of the publisher).*

examined chronic HIF-1 activation. (Nguyen et al., 2007) Specifically, in two clinical trials of cognitively impaired elderly participants, 6 months of chronic DFO injection resulted in enhanced cognitive performance and delayed cognitive decline compared with placebo (Crapper McLachlan et al., 1991; McLachlan et al., 1993). Although HIF-1 levels were not measured directly in these studies, the clinical outcomes are those we would expect from chronic HIF-1 activation and improved cerebrovascular function. Therefore, indirect human and animal evidence suggests that chronic DFO injection is associated with persistent HIF-1 activation and improved cognitive outcomes. It is important to note that while we have specifically focused on DFO-mediated HIF-1 activation, DFO has many other potentially protective effects on the vascular and neuronal system. For a detailed discussion of these effects we refer the reader to our previous review on this topic (Sorond & Ratan, 2000).

HIF-1 and Quercetin

Until recently, DFO was the only known HIF-1 activator that was safe to use in humans. However, the problem of long-term iron chelation was a significant barrier to its use over longer durations. Although novel HIF-1 activators (prolyl hydroxylase inhibitors) are being aggressively developed (Siddiq et al., 2005), currently no such compounds are available for clinical use. In 2007 the flavonoid quercetin, which has been used extensively as a nutritional supplement in humans, has been shown to activate HIF-1 (Jeon et al., 2007). Recent animal studies have shown that quercetin is also neuroprotective in animal models of cerebral ischemia (Cho et al., 2006) and improves impaired cognitive performance in animal models of repeated cerebral ischemia (Pu et al., 2007). Human studies of quercetin have been limited to its anti-inflammatory and immune-modulating activities (Nieman et al., 2007). The availability of an oral HIF-1 activator that is safe in humans allows us, for the first time, to examine the chronic effects of pharmacologic HIF-1 activation on cerebrovascular function in humans.

Flavonoids and Vascular Health

Foods and beverages rich in phenolic phytochemicals, especially the flavonoids, are being heralded as potential preventive agents for a wide range of pathological conditions, ranging from stroke to coronary artery disease (Meydani, 2002; St Leger et al., 1979). Red wine has been invoked to explain the "French paradox" (the high-fat diets in France are paradoxically not associated with excessive rates of CVD; Renaud & de Lorgeril, 1992), and ingestion of tea has been associated with a reduction in coronary events in a number of impressive epidemiological studies (Geleijnse et al., 2002; Hertog et al., 1993; Mukamal et al., 2002). One hypothesis has attributed this health benefit to the ingestion of a class of polyphenolic antioxidants called flavonoids. Flavonoids are ubiquitous in nature and can occur in large amounts in several foodstuffs, including some teas and red wines (Renaud & de Lorgeril, 1992) and, especially, cocoa (Hammerstone et al., 2000). No randomized trial has been reported for any of these foodstuffs. However, evidence is accumulating that flavonoids have beneficial effects on endothelial function. Until recently, these effects had been studied mainly in animals and in vitro (Andriambeloson et al., 1997; Diebolt et al., 2001; Fitzpatrick et al., 1995; Karim et al., 2000; Leikert et al., 2002).

In both the rabbit and the rat aorta, red wine evoked relaxation related to NO activity (Cishek et al., 1997; Diebolt et al., 2001). In human umbilical vein endothelial cells, a polyphenol extract from red wine led to increased eNOS expression and NO release (Leikert et al., 2002). The effects of specific flavonol fractions extracted from cocoa were studied by Karim and colleagues, who reported endothelium-dependent relaxation and activation of eNOS in the rabbit aorta, abolished by l-N^G-nitroarginine methyl ester (Karim et al., 2000). Our collaborators, Fisher and colleagues, have recently shown that short-term ingestion of cocoa, particularly an extract rich in the subclass of flavonoids known as flavones, induced a consistent and striking peripheral vasodilation in healthy elderly people, improving endothelial function in a NO-dependent manner (Fisher & Hollenberg, 2006). More recently we have shown that dietary intake of flavanol-rich cocoa is associated with a significant increase in CBF velocity in the middle cerebral artery as measured by TCD (Figure 16.7) (Sorond et al., 2008a). Although HIF-1 was not previously implicated in flavonol-mediated vascular changes, involvement of nitric oxide synthase, a HIF-1-regulated protein, in this process supports the possibility of HIF-1 involvement.

Inhibitors of The Renin–Angiotensin System

The renin–angiotensin system (RAS) has been extensively studied for its critical role in the regulation of sodium balance, vascular tone, and blood pressure. Recent studies have shown marked pleiotropic effects of this system, including influences on cognitive function, physical function, muscle strength, and gait speed (Carter et al., 2005; Cesari et al., 2005; Chrysant & Chrysant, 2006; Dagenais & Jamali, 2005; Di Bari et al., 2004; Papadopoulos et al., 2004). RAS activity is dependent on angiotensin II (Ang II), which binds to two receptors (types 1 and 2) in humans (Gard, 2002; Kazama et al., 2004). Both receptors are present in the brain and have opposing effects: type 1 leads to vasoconstriction, whereas the type 2 receptor leads to vasodilatation, neuronal differentiation, apoptosis, and axonal regeneration (Wilms et al., 2005). Both angiotensin-converting enzyme inhibitors (ACEI) and angiotensin receptor blockers (ARB) decrease RAS activity and produce vasodilatation. ACEIs block ACE and decrease Ang II production, whereas ARBs block the angiotensin receptor type 1 but not type 2 (Padmanabhan et al., 1999; Roig et al., 2000). In spontaneously hypertensive rats, treatment with ARB was associated with improved cerebral autoregulation (Nishimura et al., 2000), preservation of CBF after middle cerebral artery occlusion (Ito et al., 2002), and normalization of the production of nitric oxide (Yamakawa et al., 2003). Treatment with candesartan (an ARB) or captopril (an ACEI) was also associated with a smaller infarct size after middle cerebral artery occlusion (Ito et al., 2002). Unfortunately, none of these studies examined the effect of ACEI or ARB on the development of cerebral microvascular disease. Work in our laboratory has demonstrated that blood pressure lowering with an ACE-based regimen can improve CBF and reduce cerebral vascular resistance in elderly hypertensives (Lipsitz et al., 2005). ARBs have also been found to improve cerebral autoregulation in hypertensive patients with stroke (Moriwaki et al., 2004) and diabetes (Kario et al., 2005).

There is accumulating evidence that decreasing RAS activity may be protective against cognitive and physical impairment beyond lowering blood pressure. Anatomically, Ang II and its receptors are located in neurons inside the blood–brain barrier and in the cerebrovascular endothelial cells and circumventricular regions of the brain (Ando et al., 2004). Functionally, Ang II has been linked with cognitive function in animal models (Gard, 2002). For example, Ang II has been associated with poor conditioned learning, and ARB (Raghavendra et al., 1998) and ACEI (Barnes et al., 1989) may facilitate learning, independent of blood pressure.

In hypertensive humans without evidence of cognitive impairment, ACEI and ARB may be protective against cognitive and physical impairment (Benedict & Brandt, 1992; Braszko et al., 2003; Cummings & Cole, 2002; Pogosova et al., 2003; Tedesco et al., 1999; Tzourio et al., 2003). An observational study of 1220 Italian individuals with heart failure showed that treatment with ACEI was associated with improved cognitive performance (Zuccala et al., 2005). The Perindopril Protection against Recurrent Stroke Study indicated that the ACEI perindopril reduced the risk of incident cognitive impairment in those with a previous history of stroke (Tzourio et al., 2003). In the Cognition and Prognosis in the Elderly Trial, treatment with candesartan was associated with a lower rate of cognitive decline than was placebo in subjects with mild cognitive impairment (Skoog et al., 2005). This effect is likely to be specific for drugs that decrease RAS activity and is not merely related to lowering of blood pressure. Diuretics, which produce a blood pressure reduction, similar to ACEI and ARB, but activate the RAS, do not protect against cognitive decline.

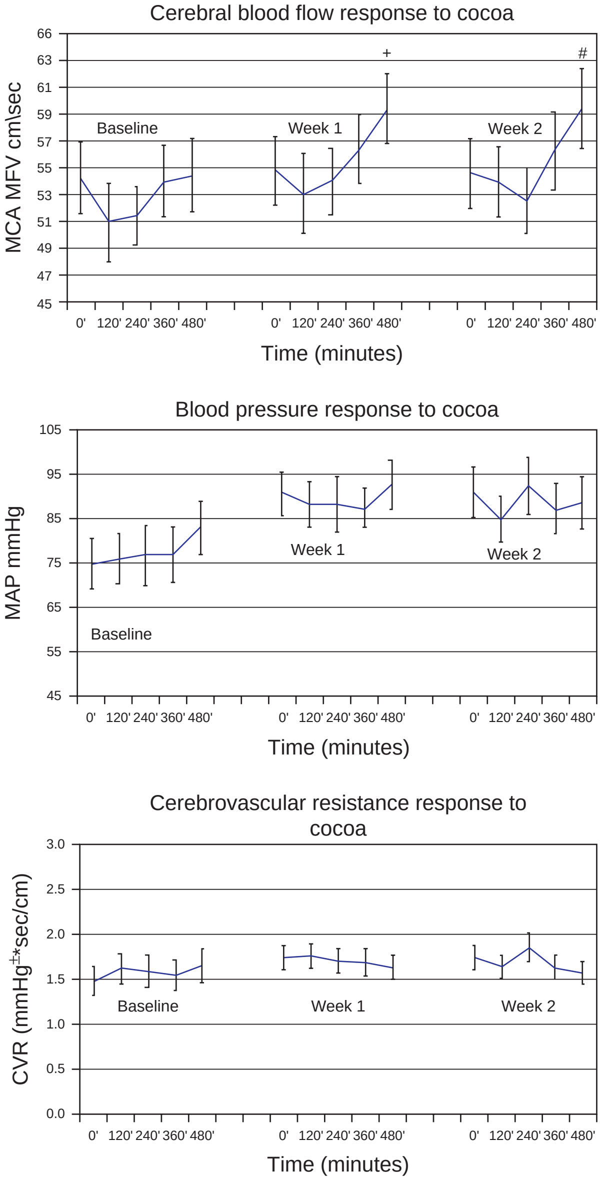

Figure 16.7 The middle cerebral artery (MCA) mean velocity (MFV), mean arterial blood pressure (MAP), and cerebrovascular resistance (CVR) response to flavanol-rich cocoa (FRC) ingestion over 8 h. Baseline denotes the response to the first dose. The same subjects were studied at days 7 and 14 of the FRC-based dietary intervention. Note the sustained increase in the acute cerebral blood flow in response to FRC intake. (*) and (#) denote points that show statistically significant changes from baseline.

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CONCLUSIONS

In this chapter we have provided evidence that aging and cardiovascular risk factors may impair cerebral blood flow regulation, produce ischemic damage to frontal subcortical periventricular watershed areas of the brain, and result in the development of cerebral microvascular disease. This, in turn, may contribute to impairments in mobility and cognition that are highly prevalent among elderly people. If this pathogenic mechanism of cerebral microvascular disease is verified in longitudinal studies, future efforts to reduce cardiovascular risk factors and improve cerebral perfusion may help reduce the burden of falls and dementia in the elderly population. Promising approaches to improving cerebral perfusion include HIF-1 activation, flavonoids, and inhibitors of the renin–angiotensin system.

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Chapter 17

Aging and Insulin Secretion

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CHAPTER CONTENTS

Introduction	**373**
Aging, Insulin Secretion, and Diabetes	**373**
Regulation of Pancreatic β Cells and Insulin Secretion	**375**
Pancreatic β Cell Function and Aging—Rodent Models	**376**
Pancreatic β Cell Function and Aging—Human Studies	**378**
Relationship to Insulin Signaling and Longevity	**380**
Conceptual Model	**381**
References	**382**

INTRODUCTION

This chapter reviews new and emerging information about important changes in the regulation of insulin secretion that occur during aging. Findings from animal models and from humans are presented and reviewed. In addition, this chapter links the age-related changes in regulation of insulin secretion to the high risk for development of diabetes mellitus observed in populations of older adults.

AGING, INSULIN SECRETION, AND DIABETES

Diabetes mellitus is one of the most common health problems of older adults, based on population studies in the United States and elsewhere (American Diabetes Association 2010; Chang & Halter, 2009). Figure 17.1 summarizes findings from the National Health and Nutrition Examination Survey, a population-based American study (Harris et al., 1998; Resnick et al., 2000). It demonstrates that the prevalence of known clinical diagnosis of diabetes increases from middle age to over age 60, for which the value is 11–12% of the population. In addition, equivalent numbers of people meet current criteria for diabetes diagnosis based on either fasting plasma glucose level greater than 125 mg/dl or a value exceeding 199 mg/dl 2 h after an oral glucose tolerance test (OGTT). Overall the prevalence rate of diabetes is approximately 25% of people over age 60. An additional 20% of the U.S. population meets criteria for impaired glucose tolerance (IGT), defined as a glucose level greater than 139 mg/dl but less than 200 mg/dl by OGTT and a fasting glucose level of less than 126 mg/dl. Other studies of people of older ages, including those over age 75, similarly demonstrate a prevalence rate in the 20–25% range (Chang & Halter, 2009). The prevalence rate includes people who have developed diabetes at a younger age and have survived into old age. However, the incidence rate of new diagnosis of diabetes also increases dramatically with age. The vast majority of older adults who meet criteria for diabetes have type 2 diabetes. However, some people with type 1 diabetes, characterized by severe insulin deficiency and damage to pancreatic β cells, survive to old age as well. The pathophysiology of type 2 diabetes is more complex. While circulating insulin levels may be variable, impaired insulin secretion in response to a glucose challenge is a universal finding in type 2 diabetes. Limited pathological studies have demonstrated diminished pancreatic β-cell mass (Butler et al., 2003).

Older adults who meet current criteria for diagnosis of diabetes mellitus are at substantial risk for complications of diabetes. These are largely grouped

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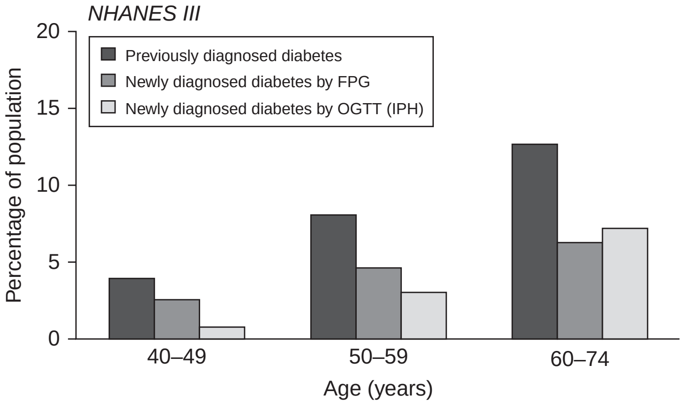

Figure 17.1 Prevalence of type 2 diabetes among elderly people in the United States according to age and American Diabetes Association diagnostic criteria, from the Third National Health and Nutrition Examination Survey (NHANES III). FPG, fasting plasma glucose; OGTT, oral glucose tolerance test; IPH, isolated postchallenge hyperglycemia (adapted from Harris et al., 1998, and Resnick et al., 2000).

as microvascular complications such as diabetic retinopathy, nephropathy, and neuropathy; and macrovascular complications, which are related to high rates of atherosclerosis leading to coronary artery disease, peripheral vascular disease, and cerebrovascular disease. For example, a diagnosis of diabetes greatly magnifies the age-related increase in acute myocardial infarction rates. There is a substantial age-related increase in risk for myocardial infarction in both men and women. However, men and women with diabetes have a much greater risk for myocardial infarction at any given age. On average, people with diabetes have myocardial infarction rates similar to those of people without diabetes who are 15 years older (Booth et al., 2006). In addition to these traditional diabetes complications, older people with diabetes are at higher risk for geriatric conditions, including decline in functional status and decreased cognitive function (Chang & Halter, 2009; Maty et al., 2004; Wray et al., 2005). It is estimated that the mortality rate associated with a diagnosis of diabetes in older adults is about twofold higher than people of the same age without diabetes.

There is now a growing body of evidence that progression from IGT or prediabetes to diabetes can be slowed substantially (Diabetes Prevention Program Research Group, 2009; Lindstrom et al., 2006). In people over age 60 at risk for progression to type 2 diabetes, lifestyle intervention to reduce body weight and increase physical activity reduced the rate of progression by 71% over 4 years. Surprisingly this effect was maintained over 10 years of follow-up, although the lifestyle intervention was much less intense during the last 6 years (Diabetes Prevention Program Research Group, 2009). A number of interventions can reduce the rate of diabetes complications (Chang & Halter, 2009; DCCT Research Group, 1993), including in older adults (Cigolle et al., 2009). These interventions include lowering of hyperglycemia and aggressive treatment of dyslipidemia and hypertension, both of which are very common in people with diabetes. Each of these intervention targets may be treated with diet, exercise programs, and one or more drugs. In combination, the overall treatment regimens may be very complex and probably need to be maintained for years for their effects to become evident (DCCT Research Group, 1993; Gaede et al., 2008). Thus, the intervention targets are difficult to achieve in the general diabetes population (Resnick et al., 2006).

Impaired pancreatic β-cell function and the resulting decline in insulin secretion contribute to the high rate of diabetes mellitus in older adults, with multiple secondary effects of diabetes on overall morbidity and mortality. In addition, there may also be a more subtle impact of impaired insulin secretion in the aging population. Insulin is a major anabolic hormone, important for storage of fuel as glycogen and fat, but also critical for protein synthesis. Diminished protein synthesis leading to a loss of muscle mass is a common finding in aging people and an important contributing factor to frailty and other functional deficits in older adults. Thus, a decline in production of insulin below the level needed for its anabolic affects could play a role in these aspects of aging as well (Chang & Halter, 2009). However, a major challenge to testing this hypothesis is that healthy, exercising, insulin-sensitive people also have low insulin levels that are adequate to meet their metabolic and anabolic needs. As described later in this chapter, comparison of insulin levels in vivo among populations requires concomitant assessment of degree of insulin sensitivity in the same people.

REGULATION OF PANCREATIC β CELLS AND INSULIN SECRETION

The primary source of insulin production is specialized neuroendocrine cells called β cells, which are located in collections of hormone-producing cells called islets of Langerhans, which are scattered throughout the pancreas. While an in-depth discussion of pancreatic islet structure and function is beyond the scope of this chapter, there is growing interest in understanding the factors important in regulation of β-cell turnover: differentiation from neural stem cells, proliferation, cell death, and regeneration (Bonner-Weir, 2001; Bouwens & Rooman, 2005). In the past, pancreatic β cells were thought to be relatively static, with low rates of turnover and limited capacity for regeneration. However, there is increasing evidence and understanding of the capacity of pancreatic β cells to grow and proliferate under some circumstances (Bouwens & Rooman, 2005; Dor et al., 2004; Meier et al., 2008). For example, in mice pancreatic islet proliferation can be induced by partial pancreatectomy, by islet injury induced by the drug streptozotocin, by the gut hormone glucagon-like peptide-1 (GLP-1), and by pregnancy (Bouwens & Rooman, 2005; Li et al., 2003; Rankin & Kushner, 2009; Tschen et al., 2009; Xu et al., 1999). In addition, obesity is known to be associated with an increase in β-cell mass, probably due to cell proliferation (Montanya et al., 2000). Obesity develops over the life span, thereby contributing to pancreatic islet compensation for obesity-related insulin resistance. However, the mechanisms of adaptation of pancreatic β-cell proliferation to obesity are not known.

Similarly there is a large and growing literature on regulation of insulin secretion by pancreatic β cells. A complete review of this process is beyond the scope of this chapter. The glucose level is known to be a key regulator of insulin secretion (Chang & Halter, 2003, 2009). The effects of glucose appear to be multiple and complex. For example, exposure of pancreatic islets in vitro or in vivo to a constant glucose stimulus leads to a complex multiphasic insulin secretory response. This response includes an acute phase occurring within minutes, thought to be largely due to an immediate release of preformed insulin granules that are available near the cell surface of pancreatic β cells, and a more prolonged second phase of insulin secretion that gradually increases in magnitude over time and is dependent on new protein synthesis.

In addition to being a direct insulin secretagogue, glucose can potentiate many other factors that can contribute to insulin secretion. Thus, nutrients such as some amino acids and fatty acids can stimulate insulin secretion, but only in the presence of adequate amounts of glucose. Similarly other hormones can stimulate insulin secretion in a glucose-dependent fashion. Prominent among such hormones are peptides derived from the GI tract such as a GLP-1 and gastric inhibitory peptide, also called glucose-dependent insulin-tropic peptide (Chang & Halter, 2003; Korosi et al., 2001; Li et al., 2003; Xu et al., 2005). Secretion of such peptides is thought to explain observations that oral glucose administration leads to greater insulin secretion over time than the same amount of glucose administered intravenously, the so-called incretin effect. In recent years, a new class of drugs called incretins have been developed as therapeutic agents for managing hyperglycemia in people with diabetes mellitus. The incretins are agents that have GLP-1-like effects (for example, exenitide); inhibit a key enzyme that rapidly degrades exogenous GLP-1, thereby prolonging GLP-1 availability in vivo (for example, the gliptin group of drugs); or are analogs of GLP-1 that are long acting because they are resistant to degradation (for example, liraglutide).

Pancreatic islets are also known to be under neural control. Insulin secretion can be activated by parasympathetic neural stimulation and influenced by sympathetic neural system stimulation as well. The sympathetic nervous system effects are complex in that β-adrenergic stimulation enhances insulin secretion, while α_2-adrenergic stimulation inhibits insulin secretion (Halter et al., 1984; Metz et al., 1978; Morrow et al., 1993). Thus during activation of sympathetic control of islet cells there is overall some inhibition of glucose-stimulated insulin secretion, but at the same time tonic enhancement mediated by β-adrenergic stimulation. Thus, when sympathetic stimulation stops, the pancreatic islets are prepared to increase insulin secretion dramatically. During stressful situations, when there is a dramatic activation of sympathetic nervous system activity, hyperglycemia develops because of multiple neural effects that increase glucose production and diminish utilization, while insulin secretion is inhibited. This hyperglycemia rapidly resolves when the sympathetic nervous system activity returns to normal as insulin secretion responds dramatically (Halter et al., 1984). Interest in α-adrenergic inhibition of insulin secretion has grown from findings that one experimental mouse model of diabetes appears to be a result of overexpression of α_2-adrenergic receptors on pancreatic islets. This type of diabetes can be prevented by knockout of the α_2-adrenergic receptors. Furthermore, in human populations an α_2-adrenergic receptor gene variant is associated with risk for type 2 diabetes (Rosengren et al., 2010).

Overall physiologic regulation of insulin secretion is most apparent in humans in response to ingestion of a meal. Meal ingestion provides a complex set of signals to pancreatic islets including an increase in glucose level, increase in other nutrients, release of GI peptides, and activation of neural signals. The net result is a finely regulated insulin secretory response that minimizes postmeal hyperglycemia under physiologic conditions as well as any overshoot and subsequent hypoglycemia. However, the complexity of these signals makes it difficult to use insulin levels after a meal as an indicator of pancreatic β-cell function in any given individual or in pathologic states. For example, one person may have a small increase in insulin levels in response to a meal, despite high postmeal glucose levels, because of impaired β-cell function. Another person may have similarly low insulin levels after a meal, but with normal β-cell function that is responding appropriately to low postmeal glucose levels and other meal-related signals. Even when a more simple stimulus is provided, such as oral glucose (e.g., OGTT), multiple neural and endocrine signals are generated in addition to the glucose level itself, and of course all of these signals, including the glucose level, are changing over time. Thus, the circulating insulin response during an OGTT is also difficult to interpret as a measure of pancreatic β-cell function.

Insulin secretion patterns after a meal are complex, but even in the absence of a major event such as meal ingestion, circulating insulin levels in vivo are challenging to interpret. One problem is that much of the secreted insulin is removed by the liver during initial delivery of insulin via the portal vein and never gets to the peripheral circulation. Under most physiological circumstances, peripheral insulin levels provide a good estimate of pancreatic secretion. However, variation of liver insulin extraction among individuals can complicate comparisons. Measurement of C-peptide levels can provide a solution to this problem. C peptide is the part of the proinsulin molecule that is cleaved off as proinsulin is converted to insulin in β-cell secretory granules. C peptide is cosecreted with insulin, but is not removed by the liver. The combination of measurement of circulating C-peptide levels with mathematical modeling of C-peptide kinetics by deconvolution analysis has provided a validated estimate of insulin secretion rate in vivo (Chang & Halter, 2003). Another problem with interpretation of insulin levels in vivo is that, as has been observed for a number of other hormones, normal basal secretion of insulin occurs in a pulsatile manner. There are rapid, low-amplitude pulses occurring every 8–15 min and in addition pulses with a longer duration of 60–140 min and larger amplitude (Chang & Halter, 2003; Matveyenko et al., 2008). Such endogenous variability of basal insulin secretion can be magnified by infusion of glucose to achieve a constant glucose level that is above normal. Thus, measurement of a single insulin level, even in the fasting state, can provide only a rough estimate of basal rates of secretion in a given individual.

A key determinant of the rate of basal insulin secretion and insulin responses to challenge such as a meal in a given individual is that individual's sensitivity to the metabolic effects of insulin. There appears to be an adaptive response to changes in body sensitivity to metabolic effects of insulin, leading to an increase in pancreatic β-cell mass, as described above, and rates of insulin secretion. The most common example of such β-cell adaptation is the response to simple obesity-related insulin resistance.

The relationship between insulin secretion and insulin sensitivity has been studied in detail in experimental animals and in humans. Among populations with varying degrees of insulin sensitivity (as quantitated by a number of methods in vivo), a surprisingly consistent relationship between insulin secretion rate and degree of insulin sensitivity has been documented (Kahn et al., 1993). As illustrated in Figure 17.2, this relationship is hyperbolic, both in humans and in rodents (Matveyenko et al., 2008). The simplest mathematical model that fits these data is the product of a measure of β-cell function and a measure of insulin sensitivity (Kahn et al., 1993). This product has been termed the Disposition Index. Thus, across a population of normal people with varying degrees of insulin resistance and appropriate compensation of insulin secretion, the Disposition Index will have the same value. A given individual may move down the hyperbolic curve by reducing weight, improving insulin sensitivity, and adapting by downregulating insulin secretion; or move back up the curve with increased weight, worsening insulin resistance, and adaptation by increasing insulin secretion. In either case, the product of insulin secretion times insulin resistance will remain unchanged.

This type of analysis has been used to identify individuals who fail to adapt normally to changes in insulin resistance by secreting inadequate amounts of insulin and have a lower product or Disposition Index. Disposition Index has been used as a way of comparing individuals who have different degrees of insulin sensitivity to identify those with poor β-cell function, in the sense of poor adaptation to changes of insulin resistance. Thus, individuals who appear to be predisposed to the development of diabetes such as women who have previously had gestational diabetes or family members of people with diabetes have been shown to have a lower Disposition Index than normal (Utzschneider et al., 2004).

PANCREATIC β CELL FUNCTION AND AGING—RODENT MODELS

A number of studies have demonstrated a decline in β-cell function and insulin secretion with age in rodents. To understand mechanisms of this age-related decline in function, studies have focused on pancreatic islet cell proliferation and β-cell turnover

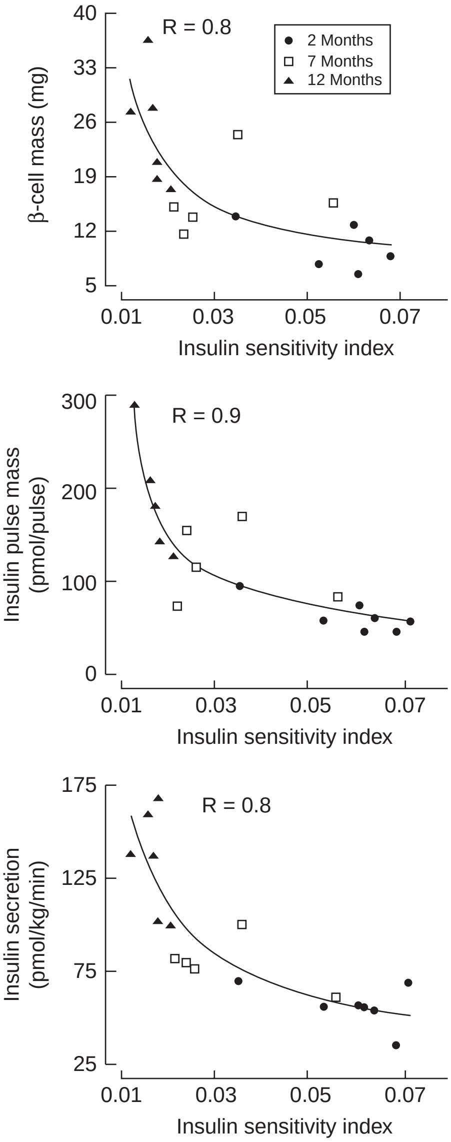

Figure 17.2 Hyperbolic relationship between insulin sensitivity vs the β-cell mass (top), pulse mass (middle), and total insulin secretion estimated by deconvolution analysis (bottom) in Sprague–Dawley rats age 2, 7, and 12 months. Each point represents data from one animal (Matveyenko et al., 2008).

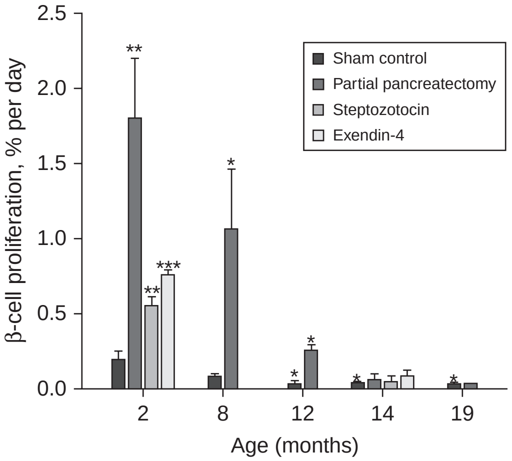

Figure 17.3 Adaptive β-cell proliferation is severely restricted with advanced age. Effects of age and of partial pancreatectomy, low-dose streptozotocin, or exendin-4 on β-cell proliferation, estimated as 5-bromo-2′-deoxyuridine (BrdU)-positive β cells (% total per day) after 14 days of continuous BrdU ingestion in mice. There is a decline in β-cell proliferation with age in the controls ($*P < 0.05$ shams at 2 months vs shams at 12, 14, or 19 months). β-Cell proliferation increased dramatically with the treatments in young mice, but the responses declined dramatically with age ($*P < 0.05$; $**P < 0.01$; $***P < 0.001$ sham vs interventions at various ages). Studies were done in male F1 hybrid B6129SF1/J mice (stock 101043), obtained at 1 and 8 months of age from The Jackson Laboratory (Bar Harbor, ME, USA). The Jackson B6129SF1/J hybrid is the product of an intercross between C57BL/6J (000664) female mice and 129S1/SvImJ (002448) male mice from The Jackson Laboratory's commercial colonies. Results are means ± SEM ($n = 4–6$ animals per group) (Rankin & Kushner, 2009).

(Bonner-Weir, 2001; Maedler et al., 2006; Rankin & Kushner, 2009; Teta et al., 2005; Tschen et al., 2009). Figure 17.3 demonstrates a modest decline in islet proliferative capacity with age in normal mice age 2 to 19 months (Rankin & Kushner, 2009). This aging effect is much more dramatic when the proliferative response of older animals to partial pancreatectomy, streptozotocin, and exendin-4 (a GLP-1 agonist) is compared to the robust responses observed in young animals. A decline in response is evident by 8–12 months of age and response is almost undetectable by 14–19 months of age. Similarly, 1- to 2-month-old mice increase islet mass and β-cell proliferation in response to high-fat diet, but 7- to 8-month-old mice cannot (Tschen et al., 2009). This study also found a lack of an islet proliferative response to streptozotocin and exendin-4 in 7- to 8-month-old mice. Thus, loss of islet proliferative capacity appears to occur early in life in rodents and perhaps would make them susceptible to impaired glucose regulation if there is future injury to islets or accelerated loss of β cells over time. Exposure to high concentrations of glucose in vitro can lead to apoptosis of β cells, evidence of glucose toxicity. Islets from adult 7- to 8-month-old Sprague–Dawley rats appear to be more sensitive to glucose-induced apoptosis (Maedler et al., 2006).

Pancreatic β-cell proliferation appears to be dependent on cell cycle regulation (Kushner et al., 2005; Park et al., 2004). Study of the cell cycle inhibitor p16 has led to some new insights on aging and

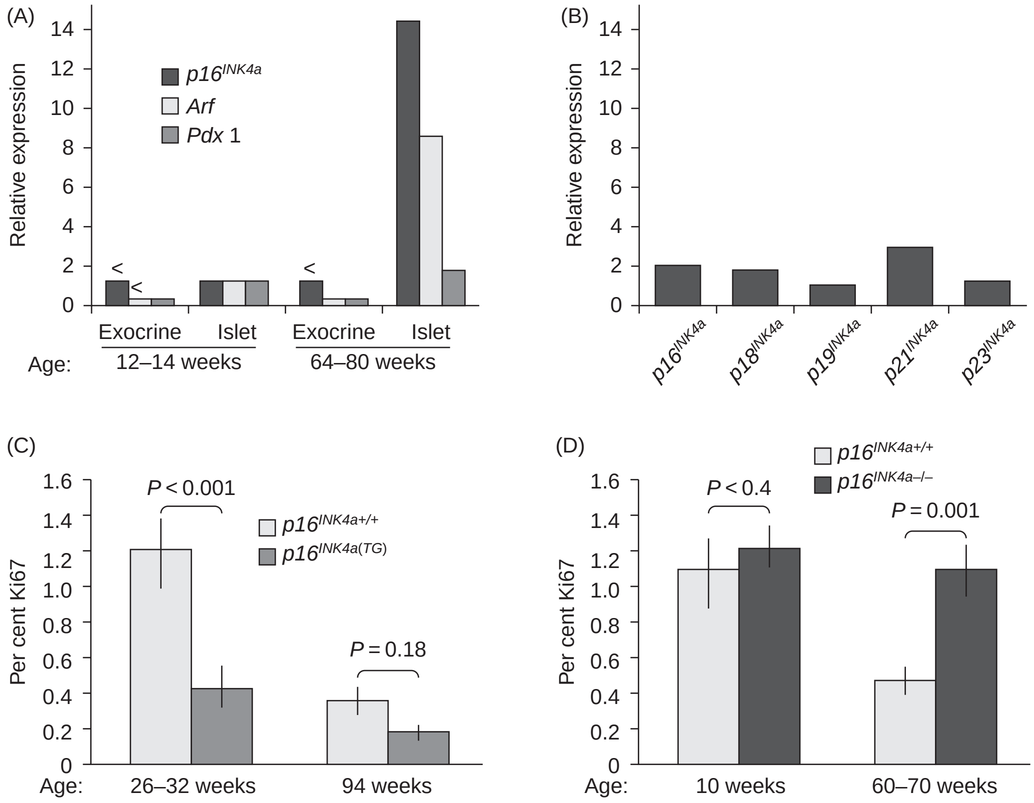

Figure 17.4 Islet gene expression and proliferation in young versus old C57BL/6 mice (The Jackson Laboratory). (A and B) Expression of transcription factors p16^{INK4a}, Arf, and Pdx1 in islets and exocrine pancreas from mice of indicated ages (A), and relative mRNA expression of other cyclin-dependent kinase inhibitors in islets with aging (B). Values are reported as mean ratio of expression in old (64–80 weeks) versus young (12–14 weeks) mice. Note the dramatic increase in p16^{INK4a} and Arf (both products of the Cdkn2a gene locus) in islets of older mice and the lack of increase in other cyclin-dependent kinase inhibitors. (C) Percentage of pancreatic islet cells positive for Ki67 (a widely used marker of islet cell proliferation) from p16^{INK4a} wild-type mice of indicated ages and from transgenic mice overexpressing p16^{INK4a} to levels comparable to those in 60-week-old wild-type mice. A reduction in proliferation index was noted in islets from transgenic mice compared to littermate controls, especially in the younger mice. (D) Percentage of Ki67-positive cells of pancreatic islets from p16^{INK4a} wild-type mice and p16^{INK4a} knockout mice of indicated ages. Note that the age-related decrease in proliferation was largely rescued by p16^{INK4a} deficiency. Values are means ± SEM (Krishnamurthy et al., 2006).

regulation of islet growth (Krishnamurthy et al., 2004, 2006). As illustrated in Figure 17.4, there is a substantial increase in expression of the cell cycle regulator p16 in islet tissue from mice age 15–18 months (Krishnamurthy et al., 2006). Increased p16 expression is associated with a substantial age-related decline in islet proliferation. Overexpression of p16 markedly reduces islet proliferation in younger mice to a level similar to that observed in older mice, and knockout of p16, thereby preventing p16 from increasing with aging, appears to reverse the age-related decline in islet cell proliferation in this model. In this context it is of great interest that p16 is one of the proteins produced from the CDKN2a gene locus (Krishnamurthy et al., 2004). Genetic variation at this locus has emerged as a consistent association with type 2 diabetes risk from genome-wide scanning studies in humans (Saxena et al., 2007; Scott et al., 2007; Steinthorsdottir et al., 2007; Zeggini et al., 2007). Thus, increased p16 production with aging is linked with decreased islet proliferation with aging, and alterations in its expression through the CDKN2a locus may contribute to the risk for type 2 diabetes.

PANCREATIC β CELL FUNCTION AND AGING—HUMAN STUDIES

Limited access to human pancreas tissue has allowed only limited exploration of regulation of pancreatic β cells in vitro, and studies of human β-cell turnover

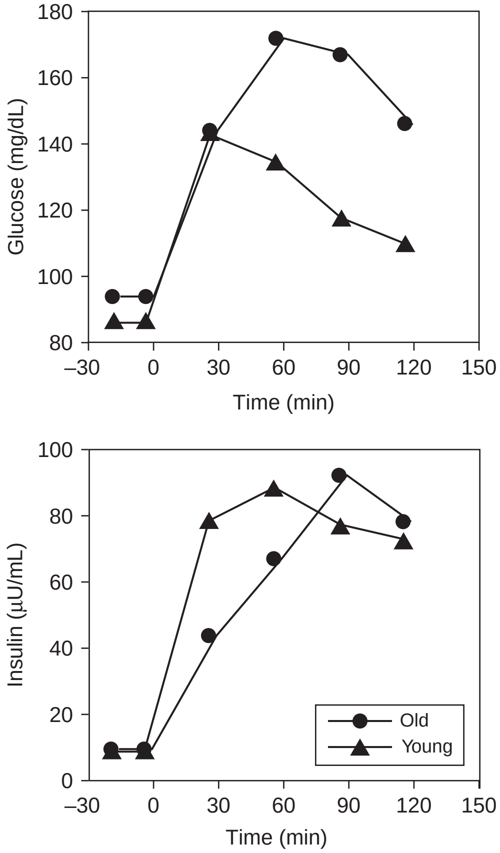

Figure 17.5 Age-related glucose intolerance. Mean plasma glucose (top) and insulin (bottom) levels before and after oral ingestion of 100 g of glucose in healthy old (n = 18) and young (n = 18) subjects matched for relative body weight and socioeconomic group. Subjects were eating an ad libitum diet. Note the slight elevation in mean fasting glucose levels in the old group and the delay in recovery of glucose levels following oral glucose. Also note the overall similarity of insulin levels between old and young subjects (Chen et al., 1987).

are also very limited. However, findings from humans appear to parallel age-related changes observed in rodents, including diminished insulin secretion in vitro, diminished proliferative capacity, and increased sensitivity to apoptotic effects of high glucose exposure (Butler et al., 2003; Ihm et al., 2006).

There is a substantial amount of information about glucose tolerance and insulin secretion as a function of age in humans (Chang & Halter, 2003). The age-related decline in glucose tolerance in nondiabetic humans has been observed in many studies (Chang & Halter, 2003; Chen et al., 1985, 1987) and is illustrated in Figure 17.5. Insulin levels have been measured during such studies of glucose tolerance. However, as indicated above, the insulin levels are very difficult to interpret because of the complexity of the stimulus and the change in glucose levels over time that is not matched between young and older subjects. This difficulty is further compounded by the challenge of studying older individuals, who often are relatively insulin resistant because of age-related obesity, decreased physical activity, and possibly other factors, but are compared to younger, more insulin-sensitive individuals. One approach to this challenge is to measure both insulin secretion under standardized conditions and insulin sensitivity in older and younger subjects and use the Disposition Index as a way of adjusting for group differences in insulin sensitivity. Multiple studies have now demonstrated that Disposition Index values are lower in older adults than in younger adults (Basu et al., 2003; Kahn et al., 1992; Szoke et al., 2008; Utzschneider et al., 2004). This indicates that overall β-cell function, at least as assessed in response to the degree of insulin sensitivity, is diminished in older people.

Another approach is to carry out controlled studies in older and younger subjects matched for degree of insulin sensitivity. In one such study, a controlled glucose stimulus was provided by varying the glucose infusion during the study to achieve a standardized rate of increase of the glucose level over time in healthy young and older subjects matched for degree of insulin sensitivity (Chang et al., 2006). Subjects meeting the criteria for diabetes were excluded. As illustrated in Figure 17.6, insulin secretion rate, estimated by deconvolution of C-peptide kinetics, increased gradually over time as the glucose level was increased by glucose infusion. Healthy older people with no abnormality of glucose tolerance had significantly lower insulin secretion in response to this same glucose challenge compared to the younger individuals. Older individuals who met the criteria for IGT, and thus may have been at risk for developing diabetes, demonstrated a further reduction in insulin secretion response. In this type of procedure with a gradual increase in glucose level in the physiologic range, people with overt type 2 diabetes would show no increase in insulin secretion. Thus, even healthy older people with normal glucose tolerance appear to demonstrate an age-related deficit of glucose-induced insulin secretion. This impairment is worse in people with IGT. The same study subjects were treated with nicotinic acid, to induce insulin resistance, for 2 weeks or with matching placebo. In response to nicotinic acid-induced insulin resistance, younger subjects consistently increased the insulin secretion response to a glucose challenge, whereas older patients with IGT were not able to do so. The Disposition Index fell slightly in all groups with nicotinic acid but the decline was greatest in the older subjects. Thus, short-term induction of insulin resistance led to expected islet function adaptation in younger subjects, but older people were less able to adapt.

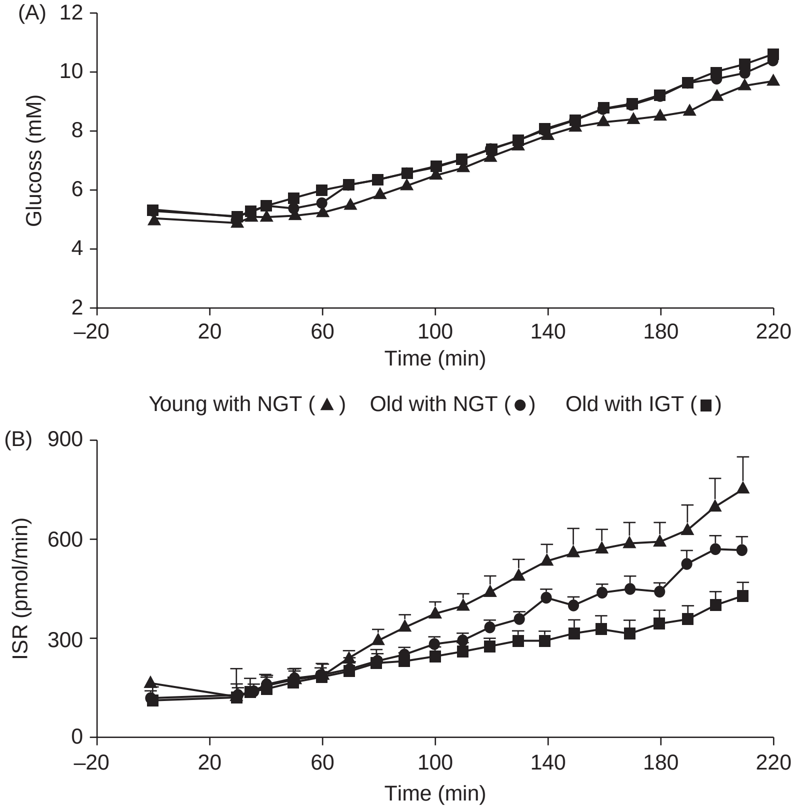

Figure 17.6 Effect of age on insulin secretion rate (ISR) in humans with normal glucose tolerance (NGT) or impaired glucose tolerance (IGT) by American Diabetes Association criteria. Plasma glucose concentrations and ISR are shown over time during intravenous glucose infusions comparing young with NGT ($n = 15$, mean age 26), old with NGT ($n = 16$, mean age 70), and old with IGT ($n = 14$, mean age 70). (A) Glucose levels during variable-rate glucose infusion begun at time 0 were well matched in the three study groups, and degree of insulin resistance was also similar in the three study groups. (B) ISR was significantly and progressively decreased in the two older groups, with the greatest impairment in old IGT ($P \leq 0.0002$, old IGT vs young and old NGT, and old NGT vs young NGT). Data are means ± SE (Chang et al., 2006).

Another study used deconvolution of C-peptide kinetics to study pulsatile insulin secretion in healthy older adults and older adults with type 2 diabetes in comparison to healthy younger people (Meneilly et al., 2005). Impairments of pulsatile insulin secretion were observed in both elderly groups and were most severe in those with diabetes. Infusion of GLP-1 partially reversed the impairments in the diabetes patients.

RELATIONSHIP TO INSULIN SIGNALING AND LONGEVITY

There has been substantial interest in the relationship between insulin signaling pathways and longevity since the unexpected findings initially from yeast and worm studies that genetic disruption of insulin and insulin-like signaling pathways can lead to a substantial increase in longevity (see Chapter 2 of this book). Additional work in *Drosophila* and some work in rodents has further demonstrated that multiple interventions to change insulin and insulin-like signaling pathways throughout life can prolong life span. However, this work is complicated by the fact that this signaling pathway is activated by a receptor mechanism that responds both to insulin and to growth factors such as insulin-like growth factor-1. In fact, in rodent models of aging it appears as though the growth effects are most important, as various models of growth deficiency are associated with longevity. The finding that impaired insulin signaling is associated with longevity and health seems at odds with findings in humans that insulin resistance and impaired insulin secretion, both leading to reduced overall insulin signaling, result in diabetes and its associated increased morbidity and mortality. This is

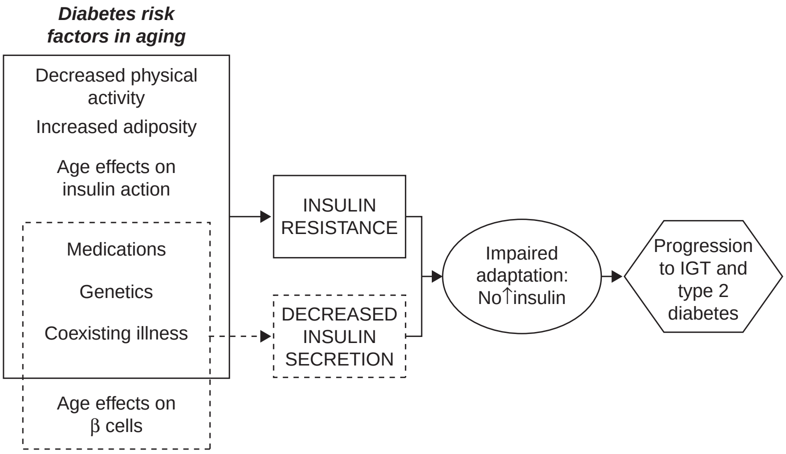
Diabetes risk factors in aging
Decreased physical activity
Increased adiposity
Age effects on insulin action
Medications
Genetics
Coexisting illness
Age effects on β cells
INSULIN RESISTANCE
DECREASED INSULIN SECRETION
Impaired adaptation: No↑insulin
Progression to IGT and type 2 diabetes

Figure 17.7 Model for age-related hyperglycemia. There are multiple risk factors associated with aging that may predispose older adults to develop diabetes. Some of these factors contribute to insulin resistance and some to decreased insulin secretion. Older individuals with impaired pancreatic β-cell function are unable to adapt adequately to insulin resistance by increasing insulin secretion. In this situation, impaired glucose tolerance (IGT) and progression to type 2 diabetes can occur. A vicious cycle may ensue as hyperglycemia develops, since "glucotoxicity" can lead to further deterioration of β-cell function and more severe insulin resistance (Chang & Halter, 2003).

obviously an area of great interest that requires substantial further investigation.

Another possible link between glucose regulation, pancreatic islet function, and longevity is the well-known effect of caloric restriction (CR) over the life span to improve longevity. Studies of CR rodents clearly demonstrate that improved glucose regulation, reduced obesity, and lower insulin levels are part of the CR phenotype (see Chapters 1 and 21). However whether these changes in glucose and insulin during caloric restriction are critical factors that contribute to longevity effects are not known. Most recently, initial CR studies in nonhuman primates have demonstrated some possible enhanced longevity, but also clear improvements in age-related changes in glucose and insulin regulation (Colman et al., 2009). Furthermore, dramatic prevention of age-related development of abnormalities of glucose metabolism was observed.

These observations also seem to be at odds with the findings that impaired insulin signaling throughout life improves longevity. However, insulin sensitivity is usually defined in relationship to metabolic effects of insulin, not its growth effects. It is thus conceivable that these two actions could be dissociated. For example, Masoro has proposed that improved sensitivity to the metabolic effects of insulin during CR leads to appropriate downregulation of insulin secretion. Low insulin levels during CR result in low insulin signaling at other sites, such as the brain, which may lead to growth retardation as a proximate cause for the enhanced longevity of CR animals (Masoro, 2009).

There is also great interest in the TOR signaling pathway as a potential mechanism by which caloric restriction leads to enhanced longevity (Kapahi & Vig, 2009; and see Chapter 9). Suppression of the TOR pathway by the drug rapamycin, even when administered to mice in middle age, enhances longevity (Harrison et al., 2009). There is evidence that a downstream ribosomal protein S6 kinase may be a key part of this pathway, and a knockout of this kinase also enhances longevity in female mice (Selman et al., 2009). Interestingly, such mice have lower body weight, less accumulation of body fat than wild-type animals, better insulin sensitivity, and improved glucose tolerance. Thus, this longevity phenotype has characteristics similar to those of caloric restriction that involve insulin regulation and its signaling pathways.

CONCEPTUAL MODEL

A conceptual model for the high rate of age-related diabetes in humans is illustrated in Figure 17.7 (Chang & Halter, 2003). This model hypothesizes that dual factors interact to lead to the development of hyperglycemia in aging: insulin resistance and decreased insulin secretion. Insulin resistance to the metabolic effects of insulin with aging appears to reflect predominantly lifestyle factors leading to increased adiposity and diminished physical activity (note that mechanisms for insulin resistance may be different for these two causal factors). Independent age effects on insulin action may also contribute, but the magnitude appears to be small. There may be genetic factors contributing to insulin resistance with aging, although these have not yet been identified. In some elderly patients, coexisting stressful illness leading to sympathetic nervous system activation and use of medications such as glucocorticoids can directly contribute to resistance to insulin action (Supiano et al., 1993). As outlined in this chapter there are aging effects on β-cell proliferation, and probably on susceptibility to apoptosis, which contribute to diminished β-cell mass and decreased insulin secretion capability with aging. Functional defects in pancreatic β-cells may also contribute to the observed age-related impairment of insulin secretion. As described earlier, sympathetic nervous system activation from coexisting illness can also contribute to impaired insulin secretion, and there is evidence for increased sympathetic nervous system activity overall in aging people even in the absence of acute illness (Supiano et al., 1990).

Finally, multiple genetic risk factors have been identified, such as the genes regulating p16 expression and the α_2-adrenergic receptor, which appear to influence diabetes risk primarily by effects on pancreatic β-cells. However, the identical and very high concordance rate for type 2 diabetes observed in both fraternal and identical twins suggests that important nongenetic factors, possibly early in life, contribute to diabetes risk (Poulsen et al., 2009). The earliest defect in pancreatic insulin secretion that is detected appears to be impaired adaptation to concomitant insulin resistance, leading to IGT and ultimately to type 2 diabetes as the age-related decline in pancreatic β-cell function and adaptation both worsen. This overly simplistic model implies that the pancreatic islet adaptation is the same regardless of the mechanisms contributing to insulin resistance. However, this hypothesis needs to be tested. The substantial preventive effects of lifestyle interventions described earlier on this progressive course appear to be due to improvement of the degree of insulin resistance, thereby reducing the need for insulin production from β cells limited by age-related changes in mass and function.

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Chapter | 18 |

Cardiovascular Effects of Aging in Primates—Gender Differences

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CONTENTS

Introduction 385
Gender Differences During Aging 386
  Gender Differences with Aging in Physiological Processes and Disease 386
  Mechanisms of Gender Difference in Aging 387
    Sexual Dimorphism from X-linked/Y-linked Genes 387
    Sex Hormones 388
  Species-Specific Differences During Aging 388
Limitations of Models for Studying Gender Differences During Aging 389
  Limitation of Aging Studies in Humans and in Rodent Models 389
  Advantages of a Primate Model 390
    In Terms of Physiology and Metabolism 390
    In Terms of Gene Expression 390
    In Terms of Life Span 390
    In Terms of Reproductive Physiology and Endocrinology 390
Studies of Aging in a Primate Model 391
  Neurobiology 391
  Reproductive Senescence 391
  Cardiovascular Disease and Diabetes 391
  Caloric Restriction 392
  Other Primate Studies 392
Gender Differences in Cardiovascular Aging in Primates 392
  Cardiomyopathy of Aging 392
  β-Adrenergic Desensitization 394
  Increased Vascular Stiffness with Aging 395
  Additional Mechanisms Mediating Increased Stiffness with Aging 396
  Summary of Gender Differences in the Primate Model of Aging 396
    Cardiomyopathy of Aging 396
    β-Adrenergic Desensitization 397
    Vascular Stiffness 397
    Molecular Mechanisms for Future Research 398
References 398

INTRODUCTION

Gender differences are widely reported in the process of biological aging and in the development of age-related diseases in humans. The primary focus of this chapter is to describe gender differences in a primate model of aging, focusing on the cardiovascular system. This is important because of the well-known gender differences in morbidity and mortality from cardiovascular disease, especially in premenopausal women, who are protected compared to age-matched men, in whom cardiovascular disease secondary to atherosclerosis and hypertension becomes increasingly more frequent. Much of this is common knowledge to physicians, e.g., symptoms of myocardial infarction (chest pain) in men 30–40 years of age are followed up more closely than in women of the same age. Over the decades after menopause, these differences become less marked and cardiovascular disease becomes more prominent in women (Akishita, 2009; Hayward et al., 2000; Takahashi & Tanaka, 2009).

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Since cardiovascular disease is a major cause of mortality in the middle-aged and elderly population, it is apparent to almost everyone that life span is greater in women than in men, and a major cause of this difference comes from the protection against cardiovascular disease in premenopausal women. Examples of this protection are shown in Figure 18.1, demonstrating that women are protected against heart disease, e.g., hypertension and hypercholesterolemia, as young adults and lose this protection around the time of menopause and actually exhibit a worse prognosis compared to the men after menopause (Gostynski et al., 2004; Lloyd-Jones et al., 2009). The hypotheses drawn to explain such differences stem from biological differences (such as genetic differences or sex hormones, for example) to acquired risks linked to differences in gender-related lifestyles and to psychosocial and behavioral causes (Verbrugge, 1985). It would be ideal to obtain information related to the mechanisms underlying gender differences in aging human subjects, but it is generally not feasible for several reasons. First, serial tissue sections are required to analyze changes in genes, proteins, and the histological composition of organs as they age, but obtaining these samples in aging humans is generally not possible. Second, the manifestations of aging per se in humans are difficult to isolate, since aging is associated with other diseases, most notably hypertension, diabetes, and atherosclerosis, which may induce parallel cardiovascular changes. Of course there are limitations to animal studies of aging as well, making it imperative to understand molecular changes integrated with what is observed in aging humans. A variety of models are therefore utilized in aging studies, including yeast, nematodes, fruit flies, rodents, and nonhuman primates. The selection of a specific species depends on many factors, including economic feasibility, husbandry, generalization of the findings, availability of background information, adaptability to experimentation, and relevance to human aging. Although each model offers strengths and limitations, nonhuman primates present both the unique advantage of close phylogenetic proximity to humans and similar menstrual and menopausal responses compared with humans. In this chapter, we first summarize the gender differences in the process of biological aging in humans. Next, we present the advantages of a nonhuman primate model of aging. Finally, we summarize the findings obtained in this model that are related to gender differences in biological aging in the cardiovascular system. Because of the genomic similarities between nonhuman primates and humans and because the aging process in nonhuman primates occurs over decades instead of a few years, in contrast to rodents, it is conceivable that the data collected in the nonhuman primate model of aging will be most applicable to understanding the aging process in humans.

GENDER DIFFERENCES DURING AGING

Gender Differences with Aging in Physiological Processes and Disease

Gender differences in human biological aging or longevity are well documented. This topic is expanded upon in depth in Chapter 23 of this book. Gender

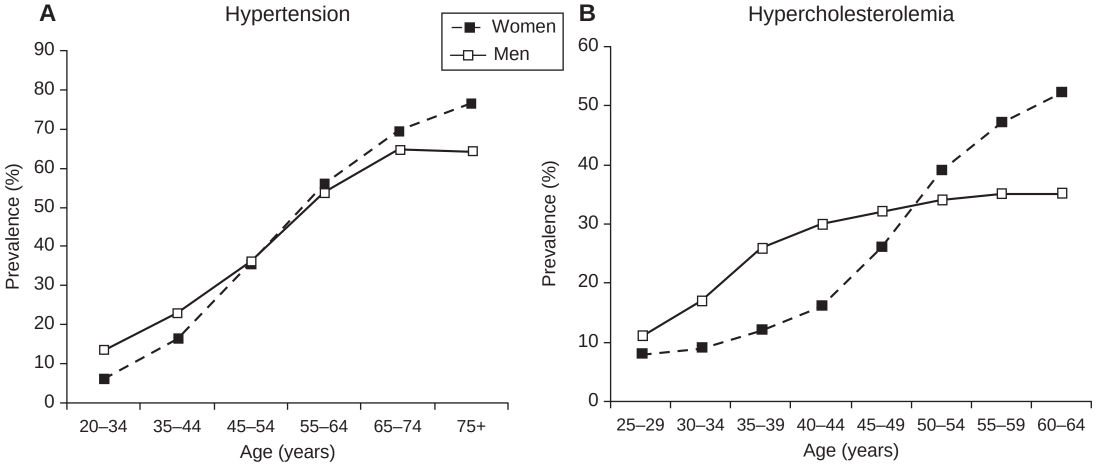


Figure 18.1 Gender differences in prevalence of cardiovascular diseases. (A) The prevalence of systemic hypertension and (B) the prevalence of hypercholesterolemia during aging in men and women are shown. Women are clearly protected as young adults but the protection is lost postmenopause, and, in later years, older women are even less protected. *Source of the data: (A) Lloyd-Jones et al. (2009) and (B) Gostynski et al. (2004).*

differences apply not only to diseases but also to physiological processes, e.g., aging. A number of reports have identified physiological differences linked to gender. For example, it is well recognized that there are significant gender differences in baseline cardiovascular function, even in healthy individuals (Hayward et al., 2000). Aging of the physiological processes is also associated with gender-specific differences in leg vascular responses during exercise. Despite reduced resting leg blood flow and vascular conductance, older men exhibit relatively preserved exercising leg hemodynamic responses, whereas older women, by contrast, exhibit attenuated vasodilator responses to exercise compared with young women (Parker et al., 2008; Sacchi et al., 1991). The effects of aging on phonatory behaviors (measured by inverse-filtered air flow, electroglottograph, and intraoral air pressure signals) also differ both qualitatively and quantitatively between men and women, because of a lower rate of age-related laryngeal degeneration in women (Higgins & Saxman, 1991). Gender differences related to the immune system have also been reported in humans (Candore et al., 2006; Clark & Peterson, 1994).

Gender differences are also well described for other pathologies, such as metabolic syndrome and osteoporosis (Pietschmann et al., 2008; Razzouk & Muntner, 2009). In addition to these differences, significant gender-specific differences have been described regarding the incidence of cardiovascular disease in patients, most importantly related to atherosclerotic vascular disease, but also including left ventricular hypertrophy, arrhythmias, and cardiac remodeling after myocardial infarction (Akishita, 2009; Hayward et al., 2000; McBride et al., 2005; Mendelsohn & Karas, 2005; Mercuro et al., 2003). Gender differences in the prevalence of cardiovascular disease vary with age but also vary according to the etiology of the disease. When considering the overall prevalence of cardiovascular disease, women show a relative protection compared to men during the first 4 decades of life. Examples are shown in Figure 18.1 for hypertension and hypercholesterolemia, from which women are protected earlier in life, lose protection at the time of menopause, and actually fare worse than men in later years (Gostynski et al., 2004; Lloyd-Jones et al., 2009). Because of this shift in cardiovascular risk between genders related to menopause and hormonal status in women, a major emphasis was put on the potentially beneficial effects of hormone replacement therapy (including conjugated equine estrogens and progestin) on the prevalence of cardiovascular disease in postmenopausal women. The Women's Health Initiative (WHI) study sponsored by the National Institutes of Health provides the most up-to-date information about this controversial therapy (Toh et al., 2010). Postmenopausal women under this form of hormone replacement have a higher risk of cardiovascular disease during the first few years that follow the initiation of the treatment, especially if such initiation occurs within the 10 years following menopause. A cardioprotective effect seems to occur only in women taking hormones at distance (more than 10 years) from the onset of menopause and for a long period of time (at least 6 years), which is unusual for the estrogen/progestin combination because such combination is usually prescribed for women with perimenopausal nefarious vasomotor effects. Similarly, a WHI parallel study using equine estrogens alone (usually prescribed to reduce the long-term consequences of menopause, such as osteoporosis) showed no protection against symptomatic coronary artery disease and a significantly increased risk of stroke and venous thromboembolism (Shuster et al., 2010).

Some prior studies also found that women are relatively protected from vascular stiffness with aging (Jonason et al., 1997) and that this protection disappears after menopause (Waddell et al., 2001). As developed in more detail in the last section of this chapter, our studies in aging monkeys (Qiu et al., 2007a; Takagi et al., 2003) also demonstrate major gender differences in vascular stiffness, in agreement with these observations in humans, and show that gender differences in the expression of genes and proteins regulating vascular function are already evident in young animals (Qiu et al., 2007b).

Mechanisms of Gender Difference in Aging

The observations summarized above lead to the question as to which mechanisms can explain the gender differences. Although this topic still remains an intense field of investigation, potential mechanisms of gender differences in longevity and aging include intrinsic differences based on gene expression, sex hormones, and reproductive physiology (Nakamura & Miyao, 2008; Verbrugge, 1982). These variables are addressed below. In addition, extrinsic factors, such as lifestyle, exercise, and nutrition, may contribute to such differences as well.

Sexual Dimorphism from X-linked/Y-linked Genes

The expression and regulation of genes linked to sex chromosomes clearly contribute to sexual dimorphism (Xu et al., 2002). To maintain a similar expression of X-linked genes between genders, gene dosage compensation mechanisms occur (Nguyen & Disteche, 2006). In females, large parts of one X allele are silenced by X inactivation, thereby reducing gene dosage to the same level as in males. However, some X-linked genes escape this process of inactivation and are therefore expressed from both X alleles in females (Carrel & Willard, 2005). Studies using gene expression profiling showed major genomic differences between genders in adipose tissue, brain, kidney, liver, skeletal muscle, and reproductive tissues in rodents (Ahluwalia et al., 2004; Rinn et al., 2004; Yang et al., 2006). However, the knowledge regarding sexual dimorphism in humans remains very limited (Rinn & Snyder, 2005). Especially in the cardiovascular system, comprehensive investigations are lacking both in rodents and in humans.

In addition, higher expression levels of X-linked genes in females can be compensated for by the expression of functionally equivalent Y-linked genes in males. The male-specific region of the human Y chromosome contains X-transposed and X-degenerated segments (Skaletsky et al., 2003). In addition to the region that encodes about 100 genes with testis-restricted expression, homologs of 29 X-linked genes have been found on the Y chromosome in the X-degenerated and the X-transposed regions. Whether protein isoforms encoded by these Y-linked genes are involved in the development of sexual dimorphism in somatic tissues is still unknown.

Interestingly, genes involved in programmed cell death, especially those with antiapoptotic functions, are reduced in abundance on the X chromosome in both aging *Drosophila* and aging mammals (Tower, 2006). The observation that antiapoptotic genes are preferentially located on autosomal chromosomes suggests some degree of sexual divergence with regard to apoptosis. Furthermore, autosomal genes encoding cytochromes of the monooxygenase family (e.g., Cyp2b10), as well as carbonic anhydrases (e.g., Car2 and Car3) and natriuretic peptides (e.g., Nppb), show gender- and/or age-specific expression levels (Isensee et al., 2008).

Another study (Tower, 2006) conducted both in *Drosophila* and in mammals, showed that the increased longevity of females compared to males may be due in part to suboptimal mitochondrial function in males. A functional link between the mitochondrial genes and the X chromosome leads to a better optimization of these genes in females compared to males (Passarino et al., 2006). Another study showed that the mechanisms by which genetic factors influence survival at an advanced age in healthy individuals are gender specific. For example, genetic variability plays a stronger role in males than in females, especially at very old age (Passarino et al., 2006).

Sex Hormones

It is generally held that gender-specific differences in the cardiovascular system are mainly due to hormones (Hayward et al., 2000). As sex steroids and growth hormone exert their biological effects via nuclear receptors and transcription factors, it is assumed that sexual dimorphism in gene expression may be observed already at a young age. Because the cardioprotection found in females predominates in premenopausal but not postmenopausal women (Hayward et al., 2000), estrogens represent the main focus of research to decipher the molecular basis of gender differences in cardiovascular disease. Of particular interest are the mechanisms by which sex steroids regulate target genes by modulating the activity of specific transcription factors (Bjornstrom & Sjoberg, 2005; Edwards, 2005).

Estrogen receptors are expressed in endothelial cells, vascular smooth muscle cells, and the myocardium in both genders (Mendelsohn & Karas, 2005). Additionally, androgen and progesterone receptors have been identified in the vasculature and myocardium of several species but have received less attention in the context of cardiovascular physiology. In animal models, estrogens modulate the response to pressure overload (van Eickels et al., 2001), myocardial ischemia and reperfusion injury (Gabel et al., 2005), vascular injury (Pare et al., 2002), and atherosclerosis (Egan et al., 2004). Moreover, estrogens play a protective role in the response of the heart to altered Ca^{2+} regulation (Xin et al., 2002). Although the direct effects of estrogens are well characterized in specific tissues (e.g., the uterus and the mammary gland), their target genes in the heart have been only partially examined, and primarily in cell culture (Mendelsohn & Karas, 2005). Some other gender differences, for example, plasma triglyceride concentration, vary in females only during pregnancy (Schwartz & Kemnitz, 1992).

Species-specific Differences During Aging

Although multiple gender differences exist in the process of aging, some of them differ among humans, nonhuman primates, and other species. Since various animal models have been used for aging studies, these species differences must be known before extrapolating the biology of aging to humans (Nadon, 2006). We provide below a few examples of these species differences in the processes of aging.

- In humans, nonhuman primates, and most species, females live longer than males. However, this major gender difference is not a universal phenomenon. For example, female Wistar rats live longer than males (Borras et al., 2003), but in C57BL6 mice, the opposite is observed (Ali et al., 2006).
- Monoamine oxidase (MAO) is expressed in two isoforms, A and B. Mice express approximately equal amounts of the two isoforms, whereas squirrel monkeys show about 10 times more B activity than A activity (Irwin et al., 1997). Mice

show a significant increase in the activity of the B isoform at middle age, but monkeys show no significant age-related variation in the pattern of expression of the two isoforms. Data from humans are conflicting because MAO can be measured only from autopsy material, which creates wide variability.

- There have also been reports of differences between rodent and nonhuman primates in terms of Parkinson disease and especially in the cellular response to toxin-induced lesions (Fitzpatrick et al., 2005).
- Species differences are also found for the expression of genes located on sex chromosomes. For example, higher expression levels of X-chromosome-linked genes were detected in female mice compared to women (Isensee et al., 2008).
- The existence of species differences is further confirmed by our previous observation of a diametrically opposite regulation of collagen expression between the monkey and the rat during aging (Qiu et al., 2007a). This important point is further developed in the last section of this chapter.

LIMITATIONS OF MODELS FOR STUDYING GENDER DIFFERENCES DURING AGING

Limitation of Aging Studies in Humans and in Rodent Models

The investigation of otherwise normal humans as they age is limited, since it is not ethical to perform studies on or take tissue samples from either younger or aged humans in the absence of a concurrent disease requiring treatment. Studies on human tissues are also complicated by the fact that aging humans are frequently afflicted with other vascular diseases, such as atherosclerosis, diabetes, and hypertension. Many individuals also receive drug therapy to treat these and other diseases, which exert additional side effects. As we detail below, in the cardiovascular system, for example, these confounding variables would complicate studies aimed at determining the mechanisms specifically leading to increased vascular stiffness as an independent risk factor.

Rodent models have provided an enormous amount of information on mammalian systems that are applicable to human biological systems (Shively & Clarkson, 2009; Williams et al., 2004). Rodent models, however, are dissimilar to primates in numerous ways, which limits the generalization to human biological systems. Therapeutic interventions in rodents and other nonprimate models may fail to show the same response as in humans. Also, substantial physiological and anatomical differences exist between rodents and primates, which limit the usefulness of rodents as a relevant model, for example, in the development of coronary artery disease (Appt et al., 2006).

A major limitation of rodent models relates to reproductive biology (Morrison et al., 2006; Wu et al., 2005). It is important to recognize that menopause is unique to species with menstrual cycles, such as women and higher primates. Although all mammals undergo decline in reproductive capacity during the aging process (Maffucci & Gore, 2006), rodents are characterized more by an "estropause" than real menopause. Indeed, rodents do not menstruate but have estrous cycles, characterized by permanently elevated estrogen concentration, low progesterone concentration, and a lack of luteinizing hormone surge and ovulation (vom Saal et al., 1994). This condition therefore markedly differs from the 28-day menstrual cycle characterizing nonhuman primates and women. In the middle-aged rodent (around 9 to 12 months), these estrous cycles become irregular, and they will eventually be totally interrupted at a later age (Felicio et al., 1984; Nelson et al., 1982). A major distinction between this rodent estropause and primate menopause is that the loss of reproductive cycles in aging rats is not accompanied by follicular atresia and a subsequent decline in estrogen production as found during menopause (Lu et al., 1979) because persistent estrus is characterized by persistently elevated estrogen concentration. Another important difference between primates and rodents is that an increase in gonadotropin-releasing hormone (GnRH) and gonadotropin production is typically found during the perimenopausal period in nonhuman primates and women (Hall & Gill, 2001; Maffucci & Gore, 2006), whereas GnRH and gonadotropin secretion decreases at estropause in rodents (Rubin & Bridges, 1989). The third difference between primates and rodents is that primates have longer gestation and lactation periods, which are similar to humans, whereas these periods are significantly shorter in rodents (Kemnitz et al, 1998).

There are two other limitations to rodent models for aging. One relates to studies on cognitive function and memory, which are difficult to perform in rodents compared with primates (Frick, 2009). In addition, many clinical studies of hormone therapy employ general tests of cognitive function (e.g., the 3MSE) (Rapp et al., 2003), for which there is no rodent equivalent. Second, differences in the types of estrogens used in many human and rodent studies may limit the applicability of the rodent to menopausal women; whereas in rodents, a form of estradiol is usually administered, in menopausal women, conjugated equine estrogens are used (Frick, 2009; Sherwin & Henry, 2008).

Advantages of a Primate Model

The limitations summarized above for rodent models are not shared by nonhuman primates. In particular, primates display physiological parameters that are close to those observed in humans (Lane, 2000; Shively & Clarkson, 2009). A variety of species have been used in aging research, including macaques (rhesus, cynomolgus, pigtails), baboons, chimpanzees, orangutans, squirrel monkeys, and lemurs. In addition to their phylogenetic proximity to humans, macaques have many other advantages for investigations related to biogerontology.

In Terms of Physiology and Metabolism

Rhesus macaque monkeys represent the most widely used species of nonhuman primates in basic and applied biomedical research (Gibbs et al., 2007) because of their genetic, physiologic, and metabolic similarities to humans. Also, their susceptibility to metabolic diseases is significantly greater than that of various New World monkeys (Tardif & Ziegler, 1992). For example, older adult macaques are characterized by a multisystem decline, which can be quantitated. Macaques can be exposed to noninvasive procedures, such as fat measurement (Marchington et al., 1989), and their decline in estimated skeletal muscle mass with age is similar to that seen in aging humans (Colman et al., 2005). Therefore, macaques have been extensively studied and thus their physiology is extremely well characterized, which paved the way to the completion of the sequencing of the rhesus macaque genome (Gibbs et al., 2007). Although most similar to humans, chimpanzees are very limited in number and require extensive requirements for housing, and invasive studies are generally not permitted.

In Terms of Gene Expression

The genetic similarity between humans and nonhuman primates makes nonhuman primates uniquely suited as models for research on complex physiological and behavioral phenotypes. The similarity of macaque to human reaches 95–99% depending on the sequences evaluated (Cline, 2007), while the rodents share 85% of human genome. From a genetic point of view, nonhuman primates have at least four advantages relative to humans (VandeBerg & Williams-Blangero, 1997). (1) Constant environmental conditions can be maintained over long periods of time, which increases the probability of detecting gene-dependent, rather than environment-dependent, effects. (2) Different environmental conditions can be imposed sequentially on individuals to characterize gene–environment interactions. (3) Various pedigrees can be generated, which are much more powerful for genetic analysis than typically available human pedigrees. For example, genetic hypotheses can be tested prospectively by selective mating. (4) Both invasive and terminal experiments can be conducted.

In Terms of Life Span

Macaques have a life span of over 30 years (Bellino & Wise, 2003), while the more commonly used rodent models have a life span of 2 to 3 years (Nadon, 2007; Nozaki et al., 1995). This long life span in primates is viewed as an additional advantage for use as a model for various aging studies, including cardiovascular and reproductive aging, the role of estrogens and other sex steroid hormones in these processes (Bellino & Wise, 2003), or the neuroendocrinology of reproductive aging (Downs & Urbanski, 2006). Conceivably, a chimpanzee model of aging would be ideal, since they live twice as long as macaques. However, like humans, the chimpanzee model is essentially not available for research involving interventional techniques.

In Terms of Reproductive Physiology and Endocrinology

The physiology of female macaques is very similar to that of women in terms of reproductive adaptation to aging, including hormone profiles during the menopausal transition and extent of age-related and menopause-associated effects of changes in hormone levels on metabolism, bone loss, and impaired cardiovascular function (Gilardi et al., 1997; Qiu et al., 2007a; Rodgers et al., 1993; Walker, 1995). Menopause in nonhuman primate species, including cynomolgus monkey, rhesus macaque (Gilardi et al., 1997), and baboon (Chen et al., 1998), tends to be similar in the age of onset, at approximately 20–25 years of age (Bellino & Wise, 2003). However, most nonhuman primates cease reproduction shortly before death (Bellino & Wise, 2003), whereas women remain postmenopausal for about a third of their life span. This may explain why menopause-associated chronic diseases, including coronary heart disease, osteoporosis, and diabetes mellitus, are more predominant in women than in nonhuman primates.

The similarities between humans and macaques also include the changes in hormonal profile during the menopausal transition, the progression to cycle termination through irregular cycles, and the protection conferred by estrogen replacement therapy following oophorectomy (Cline & Wood, 2005; Gilardi et al., 1997; Kavanagh et al., 2005; Shideler et al., 2001). The use of rodents for these purposes is limited by the period of persistent estrus, which was defined above (Felicio et al., 1984; Wu et al., 2005). Also, reproductive aging in rodents involves all parts of the hypothalamic–pituitary–ovarian axis, and the ovary is not necessarily the primary mediator (vom Saal & Finch, 1988).

Because of these limitations, it would be ideal to study the female monkey going through menopause naturally. However, the availability of these valuable animals is limited. Although ovariectomy induces an increase in follicle-stimulating hormone comparable to that in postmenopausal monkeys, major differences exist between the ovariectomized monkey and the naturally menopausal monkey, which include a different body weight, lower estradiol, and relatively higher androstenedione and estrone concentrations. Dehydroepiandrosterone sulfate concentration is known to decrease with age in primates and humans (Carlstrom et al., 1988; Kemnitz et al., 2000), such that young ovariectomized animals will produce more dehydroepiandrosterone sulfate from their adrenal glands for a significant period of time following ovariectomy (Kavanagh et al., 2005).

STUDIES OF AGING IN A PRIMATE MODEL

We review in this section the different areas of research in which a nonhuman primate model has been used successfully to determine the effects of aging. Nonhuman primate models have been employed as models for human aging in areas including neurobiology and reproductive aging, and age-related diseases such as cardiovascular disease and diabetes, and they are used for interventional studies such as caloric restriction (Colman & Kemnitz, 1998).

Neurobiology

The neurobiology of aging is one of the most studied areas of primate gerontology. Nonhuman primates have been used to study cognitive changes associated with aging and age-related diseases. The advantages of using such a model include the capacity to examine visual and nonspatial cognitive processes, as well as the ability to conduct tasks similar to those performed by humans. Old rhesus monkeys exhibit declines in cognitive function similar to those reported in humans (Peters et al., 1996). In addition, many of the same structural and biochemical changes that may underlie altered cognitive function in older humans have been observed in aged monkeys (Peters et al., 1996; Voytko, 1997; Voytko & Tinkler, 2004). Alterations in memory are the most notable presentations of cognitive decline in humans and have been extensively investigated in monkeys as well. Impaired spatial memory has been reported in older monkeys in several studies (Bachevalier et al., 1991; Bartus et al., 1978; Gulyas & Szathmary, 2002; Herndon et al., 1997; Rapp & Amaral, 1989). Also in agreement with humans, aged monkeys exhibit age-related impairments in visual recognition tasks (Albert, 1992; Oscar-Berman & Bonner, 1985). As reported in humans, older monkeys also have increased difficulty in learning new paradigms (Moss et al., 1988; Presty et al., 1987; Rapp & Amaral, 1989).

An emerging field in human aging is the exploration of the association between cognitive decline or neurodegenerative processes and the decline of estrogen levels in older females. Although human and rodent models have been more extensively studied (Sherwin, 1998; Tang, 1996; Yaffe et al., 1998), a study in rhesus monkeys provided additional evidence linking estrogen to cognitive impairment. Monkeys entering the menopause transition or those who were postmenopausal performed significantly less well than premenopausal or young control female monkeys with normal estrogen status (Lacreuse, 2006). Other studies related to the neurobiology of aging have reported age-associated neuronal atrophy, decline in nigral neuronal function, and structural changes in the cerebral cortex. Another study (Voytko, 1998) showed that changes in cognitive profiles and progression of Alzheimer disease in nonhuman primate models of aging parallel those found in humans.

Reproductive Senescence

We described above the characteristics of age-related changes in reproductive function, including hormone concentrations, menstrual cycling, and fertility, in the rhesus monkey and their similarities with their human counterparts. These hormonal changes support the use of monkeys as a model of human reproductive aging, and they are accompanied by other physiological characteristics typically associated with menopause in humans. For example, natural menopause in rhesus monkeys is followed by decreased bone mass and higher bone turnover without alteration of the calcium–vitamin D axis, which reproduces the same observations made in humans (Colman et al., 1999a). However, it is important to note that only a very limited number of studies have been done in postmenopausal monkeys. One of the reasons, explained in the previous section, is the short life span of the postmenopausal monkey. Similarly, the long-term consequences of ovariectomy in older female monkeys mimic estrogen depletion and postmenopausal bone loss occurring in women (Smith et al., 2009). Although the monkeys lose bone mass after ovariectomy, the extent of such loss cannot be described as osteoporotic.

Cardiovascular Disease and Diabetes

Primate models have been used extensively in studies of cardiovascular disease (Cefalu, 1997; Clarkson, 1988; Clarkson et al., 1987, 1995). Most research on cardiovascular disease in nonhuman primates has focused on the effects of various dietary interventions on the development of disease markers or risk factors. A limited number of studies have examined cardiovascular risk factors in older animals. It has been reported that serum triglyceride levels increased with age in both male (Verdery et al., 1997) and female (Lane et al., 1999a,b) rhesus monkeys. Adiposity is also increased in older animals (Kemnitz et al., 1993; Lane et al., 1999a,b). Our group also performed several studies addressing cardiovascular aging in the monkey. For example, we showed that aging increases the activity of matrix metalloprotease-2 and of angiotensin II in aorta from nonhuman primates (Wang et al., 2003). We also described alterations in endothelial function and vasoreactivity in this model (Asai et al., 2000, 2001; Sato et al., 1995).

Diabetes may occur spontaneously in many species of nonhuman primates (Hansen & Bodkin, 1986; Howard, 1982, 1983, 1984; Howard & Yasuda, 1990; Howard et al., 1986), reproducing the type 2 (non-insulin-dependent) diabetes mellitus in humans, characterized by fasting hyperglycemia and glucose intolerance. Many of the clinical findings associated with the disease can be reduced by weight loss (Hansen & Bodkin, 1993) and by caloric restriction *(*Bruns & Kemnitz, 2004; Hudson et al., 1996; Kaplan, 2004; Kemnitz et al., 1993; Lane et al., 1999a,b), as described further below.

Caloric Restriction

Caloric (or dietary) restriction (CR) has been extensively studied because it reproducibly limits the consequences of aging, in terms of life span and age-related progression of disease. Once initiated in rodents (Weindruch, 1988; Yu, 1994), this field of investigation more recently extended to nonhuman primates. Two major studies in rhesus monkeys, one at the National Institute of Aging and the other at the University of Wisconsin, spanned a period of up to 20 years and showed effects similar to those seen in rodents in terms of reduction of oxidative stress and metabolic improvement (better maintenance of insulin sensitivity with aging and lower total cholesterol and triglyceride levels, for example) (Rezzi et al., 2009). There was also a significant reduction in the incidence of aging-related deaths and pathologies (such as diabetes, cardiovascular disease, cancer, and brain atrophy), as well as a better preservation of brain function, such as locomotor performance (Colman et al., 2009; Ingram et al., 2007; Wanagat et al., 1999). Although CR increases life span in rodents (Roth et al., 2001), conflicting results remain regarding the monkey (Mattison, 2005), especially because the precise consequences of CR on longevity in a monkey model will require a much longer time of investigation, as rhesus monkeys can live up to 40 years.

Other Primate Studies

Nonhuman primates have been used for various additional investigations, including visual system anatomy and function (Bito et al., 1982; Croft et al., 1998; Koretz et al., 1987) and macular degeneration (Dawson et al., 1989; Engel et al., 1988), endometriosis (Coe et al., 1998; Hadfield et al., 1997), osteoarthritis (Carlson et al.,1996; Colman et al., 1999a,b; Grynpas et al., 1993), mitochondrial abnormalities (Lee et al., 1998; Schwarze et al., 1995), and biomarkers of aging (Lane et al., 1997; Nakamura et al., 1994, 1998; Short et al., 1997, 1987). Several studies have focused on the immunological system (Attanasio et al., 2001; Ebersole et al., 2008; Stacy et al., 2008) or on endocrine dysfunction (Goncharova & Lapin, 2002, 2004; Lacreuse et al., 2007). Several species of nonhuman primates exhibit changes in bone mineral content and architecture similar to those reported in humans. Both male and female monkeys show a significant decline in bone mass during aging (Colman et al., 1999b). Monkeys also develop symptoms similar to human osteoarthritis.

GENDER DIFFERENCES IN CARDIOVASCULAR AGING IN PRIMATES

Some of the features of cardiovascular aging related to atherosclerosis and diabetes have been reviewed above. However, there are two pathophysiological hallmarks of aging that are observed in most species, most prominently in humans. These involve (1) changes in the structure and function of the heart, resulting in the cardiomyopathy of aging, and (2) increased vascular stiffness. One question persists from studies in older patients, i.e., whether these changes are independent of or associated with other disease states of aging, e.g., atherosclerosis or diabetes. To address this, the primate model is ideal since it does not develop atherosclerosis on a normal, vegetarian diet, and diabetes occurs in only a small fraction of aging macaques. In our studies described below, we compared young (around 3–7 years) and old (around 19–25 years) cynomolgus monkeys (*Macaca fascicularis*) of both genders, and all groups showed normal levels of blood lipids and sugar.

Cardiomyopathy of Aging

This is characterized by myocyte hypertrophy, myocyte apoptosis, and cardiac dysfunction. All of these features are shared between species, from old mice (Vatner et al., 2009) and rats (Roberts & Goldberg, 1976; Sebban & Yazdani, 1992) to humans (Lakatta, 1994, 1998, 2002a; Lakatta & Levy, 2003b). In our monkey population, there was also a significant increase in left ventricle (LV) myocyte hypertrophy and apoptosis in aging males (Zhang et al., 2007; Figure 18.2), but LV dysfunction was not yet present. It is likely that decreased contractility is a later development in the monkey model of aging. Interestingly, LV/body weight did not increase in the old male monkeys, which is opposite to that observed in rodents (Lakatta, 2002b, 2003; Lakatta & Levy, 2003a,b; Zhang et al., 2007), despite a significant increase in LV myocyte cross-sectional area. There are two reasons for such discrepancy. First, body weight increased similarly in old monkeys independent of gender (Qiu et al., 2007b), and this increase was mainly due to body fat. Thus, the increased body weight automatically resulted in reduced ratio of LV/body weight. Therefore, it is likely that the LV/body weight ratio in aging monkeys is not as accurate as the same measurement in rodents, with much shorter life span. An alternative measurement commonly performed in rodents, LV/tibial length ratio, is much more accurate but it is practically difficult to measure in monkeys. Second, as noted above, there was a significant increase in myocyte apoptosis with aging, which reduces the number of cardiac myocytes and thereby offsets the relative increases in mass due to hypertrophy of the myocytes. Another interesting feature of the LV hypertrophy of aging in monkey is that the increase in myocyte cross-sectional area is not uniform, but occurs only in a fraction of the myocytes, causing a shift in the Gaussian distribution of myocyte cross-sectional area normally observed in hearts from young monkeys (Figure 18.2). This observation may be due to cell-to-cell differences in gene expression, although this attractive possibility remains to be tested experimentally. Also, hearts from aging monkeys did not show signs of increased cell proliferation, suggesting that myocytes themselves, rather than proliferative cells, such as stem cells and progenitor cells, are responsible for most of the histological changes.

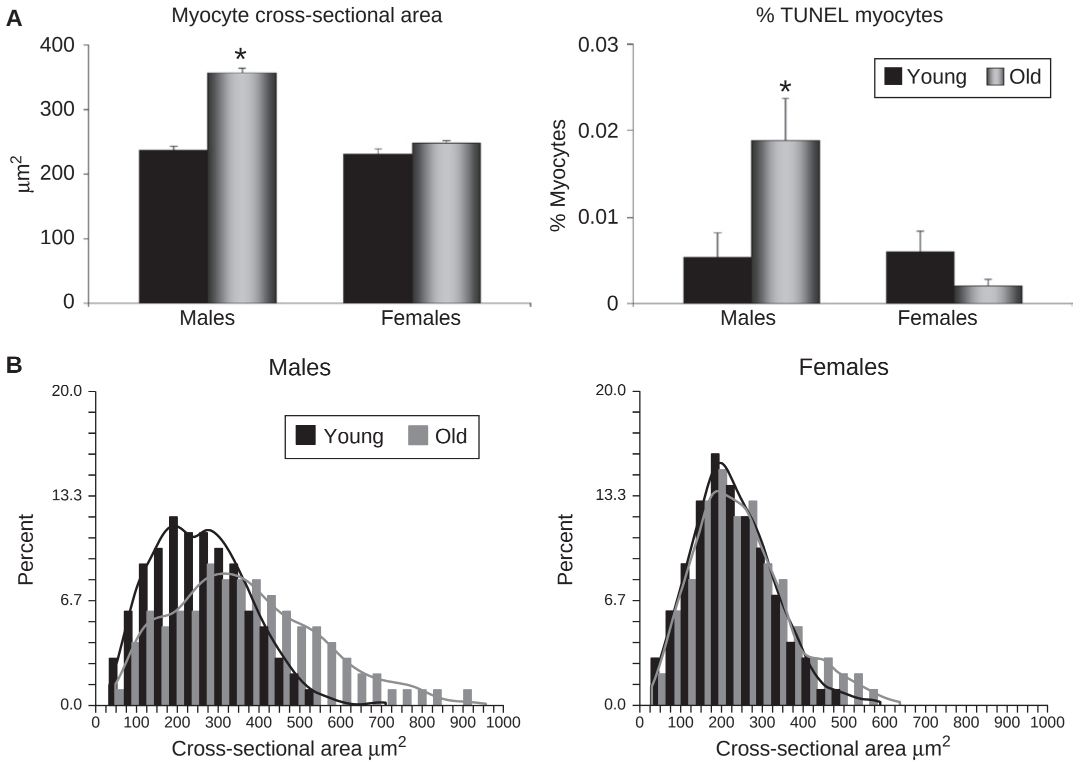


Figure 18.2 Gender differences in cardiomyopathy of aging in primates. Two components of the cardiomyopathy of aging include (A, left, and B) myocyte hypertrophy, as reported by myocyte cross-sectional area, and (A, right) apoptosis of myocytes. The older monkeys were 19–25 years of age, and the older females were of such age, but were perimenopausal or had just started menopause. (A) The myocyte cross-sectional area and the percentage of apoptotic cardiac myocytes increase in older male monkey hearts more than in older female hearts. *$P < 0.05$ versus corresponding young animals. (B) The sex difference in the distribution of cardiac cell size (as measured by cross-sectional area) between aging male and aging female monkeys. Note that the distribution of myocyte cross-sectional area was Gaussian in young and old female monkeys, but in old male monkeys, the distribution was shifted to the right, reflecting the increased percentage of hypertrophied myocytes *(reprinted from Zhang et al., 2007, by permission of the publisher).*

β-Adrenergic Desensitization

Sympathetic regulation is an important component mediating LV function. Although LV function remains normal in the old monkeys, before the onset of aging cardiomyopathy, the response to sympathomimetic amines, e.g., norepinephrine and isoproterenol, is depressed significantly (Takagi et al., 2003). This phenomenon precedes the functional state of aging cardiomyopathy and most likely is involved in its pathogenesis. Isoproterenol normally increases myocardial inotropy, through both β_1- and β_2-adrenergic receptor stimulation, and also induces marked systemic vasodilation, primarily by stimulating β_2-adrenergic receptors (Takagi et al., 2003). A unique feature of our studies in aging monkeys is that measurements of LV function were made in chronically instrumented conscious animals with continuous measurements of LV pressure and dimensions (Figure 18.3). Calculation of total peripheral resistance was made from measurements of cardiac output and aortic pressures, assessed by implanted aortic flow probes and implanted aortic pressure gauges or catheters (Figure 18.3). The increases in both myocardial contractility and peripheral vasodilation induced by isoproterenol were attenuated in old male monkeys (Figure 18.4). LV inotropic response to norepinephrine was also diminished in old male monkeys (Takagi et al., 2003). This is due in part to β-adrenergic receptor desensitization, which has also been observed in aging rodent models (Borton & Docherty, 1989; Liggett et al., 2000). However, it is also probably due in part to reduced inotropic reserve in old monkeys. Interestingly, the response to forskolin, which stimulates adenylyl cyclase distal to the β-adrenergic receptor, was also markedly diminished in old male monkeys. As with isoproterenol, both the increase in LV contractility and the decrease in total peripheral resistance induced by forskolin were diminished in old male monkeys. These data imply that the mechanism of β-adrenergic desensitization of aging is not solely at the level of the β-receptor, but may be also at the level of adenylyl cyclase.

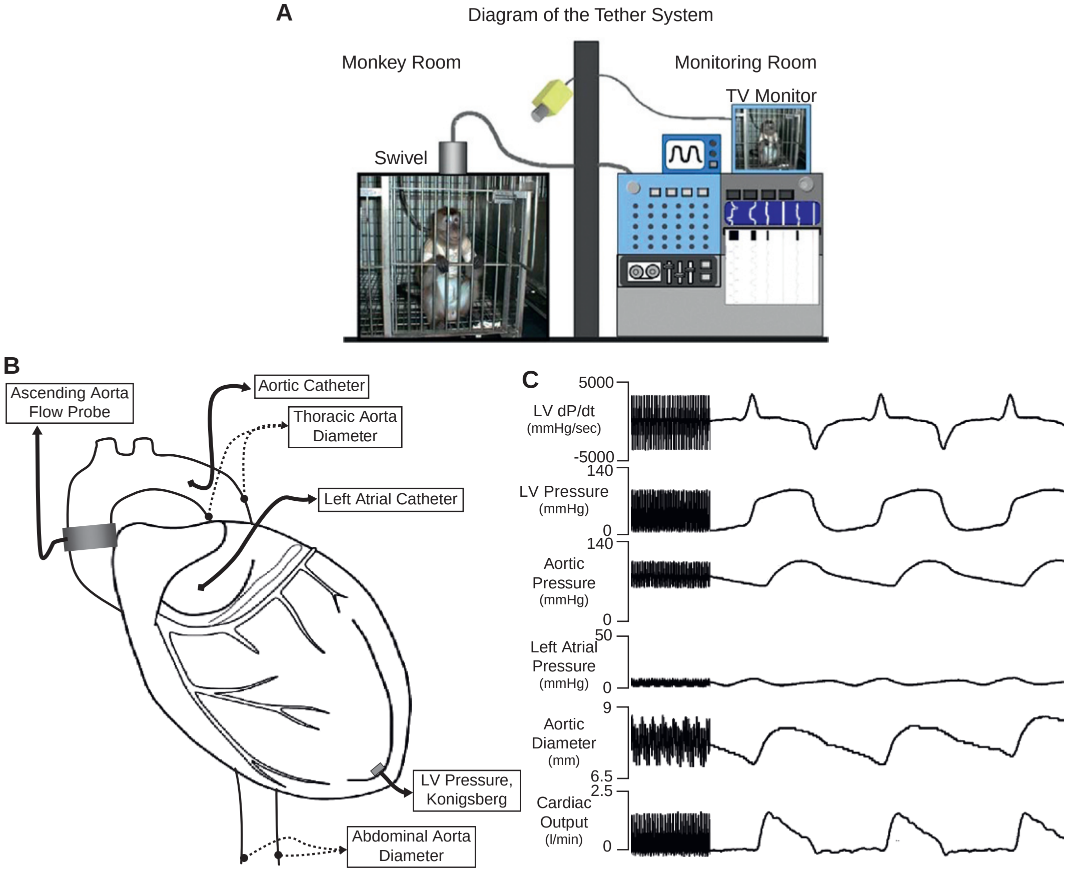

A
Diagram of the Tether System
Monkey Room
Monitoring Room
TV Monitor
Swivel
B
Ascending Aorta Flow Probe
Aortic Catheter
Thoracic Aorta Diameter
Left Atrial Catheter
LV Pressure, Konigsberg
Abdominal Aorta Diameter
C
5000
LV dP/dt (mmHg/sec)
-5000
140
LV Pressure (mmHg)
0
140
Aortic Pressure (mmHg)
0
50
Left Atrial Pressure (mmHg)
0
9
Aortic Diameter (mm)
6.5
2.5
Cardiac Output (l/min)
0

Figure 18.3 Physiological measurements of cardiovascular function in the conscious monkey. This figure illustrates how the physiological measurements were recorded in the conscious monkeys, i.e., (A) the tether system, (B) the instrumentation of the monkey heart, and (C) the real-time measurement of contractility, pressures, aortic diameter, and cardiac output in the conscious monkey.

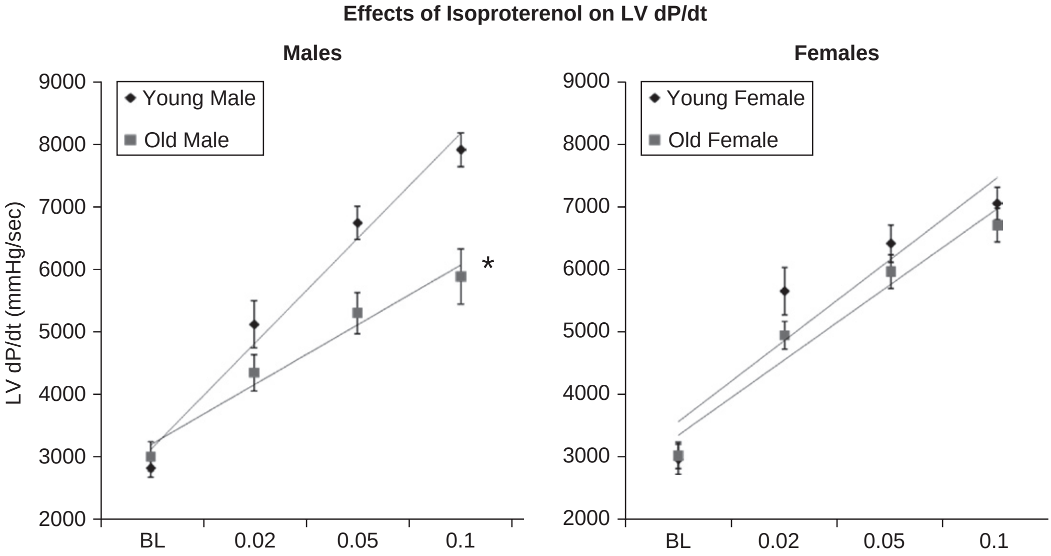


Figure 18.4 Gender differences in the cardiovascular response to sympathetic stimulation with aging. Differences in increases in left ventricular +*dP/dt,* i.e., LV contractility, between young and old male and female monkeys, upon stimulation with increasing doses of the β-adrenergic agonist isoproterenol, are shown. β-Adrenergic receptor stimulation increases cardiac contractility (increased *dP/dt*), which is desensitized in older male monkeys, and less so in older female monkeys, indicating protection against β-adrenergic receptor desensitization in old females *(reprinted from Takagi et al., 2003, by permission of the publisher).*

Increased Vascular Stiffness with Aging

Vascular changes with aging are even more prominent than cardiac changes. As patients progress from middle to older age, an increase in systolic arterial pressure is observed. This is due to increased stiffness of the arterial tree. Increases in vascular stiffness have been documented in older humans. In our monkey model of aging, we utilized direct and continuous measurements of aortic dimensions and pressures to assess vascular stiffness. Aortic dimensions were measured with ultrasonic dimension crystals implanted on opposing surfaces of the thoracic aorta, along with implanted miniature pressure gauge aortic catheters (Figure 18.3). Aortic stiffness was assessed using the direct measurements of aortic pressure and diameter, during which aortic pressure was decreased by either hemorrhage or nitroprusside and increased by either volume overload or phenylephrine; and stiffness was calculated as the relationship between aortic pressure and dimensions. When aortic diameter is plotted as a function of aortic pressure over a wide range of pressures, the resulting slope of the pressure–dimension relationship is a direct index of vascular stiffness, i.e., in younger animals with a more compliant aorta, diameter changes directly with pressure, whereas in old monkeys with a stiffer aorta, the increase in arterial pressure is accompanied by a smaller increase in aortic diameter (Figure 18.5B). Vascular stiffness, whether assessed by pressure–diameter relationship (Figure 18.5C) or by calculation of β or other stiffness parameters (Figure 18.5A), increased significantly in old male monkeys (Qiu et al., 2007a).

The mechanisms mediating this increased stiffness with aging are complex. The most obvious are the structural changes that occur in the arteries with aging (Qiu et al., 2007a). Although there is no evidence of atherosclerosis in the old monkeys, either by blood lipids or by intimal deterioration, there was an impairment of endothelial function and consequently reduced vasodilation in response to endothelial vasodilators, e.g., acetylcholine (Asai et al., 2000). The reduced ability of the endothelium to induce vasorelaxation may contribute to the increased vascular stiffness of aging. Another cause relates to changes in the vascular extracellular matrix, i.e., collagen and elastin. Figure 18.6 shows an example of the increase in collagen and decrease in elastin in the aging male monkey aorta. Interestingly, although total collagen increases, this is due primarily to the increased medial cross-sectional area, and collagen density in itself does not increase significantly (Qiu et al., 2007a). There are also changes in collagen synthesis, which contrasts with the increased stiffness. In particular, the abundance of collagen types 1 and 3 decreases with aging in monkeys, which is opposite to the findings in rodents (Qiu et al., 2007a). In our view, the most important

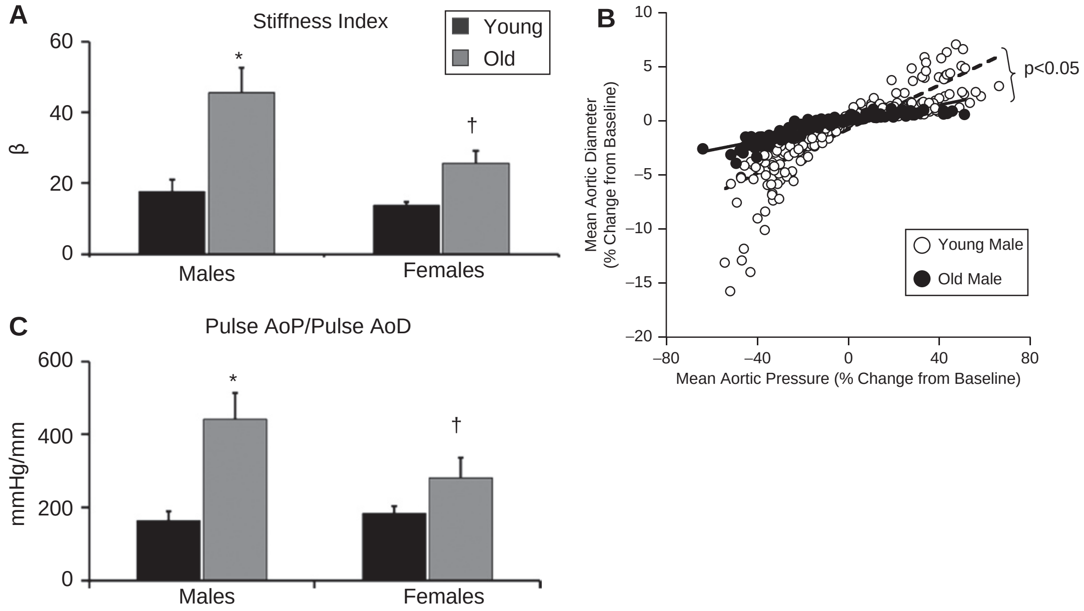


Figure 18.5 Gender differences in aortic stiffness with aging. (A) The stiffness index (β), (B) the aortic pressure–dimension relationship over a wide range of pressures in an old vs young male monkey, and (C) the ratios of pulse aortic pressure (AoP) and pulse aortic diameter (AoD) are compared. $*P < 0.05$ vs corresponding young animals; $\dagger P < 0.05$ vs corresponding old male monkeys. All indices of vascular stiffness were increased more in old male monkeys than in old female monkeys *(reprinted from Qiu et al., 2007a,b, by permission of the publisher).*

architectural change responsible for increased stiffness is the decrease in elastin density (Figure 18.6) and the resulting increase in collagen/elastin ratio. This observation, together with the observed destruction of elastin architecture in the vessel wall (Figure 18.6), represents a primary mechanism underlying vascular stiffening. As shown in Figure 18.6, the decreases in elastin density are not observed in old female monkeys, whereas increases in vascular stiffness with aging in females are markedly diminished compared to age-matched male monkeys.

Additional Mechanisms Mediating Increased Stiffness with Aging

It is our hypothesis that increases in stiffness not only are due to changes in the extracellular matrix, but also involve structural changes with increased stiffness in vascular smooth muscle cells. This is based on a study from our laboratory using atomic force microscopy to assess the mechanical properties of aging LV myocytes from rats (Lieber et al., 2004). In that study there was a significant increase in stiffness of LV myocytes, which contributes to the increased LV stiffness in the heart muscle. More recently we have preliminary data in our monkey model of aging showing parallel findings in vascular smooth muscle cells.

The cellular/molecular mechanisms mediating these changes in the cells and not in the extracellular matrix need to be elucidated. Insight into these molecular mechanisms may be obtained from DNA microarray studies. Indeed, microarrays from aging male monkey aortas (Figure 18.7) demonstrate that more than 400 genes are differentially regulated compared to aortic samples from young male and female monkeys (Qiu et al., 2007b). The reasons for this gender difference are not entirely clear; however, the fact that this difference is already observed in young animals argues for genetic (for example, sex chromosomes) rather than epigenetic or environmental causes. In that study, whole aortic homogenates minus the endothelium were used for microarrays (Qiu et al., 2007b). It will be important to isolate vascular smooth muscle cells to perform cell-specific microarrays to avoid complications introduced by other cell types from the aortic wall.

Summary of Gender Differences in the Primate Model of Aging

Cardiomyopathy of Aging

In older female monkeys, not only was LV hypertrophy not observed, in contrast to males, but also the normal Gaussian distribution of myocyte

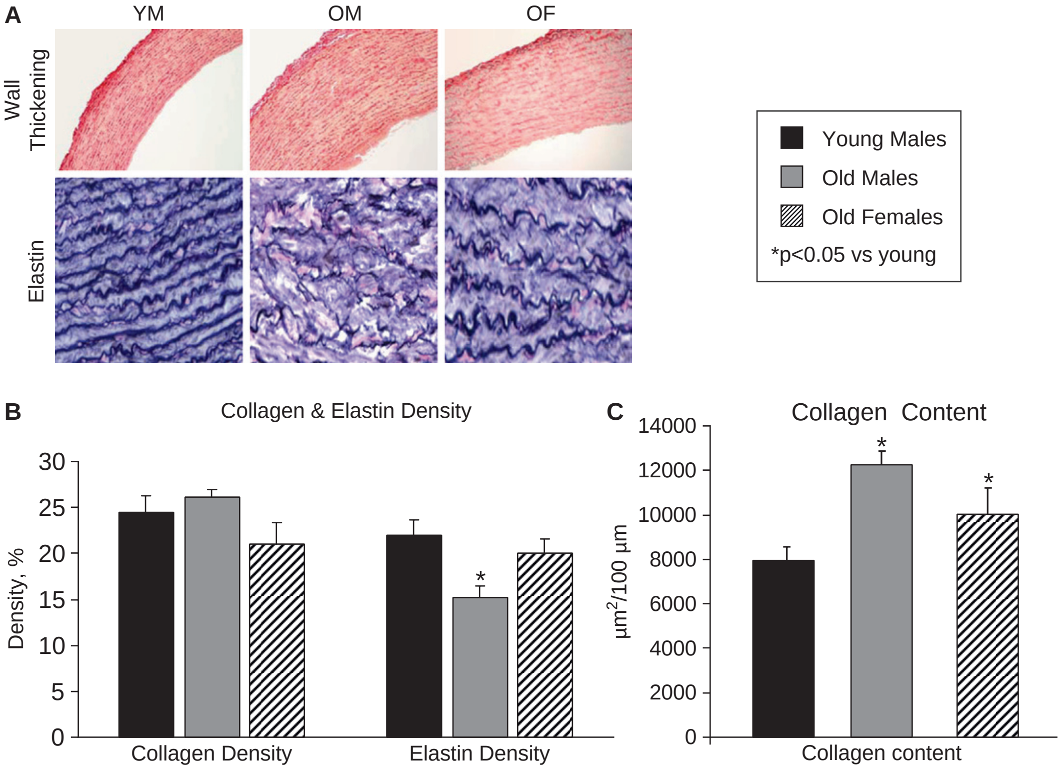


Figure 18.6 Gender differences in aortic structure with aging. (A) The relative change in aortic wall thickness in young male (YM), old male (OM), and old female (OF) monkeys is shown. Note that aortic thickness was increased in OM and less so in OF. The elastin is shown below. The decrease in elastin density was observed only in OM and there was also greater disruption of elastin architecture in OM than OF. (B) Mean ± SEM values for collagen and elastin density in young male, old male, and old female monkeys. Elastin density decreased only in older male monkeys, while collagen density did not increase. (C) Total collagen increased, however, because of increased vascular thickness with aging; $*P < 0.05$ *(adapted from Qiu et al., 2007a, by permission of the publisher).*

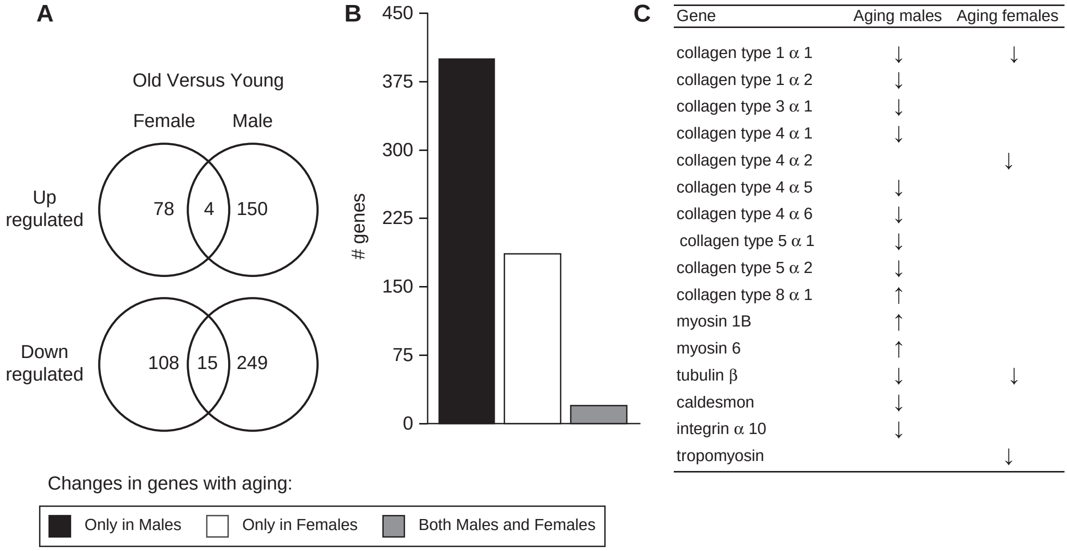
cross-sectional area was preserved, as opposed to old male monkeys, for which it was shifted to the right compared to the distribution in young monkeys (Zhang et al., 2007). A shift in myocyte distribution according to their size is known to occur in myocardial hypertrophy, in which not all myocytes experience hypertrophy to the same extent. Apoptosis in myocytes from aging female hearts was also reduced compared to males. This protection in female monkeys can be due to the fact that either menopause had not occurred or was too recent to allow for the protective effects of hormones to dissipate. Of interest, mitochondrial oxidative function in LV muscle was also preserved with aging in females compared to males (Yan et al., 2004). Most likely, these changes may reflect the perimenopausal changes observed in women. Even in the older male monkeys, aging cardiomyopathy had been initiated, but did not progress to severe impairment of cardiac function. This suggests that the more severe cardiomyopathy in patients occurs in the very elderly, or is attributable in part to underlying nascent atherosclerosis, hypertension, or diabetes.

β-Adrenergic Desensitization

The reduction in inotropic response observed with isoproterenol in old male monkeys was markedly attenuated in old female monkeys (Figure 18.4). Preserved inotropic and vascular responses to forskolin were also observed in aging females. The adrenergic desensitization may contribute to aging cardiomyopathy, since it limits inotropic and chronotropic responses to sympathetic stimulation, e.g., with exercise.

Vascular Stiffness

Vascular stiffness showed some increase in old female monkeys compared to young animals, but it was far less pronounced than in age-matched male monkeys (Figure 18.5) (Qiu et al., 2007a). As noted above,



Gene	Aging males	Aging females
collagen type 1 α 1	↓	↓
collagen type 1 α 2	↓	
collagen type 3 α 1	↓	
collagen type 4 α 1	↓	
collagen type 4 α 2		↓
collagen type 4 α 5	↓	
collagen type 4 α 6	↓	
collagen type 5 α 1	↓	
collagen type 5 α 2	↓	
collagen type 8 α 1	↑	
myosin 1B	↑	
myosin 6	↑	
tubulin β	↓	↓
caldesmon	↓	
integrin α 10	↓	
tropomyosin		↓

Figure 18.7 Gender differences in aortic gene expression with aging. (A and B) Total numbers of genes differentially regulated (either up or down) between young and old females and males, respectively. Note that approximately 400 genes were changed in old males, only 19 of which were regulated similarly in old females. (C) Examples of genes involved in cell–matrix interactions and vascular smooth muscle cell phenotype showing gender-specific regulation with aging *(adapted from Qiu et al., 2007b, by permission of the publisher).*

elastin density was not diminished in old females (Figure 18.6). Female monkeys also showed differences in the types of collagen expressed in the vasculature. In particular, collagen type 8, which promotes the migration of vascular smooth muscle cells in the neointima, was expressed significantly more in old males than in old females (Qiu et al., 2007a).

Molecular Mechanisms for Future Research

Microarray data also provide insight into the mechanisms of preservation of the vascular function of aging females (Qiu et al., 2007b). Of the more than 400 genes that change in aging male monkey aortas, only 19 of these genes also changed in a similar direction in aging females (Figure 18.7). Multiple gene categories were differentially regulated between males and females, including genes participating in extracellular matrix composition, phenotype of vascular smooth muscle cells, metabolism, protein synthesis, and resistance to apoptosis, among others. In addition, specific categories of genes showed opposite regulation between males and females. Thus, the preservation of vascular compliance with aging in older female monkeys is more complicated than simply preserved elastin synthesis and structure (Figure 18.7). Importantly, the analysis of the microarrays also suggested that gender differences in vascular gene expression are already imprinted at an early age and therefore precede the gender differences in vascular stiffening. For example, genes participating in vascular structure, receptor signaling, cell adhesion, and transcriptional control showed a significantly different regulation between young male and female monkeys (Qiu et al., 2007b). These studies point out that, although much has been elucidated about gender differences in aging, additional mechanisms need to be unraveled.

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Chapter | 19 |

Cerebral Vascular Dysfunction with Aging

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CHAPTER CONTENTS

Introduction 405
Vascular Remodeling 405
Blood–brain Barrier 406
Cerebral Blood Flow 407
Endothelium-dependent Responses 408
Oxidative Stress 410
Peroxisome Proliferator-activated Receptor γ 412
Vascular Inflammation 412
Renin–angiotensin System 413
Conclusions 414
Acknowledgments 414
References 414

INTRODUCTION

Aging is associated with a variety of changes in the cardiovascular system, including alterations within the vascular tree (Faraci, 1993; Folkow & Svanborg, 1993; Marin, 1995). Such changes have been observed in various vascular beds, including coronary, cerebral, and mesenteric arteries, as well as blood vessels that supply skeletal muscle (Muller-Delp, 2006; Hatake et al., 1990; Rodriguez-Manas et al., 2009; Brandes et al., 2005; Zeiher et al, 1993). Although aging affects blood vessels in many vascular beds, one region where aging has particularly profound effects is the cerebral circulation, in which aging is the major risk factor for vascular disease and stroke and is the leading cause of cognitive decline (Faraci, 1993; Farkas & Luiten, 2001; Iadecola, 2009; Park et al., 2007; Rothwell et al., 2005). Vascular dysfunction is a term commonly used to describe abnormalities in vascular structure and function that collectively increase vascular tone and permeability, promote thrombosis, and alter vascular growth. The impact of vascular dysfunction is profound in the brain (Chrissobolis & Faraci, 2008; Faraci, 1993; Farkas & Luiten, 2001; Iadecola, 2009; Modrick et al., 2009; Park et al., 2008). In addition to the traditional view that vascular disease promotes thrombosis and increases in vascular tone, vascular disease in the brain can have additional consequences, including detrimental effects on delivery of metabolic substrates, vascular permeability and brain fluid balance, neural function, and recovery following stroke and other forms of brain injury (Faraci, 1993; Chrissobolis & Faraci, 2008; Cipolla, 2010; Farkas & Luiten, 2001; Iadecola, 2009; Zlokovic, 2008) (Figure 19.1).

This chapter summarizes select changes in blood vessels during aging. Because the impact of these vascular changes and the subsequent end-organ damage is great in the brain, the discussion focuses much of its attention on alterations in the cerebral circulation. Despite its prominence as a cardiovascular risk factor, the impact of aging is one of the least studied areas in vascular biology. By far, most studies in people and experimental models of vascular disease and stroke are not performed in aged subjects.

VASCULAR REMODELING

Vascular structure can change during aging. For example, changes in the microcirculation and capillary bed occur with age, resulting in impaired delivery of glucose and other metabolic substrates. These changes include reductions in capillary density, thickening

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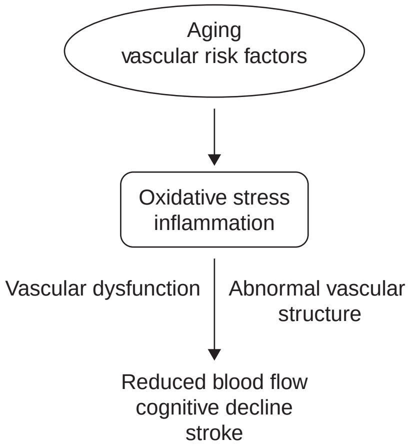


Figure 19.1 Schematic illustration of how aging, either alone or in combination with risk factors for vascular disease, may produce reductions in cerebral blood flow, stroke, and cognitive decline. The two major mechanisms that appear to underlie progression of vascular disease, including impairment of vascular function and abnormal vascular growth, are oxidative stress and inflammation.

of the basement membrane, loss of mitochondria in endothelium, and perivascular accumulation of collagen (Farkas & Luiten, 2001). With normal aging in humans, magnetic resonance angiography-based measurements suggest a loss of blood vessels overall and outward remodeling of large cerebral arteries in older individuals (Bullitt et al., 2009). Remodeling of blood vessels (inward or outward remodeling) involves structural alterations and changes in the vascular lumen that cannot be accounted for by differences in vascular tone or mechanical characteristics of the vessel wall (Faraci, 2008).

In both humans and experimental animals, blood vessels become stiffer or less distensible with age (Dupuis et al., 2004; Hajdu et al., 1990; Nagasawa et al., 1979). In the cerebral circulation, increased stiffness of the vasculature is associated with decreases in the relative levels of distensible elements of the vessel wall, including smooth muscle and elastin (Hajdu et al., 1990). Expression of collagen, a nondistensible component of the vessel wall, increases with age in cerebral arteries (Nagasawa et al., 1979; Gudiene et al., 2007). Decreased distensibility of cerebral blood vessels may contribute to the impaired vascular responses that have been described with aging (discussed below). In the human basilar artery, for example, responses to several vasoconstrictors are impaired with aging, a change that could reflect nonspecific effects of alterations in vascular mechanics on vascular tone. Structural changes in blood vessels have functional consequences because vascular remodeling and changes in vascular mechanics affect vasodilator capacity and minimal vascular resistance. As a result, changes in the composition and mechanical properties of the vessel wall may influence vascular responses and thus the regulation of cerebral blood flow. Measurements of the cross-sectional area of the vessel wall in rats indicate that smaller cerebral arterioles atrophy (become thinner) with aging (Dupuis et al., 2004; Hajdu et al., 1990). Thinning of the wall in cerebral microvessels may contribute to aneurysm formation or microvascular hemorrhage, particularly during hypertension, which is relatively common in older individuals.

BLOOD–BRAIN BARRIER

The blood–brain barrier (BBB) is formed by the continuous layer of cerebral endothelial cells anchored to each other through an array of tight-junction proteins that limit the entry of cells and many blood-borne substances into the brain (Abbott et al, 2010; Cipolla, 2010; Zlokovic, 2008). The BBB is often discussed in relation to cerebral capillaries but is also present and functional in cerebral arteries and arterioles as well as cerebral venules (Butt et al., 1990; Cipolla, 2010; Mayhan & Heistad, 1985). The BBB is a unique structure with very specific structural and functional characteristics (Abbott et al, 2010). The many functions and selectivity of the BBB include the control of ion and neurotransmitter levels in the extracellular fluid. Precise homeostasis of local concentrations of these ions and molecules is important because they have a major impact on cellular excitability. An array of transporters are expressed within the BBB and involved in the transport of nutrients, amino acids, and ions, as well as select peptides and proteins. For example, the glucose transporter GLUT1 is key for providing the constant supply of glucose needed for normal cellular function.

Alterations in BBB properties occur in a variety of disease states (Abbott et al, 2010; Cipolla, 2010; Zlokovic, 2008; Jacob et al., 2010). Dysfunction of the BBB may result as a consequence of disease but may also contribute to neurological disease, particularly for pathologies with a vascular origin (Abbott et al, 2010; Cipolla, 2010; Zlokovic, 2008). Importantly, the properties of the BBB change with age (Farrall & Wardlaw, 2009; Popescu et al., 2009). In both people and animal models of aging, the permeability of the BBB increases with age (Farrall & Wardlaw, 2009; Popescu et al., 2009). Transport of glucose may be altered, as there is evidence for reduced expression of GLUT1 in the vasculature during aging (Popescu et al., 2009; Vorbrodt et al., 1999). Mechanisms that account for these changes are poorly defined at present, but oxidative stress and inflammation, both key underlying mechanisms for vascular disease in general (see discussion below), may be involved (Popescu et al., 2009). As will be discussed below, oxidative stress and formation of reactive oxygen species (ROS) occur in the vasculature with aging. ROS inhibit normal expression and function of tight-junction proteins and disrupt the integrity of the BBB (Pun et al., 2009; Schreibelt et al., 2007). In older females, loss of estrogen may also contribute to BBB dysfunction (Popescu et al., 2009). For example, a 2010 study indicates that estrogen normally increases claudin-5 gene transcription, increasing the expression of this protein at the BBB (Burek et al., 2010). Because claudin-5 is a key tight-junction protein within the BBB, the absence of claudin-5 greatly increases BBB permeability (Nitta et al., 2003).

CEREBRAL BLOOD FLOW

Because the brain lacks energy reserves, its normal function is dependent upon a level of perfusion that matches the level of cellular activity. Blood flow to the brain is highly regulated, involving multiple, intimately coordinated mechanisms. This regulation includes the integration of both regional and segmental changes in vascular tone, as well as major interactions and communication between various cell types.

Blood flow through individual blood vessels is proportional to the drop in pressure along that vessel and the resistance to blood flow that the vessel provides. Because arterial and venous pressure are normally relatively stable, the key determinant of vascular resistance and hence blood flow is vessel diameter. Blood flow through most blood vessels is proportional to the fourth power of the radius, meaning that changes in the diameter of a blood vessel have a substantial effect on blood flow. This concept is important because it illustrates how local blood flow can be determined by changes in local vessel diameter even in the absence of changes in blood pressure.

Major determinants of cerebrovascular resistance and thus cerebral blood flow include blood pressure, local pH (hypercapnia and hypocapnia), and levels of oxygen (the partial pressure of O_2 or blood O_2 content) (Figure 19.2). Changes in blood flow itself affect vascular tone via flow-mediated or propagated vasodilation (Fujii et al., 1991). Other cell types, particularly neurons and endothelial cells, affect the tone and structure of vascular smooth muscle through diverse mechanisms, including the release of vasoactive and/or trophic molecules, coupling and spread of electrical signals from one cell to another via gap junctions, and so on. Glial cells release vasoactive molecules (Murphy et al., 1994) and there is increasing interest in the role of astrocytes in regulating local resistance of arterioles within the neurovascular unit in the brain parenchyma (Iadecola & Nedergaard, 2007; Koehler et al., 2009). Mechanisms that control communication along vascular segments, including between upstream and downstream blood vessels, are poorly defined but likely to be very important in brain. The cerebral circulation is relatively unique in that large cerebral arteries (middle and posterior cerebral arteries, basilar artery, etc.) and cerebral (pial) arterioles on the surface of the brain account for 50–60% of vascular resistance (Faraci & Heistad, 1990; Baumbach et al., 2003, 2004). About one-third of vascular resistance resides in small vessels within the brain parenchyma (Faraci & Heistad, 1990). This arrangement necessitates integration between different vascular segments for optimal regulation of local perfusion pressure (microvascular pressure) and local blood flow (Faraci & Heistad, 1990; Fujii et al., 1991).

Resting levels of cerebral blood flow in humans change as individuals age. A series of studies has indicated that there is a reduction in the level of resting blood flow to brain (per unit of tissue weight) with aging (Bertsch et al., 2009; Faraci, 1993; Farkas & Luiten, 2001; Sonntag et al., 2007; Stoquart-El Sankari et al., 2007). For example, a negative correlation of local blood flow with age has been described in brain regions associated with memory and behavior, such as the cingulate gyrus and the gray matter of the cerebrum (Faraci, 1993). Blood flow to gray matter has been estimated to decrease at a rate of ≈0.5% per year in humans (Faraci, 1993). This gradual decline in cerebral blood flow is associated with a similar decrease in cerebral oxygen metabolism (Faraci, 1993; Farkas & Luiten, 2001). It is increasingly becoming apparent that relatively modest, but chronic, reductions in cerebral blood flow (which do not produce ischemia) have functional consequences such as reductions in protein synthesis (Hossmann, 1994). Early structural and functional changes in the vasculature may lead to hypoperfusion, impairment of vasodilator responses, and subsequent cellular injury (Joutel et al., 2010).

In addition to changes in resting blood flow, vascular responses to diverse vasoactive stimuli change with age. For example, through effects on extracellular pH, an increase in arterial CO_2 partial pressure (hypercapnia) is a potent dilator of cerebral blood vessels (Brian, 1998; Cipolla, 2010). The normal increase in cerebral blood flow that occurs during hypercapnia is impaired with aging (Faraci, 1993; Ito et al., 2002; Park et al., 2007; Shin et al., 2007) (Figure 19.2). Cerebral vasodilation in response to hypoxia or reductions in cerebral vascular resistance with decreases in arterial blood pressure (autoregulation) (Cipolla, 2010) is also impaired with aging (Faraci, 1993; Dupuis et al., 2004) (Figure 19.2). Vasodilation in response to activation of ATP-sensitive potassium channels in vascular muscle, a mechanism thought to contribute to autoregulation of blood flow during reductions in arterial pressure, is impaired with aging (Toyoda et al., 1997).

Increases in cellular activity in brain are normally closely matched with increases in local blood flow and

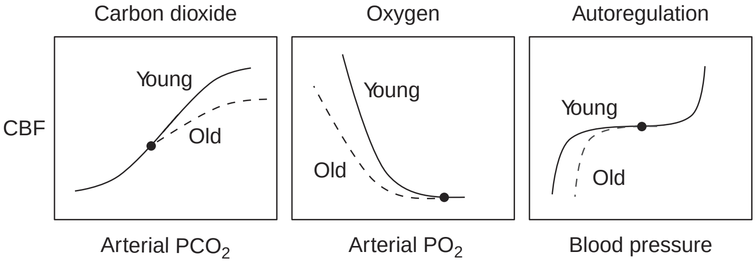


Figure 19.2 Schematic illustrating the effects of changes in the partial pressure of arterial CO_2 (PCO_2) and arterial O_2 (PO_2) and in the arterial blood pressure on cerebral blood flow (CBF) in young and old individuals. For each curve, the solid circle represents the value for CBF under normal conditions.

oxygen delivery via a mechanism called functional hyperemia or neurovascular coupling (Iadecola, 2004, 2009). This increase in local blood flow ensures adequate delivery of oxygen, glucose, and other nutrients to various brain regions, as well as efficient removal of metabolic by-products. Neurovascular coupling is impaired with aging (Park et al., 2007; Shin et al., 2007; Tsukada et al., 2000). An attractive hypothesis is that reductions in resting blood flow and impairment of increases in blood flow during enhanced cellular activity result in a mismatch between energy requirements, substrate delivery, and clearance of cellular by-products. If present chronically, these changes, along with increases in the permeability of the BBB, may contribute to cognitive decline or dementia with age (Farkas & Luiten, 2001; Iadecola, 2009; Zlokovic, 2008).

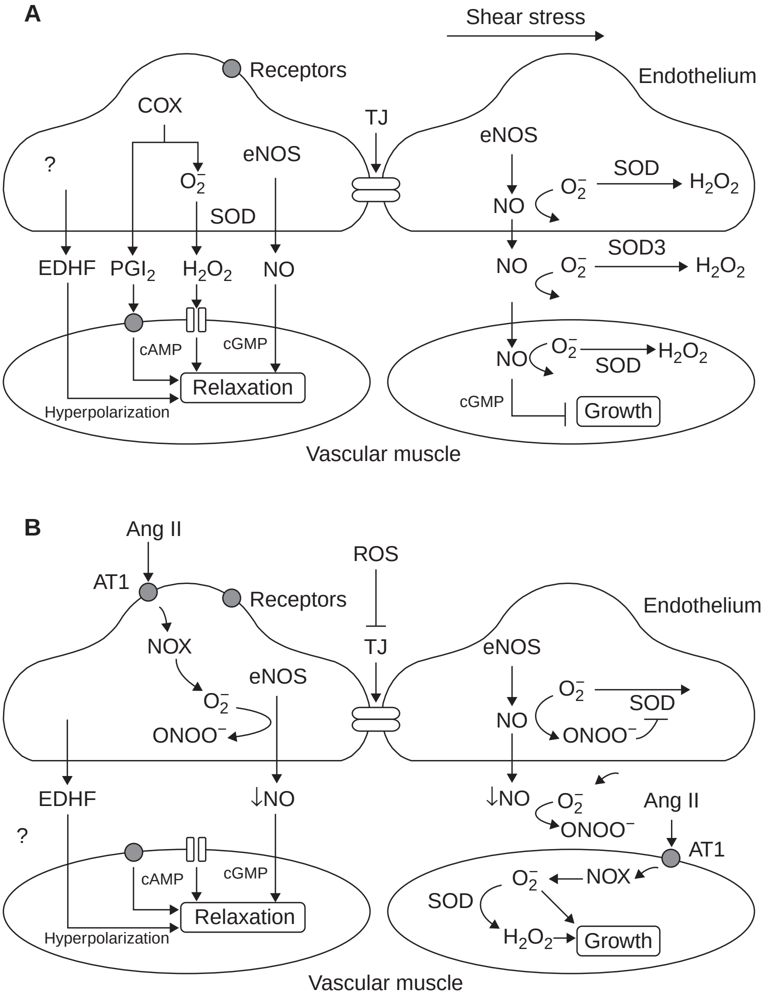
ENDOTHELIUM-DEPENDENT RESPONSES

Vascular endothelium plays a fundamental role in both health and disease throughout the circulation (Brandes et al., 2005; Chrissobolis & Faraci, 2008; Faraci & Heistad, 1998; Feletou & Vanhoutte, 2009). In addition to the traditional view that endothelial cells affect thrombosis, vascular tone, and vascular permeability, endothelium in the brain affects glia, neuronal function, and neurogenesis (Arai & Lo, 2009; Chen et al., 2005; Faraci, 2006b; Garthwaite et al., 2006; Gertz et al., 2006). Accordingly, endothelial dysfunction in the brain produces deleterious vascular effects, but also potentially has major detrimental effects on neural function as well as recovery following stroke and other forms of brain injury.

Cerebral endothelial cells influence resting vascular tone and mediate vasodilator responses to endothelium-dependent agonists as well as increases in blood flow (Ahmed & Hamel, 2000; Andresen et al., 2006; Faraci & Heistad, 1998; Faraci, 2002; Ngai & Winn, 1995) (Figure 19.3). As noted above, brain endothelial cells form the BBB, which has many unique properties and functions (Abbott et al, 2010; Cipolla, 2010). Endothelium also normally inhibits growth of vascular muscle (Baumbach et al., 2004) (Figure 19.3). The impact of endothelial dysfunction in brain is diverse, as it can cause or promote reductions in cerebral blood flow, or stroke, and may well contribute to cognitive decline (Chrissobolis & Faraci, 2008; Iadecola, 2009; Faraci, 2005).

A key mechanism by which endothelium affects other cells is via the production of nitric oxide (NO) by endothelial NO synthase (eNOS) (Faraci & Heistad, 1998; Faraci, 2006b) (Figure 19.3). In addition to being a potent vasodilator, NO acts as a messenger molecule and mediates the majority of endothelium-dependent responses in the brain (Faraci & Heistad, 1998; Faraci, 2002, 2006b). In addition to exerting effects on resting vascular resistance, endothelial cells mediate vasodilator responses to a diverse group of receptor-mediated agonists including acetylcholine, substance P, thrombin, and ADP. Vasodilation in response to increases in blood flow is also mediated by NO in cerebral blood vessels (Ngai & Winn, 1995).

The prominent role of NO has been observed in a variety of both large and small blood vessels from multiple species, including humans (Ahmed & Hamel, 2000; Baumbach et al., 2004; Faraci & Heistad, 1998; Faraci, 2002, 2006b; Geddawy et al., 2010). Most of the vascular effects of NO are mediated by activation of soluble guanylate cyclase and production of cGMP (Didion et al., 2001; Faraci et al., 1998; Sobey & Faraci, 1997) (Figure 19.3). Endothelial cells affect basal tone of blood vessels throughout the vasculature by inhibiting myogenic tone. Under resting conditions, constitutive production of NO by eNOS exerts a dilator influence throughout all segments of the brain circulation. These include large arteries and pial vessels on the surface of the brain as well as smaller arterioles in the parenchyma (Ahmed & Hamel, 2000; Cipolla et al., 2004, 2009; Cipolla & Bullinger, 2008; Faraci & Heistad, 1998; Faraci, 2006).


A
Shear stress
Receptors
Endothelium
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cAMP
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Relaxation
Hyperpolarization
NO
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Relaxation
SOD
Hyperpolarization
H_2O_2
Growth
Vascular muscle

Figure 19.3 Schematic illustrations of some of the effects on endothelial cells within the vessel wall under normal conditions and with aging. (A) Under normal conditions, endothelial NO synthase (eNOS) produces NO under basal conditions and in response to a variety of receptor-mediated agonists or increased shear stress. eNOS-derived NO produces relaxation of vascular muscle and inhibits vascular growth by activating soluble guanylate cyclase causing production of cGMP. Endothelium-derived hyperpolarizing factor (EDHF) is a substance (or mechanism) that produces vasodilation by hyperpolarizing vascular muscle. This hyperpolarization is initiated within endothelial cells but may spread to vascular muscle via myoendothelial gap junctions (Andresen et al., 2006; Feletou & Vanhoutte, 2009). Local release of K^+ or an unidentified diffusible factor from endothelium may also produce hyperpolarization of underlying smooth muscle (not shown). Cyclooxygenase (COX) produces prostacyclin (PGI_2) but also O_2^- during the metabolism of arachidonic acid. After conversion of O_2^- to H_2O_2 by superoxide dismutase (SOD), H_2O_2 relaxes vascular muscle via effects on K^+ channels. Vasodilation of cerebral microvessels in response to bradykinin and arachidonic acid is mediated by H_2O_2. PGI_2 is produced by cerebral endothelium and is known to cause vasodilation via formation of cAMP. Unlike NO, EDHF, and H_2O_2, the functional importance of PGI_2 as an endothelium-derived relaxing factor remains unclear, however. Tight-junction proteins (TJ) anchor endothelial cells to one another and are a critical determinant of normal BBB integrity. Steady-state levels of O_2^- are normally relatively low, in part because of activity of intracellular and extracellular forms of SOD. (B) With aging, there are multiple potential sources of O_2^- in brain vascular cells. One key source of O_2^- that has been shown to be functionally important during aging is NADPH oxidase (NOX), which may be activated by angiotensin II (Ang II) acting on AT1 receptors as well as other stimuli. ROS may decrease expression or function of TJ proteins, resulting in an increase in the permeability of the BBB. The very efficient interaction of O_2^- and NO decreases the bioavailability of NO and thus creates a loss of the normal NO-mediated signaling, including the protective effects of NO. H_2O_2 and/or O_2^- may also stimulate growth or remodeling of vascular muscle.

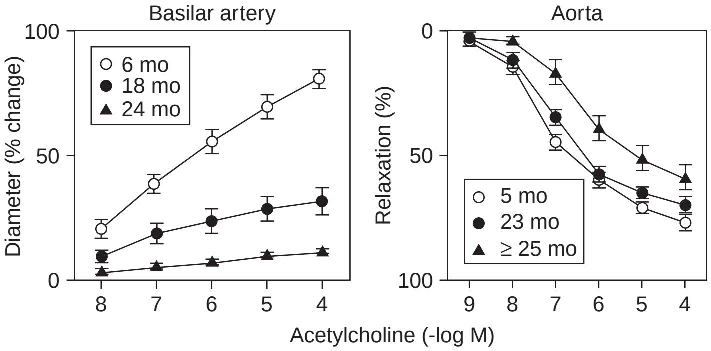

Figure 19.4 Differences in the impact of age on responses to the endothelium-dependent agonist acetylcholine in a cerebral artery (the basilar artery) (left) and the aorta (right). Graphs are redrawn from data presented in Modrick et al. (2009) and Brown et al. (2007). The ages for each group of mice are shown in the keys. Values are means ± SE.

Other endothelium-derived relaxing factors or vasodilator mechanisms, including endothelium-derived hyperpolarizing factor (EDHF), may play an important role in regulation of vascular tone in some segments of the circulation (Andresen et al., 2006; Cipolla et al., 2009) (Figure 19.3). A 2009 study has provided the first evidence that EDHF affects resting tone in parenchymal arterioles (Cipolla et al., 2009). In the microcirculation, responses of cerebral arterioles to bradykinin are cyclooxygenase dependent and mediated by hydrogen peroxide (H_2O_2) (Sobey et al., 1997) (Figure 19.3). Whether the loss of these non-NO-mediated vasodilator influences on blood vessels contributes to the reductions in cerebral blood flow that occur with aging is not clear.

One of the major sites of cellular damage during aging is endothelium. Changes in endothelial cells with age occur in multiple vascular beds, including blood vessels supplying heart, mesentery, and skeletal muscle (Muller-Delp, 2006; Rodriguez-Manas et al., 2009; Brandes et al., 2005; Zeiher et al, 1993). The changes that occur in this cell type in brain are a striking example of this concept, because the influence of endothelium is great in this organ. Impairment of endothelium-dependent regulation of vascular tone occurs with aging in cerebral blood vessels from experimental animals and in humans (Arribas et al., 1997; Faraci, 1993; Geary & Buchholz, 2003; Hatake et al., 1990; Mayhan et al., 1990, 2008; Park et al., 2007; Modrick et al., 2009). Such impairment is seen with agonists that affect vascular tone via NO- and H_2O_2-dependent mechanisms (Arribas et al., 1997; Faraci, 1993; Geary & Buchholz, 2003; Mayhan et al., 1990, 2008; Park et al., 2007; Modrick et al., 2009). This form of vascular dysfunction worsens with increasing age in brain and other regions (Hatake et al, 1990; Park et al., 2007; Modrick et al., 2009; Rodriguez-Manas et al., 2009; Zeiher et al, 1993). It is very likely that other risk factors for vascular disease (hypertension, diabetes, hypercholesterolemia, etc.) interact with and accelerate age-induced vascular abnormalities.

A key observation that has emerged is that age-induced endothelial dysfunction occurs earlier and may be larger in magnitude in the cerebral circulation than in blood vessels outside of the brain (Modrick et al., 2009) (Figure 19.4). Thus, although abnormalities develop with age throughout the vascular tree, the circulation of the brain may be particularly sensitive to age-induced endothelial dysfunction.

OXIDATIVE STRESS

Bioassay studies have provided evidence that production of NO by endothelium is not impaired with aging (Paterno et al., 1994). These findings suggest that impaired responses to endothelium-dependent stimuli in older subjects may be due to other mechanisms, including the inactivation of NO (loss of NO-mediated signaling) due to oxidative stress. Oxidative stress occurs as the result of an imbalance between the generation of ROS and the antioxidant defense mechanisms (Faraci & Didion, 2004). ROS have direct effects on vascular tone but are also key mediators of vascular dysfunction and potent stimulators of vascular growth (Baumbach et al., 2006; Chrissobolis & Faraci, 2008; Faraci, 2005, 2006a,c; Modrick et al., 2009) (Figure 19.3). ROS may be particularly important in the cerebral circulation. Cerebral arteries have the capacity to generate relatively high levels of superoxide compared to extracranial blood vessels and may be particularly sensitive to ROS (Baumbach et al., 2006; Chrissobolis & Faraci, 2008; Faraci, 2006a,c; Miller et al., 2005; Ogawa et al., 2005).

There has been considerable interest in the role of ROS in relation to aging in general (Finkel, 2005; Lenaz et al., 2006). In the peripheral vasculature, oxidative stress occurs and plays a key role in mechanisms that promote vascular dysfunction with aging (Brandes et al., 2005; Brown et al., 2007; Csiszar et al., 2008; Herrera et al., 2010; Rodriguez-Manas et al., 2009; Van der Loo et al., 2000). There has been similar interest in the impact of ROS on the cerebral vasculature (Chrissobolis & Faraci, 2008; Faraci, 2005; Iadecola, 2009). For example, NO reacts extremely efficiently with superoxide (Faraci & Didion, 2004), resulting in the loss of normal NO-mediated signaling (Figure 19.3). Since NO plays a major role in vascular biology, the loss of NO bioavailability has far-reaching implications and is thought to contribute to vascular disease, with both short- and long-term consequences (Chrissobolis & Faraci, 2008; Faraci, 2005). These changes include reductions in resting blood flow and abnormal vascular growth, as well as impairment of NO-mediated responses and neurovascular coupling. ROS alter the extracellular matrix and vascular structure via the activation of matrix metalloproteinases, potentially increasing vascular permeability or contributing to vascular remodeling. Many of these changes may occur in response to activation of redox-sensitive transcription factors [including nuclear factor-κB (NF-κB), activator protein-1, and hypoxia-inducible factor-1α] and subsequent changes in gene expression (Csiszar et al., 2008; Faraci & Didion, 2004). Because endothelium normally promotes neurogenesis (Chen et al., 2005; Shen et al., 2004), endothelial dysfunction and the loss of eNOS-mediated signaling in the presence of vascular disease may impair neurogenesis and, thus, recovery from other forms of brain injury.

There are multiple potential sources of superoxide and other ROS in the vasculature. These sources include mitochondria, NADPH oxidase, cyclooxygenase, myeloperoxidase, xanthine oxidase, and lipoxygenase (Chrissobolis & Faraci, 2008; Leopold & Loscalzo, 2009). Under special circumstances, such as after oxidation of tetrahydrobiopterin, eNOS may become “uncoupled,” by which an abnormal flow of electrons through the enzyme results in production of superoxide instead of NO (Faraci, 2006a). Studies have focused mainly on the role of NADPH oxidase as a source of superoxide that promotes vascular dysfunction in a variety of diseases and with aging. Several lines of evidence support such a role for ROS and this enzyme complex in the cerebral circulation (Chrissobolis & Faraci, 2008). With aging, increases in superoxide occur in blood vessels (Park et al., 2007), and impairment of endothelial function and neurovascular coupling is restored to normal with scavengers of superoxide, pharmacological inhibitors of NADPH oxidase, or genetic deletion of the Nox2 catalytic component of NADPH oxidase (Mayhan et al., 2008; Modrick et al., 2009; Park et al., 2007). These results all support the concept that a key source of superoxide in blood vessels during aging is NADPH oxidase (Figure 19.3).

The interaction of NO with superoxide results in the formation of peroxynitrite, and increases in peroxynitrite in the vasculature have been described in models of aging and in blood vessels from humans (Donato et al., 2007; Rodriguez-Manas et al., 2009; Vaishnav et al., 2007; Van der Loo et al., 2000). Peroxynitrite produces several forms of cellular injury, including activation of the nuclear enzyme poly(ADP-ribose) polymerases (PARP) (Jagtop & Szabo, 2005; Maneen et al., 2006). Peroxynitrite is a potent inducer of DNA strand breaks, which result in PARP activation. Excessive activation of PARP depletes cellular energy pools and, as a consequence, produces cellular dysfunction (Jagtop & Szabo, 2005). Studies utilizing pharmacological inhibitors of PARP suggest that activation of this enzyme contributes to vascular dysfunction with aging (Didion et al., 2006; Modrick et al., 2009).

Blood vessels are protected from oxidative stress by an array of antioxidant enzymes including the superoxide dismutases (SODs) (Faraci & Didion, 2004). The three isoforms of SOD in mammals are cytosolic or copper–zinc SOD (Cu,Zn-SOD or SOD1), manganese SOD (Mn-SOD or SOD2) localized in mitochondria, and an extracellular form of Cu,Zn-SOD (EC-SOD or SOD3) (Faraci & Didion, 2004). Studies utilizing mice genetically deficient in SOD1 or SOD2 have suggested important roles for both enzymes in protecting the vasculature during aging. For example, vascular superoxide levels increase in old wild-type mice but increase even further in heterozygous SOD1-deficient mice ($SOD1^{+/-}$) with aging (Didion et al., 2006). Endothelial function is markedly impaired in old $SOD1^{+/-}$ mice at an age at which endothelial function was still normal in wild-type mice (Didion et al., 2006). Thus, oxidative stress and vascular dysfunction with aging are accelerated in the face of SOD1 deficiency.

In relation to SOD2, a protective role for the vasculature seems likely because this isoform of SOD is expressed in relatively high levels in endothelial cells (Faraci & Didion, 2004). Although endothelial function is similar in young wild-type and $SOD2^{+/-}$ mice, this phenotype is selectively impaired in old wild-type mice, but impaired to the greatest degree in old $SOD2^{+/-}$ mice (Brown et al., 2007). Superoxide levels in the vasculature are increased to the highest level in old $SOD2^{+/-}$ mice compared with old wild-type or young mice of either genotype. Thus, SOD2 haploinsufficiency results in increased vascular oxidative stress and augmented endothelial dysfunction with aging. Although protein levels for SOD2 may not change with age (Brown et al., 2007; Donato et al., 2007), the activity of SOD2 in cerebral arteries declines with age (D’Armiento et al., 2001), and genetic deficiency in SOD2 increases deposition of β-amyloid in the vasculature of a mouse model of Alzheimer disease (Esposito et al., 2006). It is noteworthy that peroxynitrite and other ROS may reduce the activity of SODs (Faraci & Didion, 2004), further amplifying the level of oxidative stress even if levels of SOD protein are not reduced (Figure 19.3).

Along with SOD1 and SOD2, the extracellular form of SOD (SOD3) may also protect the vasculature.

In both old rats and old mice, studies indicate that SOD3 inhibits increases in vascular superoxide and endothelial dysfunction with aging (Brown et al., 2006; Lund et al., 2009).

In addition to impairing vasodilator responses, oxidative stress during aging can affect vasoconstrictor responses. For example, constriction of large cerebral arteries to serotonin is increased in aged rats (Hajdu et al., 1993). Serotonin-induced vasoconstriction is also enhanced by deficiency in SOD1 in old mice (Didion et al., 2006). Serotonin is an important stimulus that can exert marked effects on vascular tone. The vasoconstrictor effect of aggregating platelets is mediated by serotonin (Kaul et al., 1993). Enhanced vasoconstrictor effects of serotonin during aging may contribute to vasospasm or transient ischemic attacks.

Combined, these findings provide direct evidence that selective reductions in expression or activity of SODs accelerate vascular dysfunction with aging. It should be noted that the level of vascular dysfunction seen in old SOD1$^{+/-}$ or SOD2$^{+/-}$ mice was similar to that seen in old SOD3$^{-/-}$ mice. Therefore, it appears that the loss of one copy of either the SOD1 or the SOD2 gene affects vascular aging to approximately the same extent as complete loss of SOD3. These findings imply that while all three forms of SOD are important, the loss of SOD1 or SOD2 activity is accompanied by a greater vascular phenotype during aging.

In relation to oxidative stress and possible therapy or prevention, exercise (voluntary running) has been shown to decrease oxidative stress in the vasculature and improve endothelial function in old mice (Durrant et al., 2009). These beneficial effects appear to result from a combination of increased SOD activity and reduced expression of NADPH oxidase (Durrant et al., 2009). Short-term caloric restriction also reduces oxidative stress and improves endothelial function during aging (Rippe et al., 2010). Conceptually, these results are interesting because they provide evidence that some changes in vascular function with age are not fixed and may be reversed with appropriate treatment or intervention.

PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR γ

In addition to antioxidants such as SODs, other mechanisms may protect the vasculature from oxidative stress with aging. Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor. While the biological importance of PPARγ has been well defined in adipose tissue, more recent work indicates that PPARγ is also expressed in vascular cells, where it may protect blood vessels (Ketsawatsomkron et al., 2010). PPARγ regulates the expression of clusters of target genes primarily by binding to PPAR-response elements or through protein–protein interactions with other transcription factors including NF-κB (Berger & Moller, 2002; Buchan & Hassall, 2000). Antioxidant effects of PPARγ can occur in blood vessels. For example, genetic interference with PPARγ in otherwise normal mice produced increased levels of superoxide and impairment of endothelial function, as well as hypertrophy and inward remodeling in cerebral blood vessels (Beyer et al., 2008). Activators of PPARγ prevent vascular remodeling during hypertension and decrease expression of NADPH oxidase and receptors for angiotensin II (Ang II; AT1 receptors) while increasing the expression of SOD1 (Inoue et al., 2001; Schiffrin, 2005; Sugawara et al., 2001; Cipolla et al., 2010). As is discussed below, Ang II is a key stimulus that promotes oxidative stress in vascular cells.

In relation to aging, mRNA, protein, and the DNA-binding activity of PPARγ decrease with age in the kidney (Sung et al., 2004), and treatment with a synthetic activator of PPARγ reduces renal injury with age, possibly by inhibiting oxidative stress (Yang et al., 2009). Reductions in PPARγ expression have been reported to reduce life span (Argmann et al., 2010). In a model of senescence using endothelial cells in culture, increased age was associated with reduced expression of PPARγ protein and increased expression of AT1 receptors (Scalera et al., 2008). Pharmacological activation of PPARγ reduced expression of AT1 receptors and inhibited oxidative stress in this model (Scalera et al., 2008).

Klotho is a gene known to reduce oxidative stress and age-related symptoms as well as extending life span (Kuro-o, 2008). The expression of klotho is increased by pharmacological activation of PPARγ (Zhang et al., 2008) and thus may contribute to the protective effects of PPARγ with aging (Yang et al., 2009; Argmann et al., 2010). In heterozygous knock-in mice expressing the P465L dominant-negative mutation in PPARγ, age-related endothelial dysfunction occurred earlier and to a greater extent after interference with normal PPARγ function (Modrick et al., 2010). The mechanism that accounts for accelerated age-induced vascular dysfunction involved oxidative stress, as it was prevented by a scavenger of superoxide. These preliminary findings suggest a novel yet fundamental role for PPARγ in protection against age-induced oxidative stress and vascular dysfunction. Consistent with these results, vascular dysfunction in aged mice overexpressing amyloid precursor protein, a model of Alzheimer disease, is restored toward normal by treatment with a synthetic activator of PPARγ (Nicolakakis et al., 2008).

VASCULAR INFLAMMATION

Components of the inflammatory response are activated within the vasculature in diseases including atherosclerosis and hypertension as well as during aging (Csiszar et al., 2008; Didion et al., 2009; Savoia & Schiffrin, 2007). For example, NF-κB is a key transcription factor in relation to inflammatory responses in general (Csiszar et al., 2008). During normal aging, there is increased activation of NF-κB and increased production of proinflammatory cytokines within the vessel wall, including tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Csiszar et al., 2008; Donato et al., 2007, 2008; Rodriguez-Manas et al., 2009). Inflammation and oxidative stress may function synergistically in vascular disease. Both TNF-α and IL-6 promote oxidative stress and are established mediators of vascular dysfunction (Schrader et al., 2007; Zhang et al., 2009).

Although inflammatory mechanisms are thought to be key contributors to vascular disease (Csiszar et al., 2008; Didion et al., 2009; Savoia & Schiffrin, 2007; Zhang et al., 2009), little is known about the functional importance of anti-inflammatory mechanisms in aging. Several studies have led to the concept that the anti-inflammatory cytokine IL-10 may be a key mediator of vascular protection (Caligiuri et al., 2003; Didion et al., 2009; Gunnett et al., 1999, 2000, 2002; Mallat et al., 1999). IL-10 attenuates increases in vascular superoxide and vascular dysfunction in response to lipopolysaccharide as well as during diabetes, hypertension, and atherosclerosis (Caligiuri et al., 2003; Didion et al., 2009; Gunnett et al., 1999, 2000, 2002; Mallat et al., 1999). A 2009 study tested the hypothesis that IL-10 protects against aging-induced vascular dysfunction and found that age-related and superoxide-mediated endothelial dysfunction occurred much earlier and to a greater extent in mice genetically deficient in IL-10 (Didion et al., 2009). These findings suggest a novel role for IL-10 in oxidative stress and age-induced inflammation and vascular dysfunction.

Other mechanisms may protect against vascular inflammation during aging. Both eNOS-derived NO and PPARγ decrease activation of NF-κB and expression of inflammatory genes (Berger & Moller, 2002; Buchan & Hassall, 2000; De Caterina et al., 1995). Loss of bioavailability of NO because of reduced expression of eNOS, eNOS uncoupling, or inactivation of NO by superoxide therefore promotes vascular inflammation and dysfunction (Chrissobolis & Faraci, 2008). As noted above, PPARγ protects against vascular dysfunction with aging, and the inhibitory effects of PPARγ on vascular inflammation may contribute to this mechanism of vascular protection.

RENIN–ANGIOTENSIN SYSTEM

The renin–angiotensin system plays a major role in changes in vascular structure and function in pathophysiological conditions (Lyle & Griendling, 2006; Mehta & Griendling, 2007). Ang II is a major promoter of oxidative stress and inflammation in the vasculature (Chrissobolis & Faraci, 2008; Lyle & Griendling, 2006; Mehta & Griendling, 2007; Savoia & Schiffrin, 2007). In the cerebral circulation, Ang II produces diverse effects, including oxidative stress, endothelial dysfunction, impairment of neurovascular coupling, vascular hypertrophy, and inward vascular remodeling (Baumbach et al., 2003; Chrissobolis & Faraci, 2010; Faraci et al., 2006; Girouard et al., 2006, 2007; Kazama et al., 2004). Increases in superoxide in response to Ang II are produced mainly by NADPH oxidase and are relatively large in cerebral arteries compared to extracranial vessels (Chrissobolis & Faraci, 2008; Miller et al., 2005) (Figure 19.3). Ang II and ROS activate NF-κB, resulting in production of IL-6, a key mediator of Ang II-induced vascular dysfunction and hypertrophy (Didion et al., 2009; Savoia & Schiffrin, 2007; Schrader et al., 2007). Vascular expression of IL-6, TNF-α, and p22^{phox} (a component of NADPH oxidase) increases during Ang II-dependent hypertension (Didion et al., 2009). Detrimental effects of Ang II are generally mediated by activation of AT1 receptors (Lyle & Griendling, 2006; Mehta & Griendling, 2007).

The role of angiotensin II in aging has become a focus of attention (Cassis et al., 2010), including the contribution of this system to vascular changes. Expression of angiotensin-converting enzyme (ACE) and AT1 receptors, as well as the tissue levels of Ang II, is increased in the aorta in old monkeys and people (Wang et al., 2003, 2007). The effects of Ang II and activation of AT1 receptors may be antagonized by a second form of ACE (ACE2), which produces the peptide Ang (1-7), a vasodilator (Santos et al., 2008). Expression of ACE2 decreases markedly with age (Xudong et al., 2006), which represents the loss of a potential protective mechanism for the cardiovascular system.

Chronic pharmacological inhibition of the renin–angiotensin system (inhibition of Ang II formation or AT1 receptors) attenuates vascular growth (Basso et al., 2007), and genetic deficiency in AT1 receptors attenuates peroxynitrite formation during aging (Benigni et al., 2009). As discussed earlier, aging produces marked superoxide-mediated endothelial dysfunction in cerebral blood vessels (Modrick et al., 2009). This effect of aging on cerebral endothelial cells did not occur in animals lacking AT1 receptors, providing direct evidence for a novel and key role for Ang II and AT1 receptors in age-related vascular dysfunction (Modrick et al., 2009) (Figure 19.3). In this regard, it is important to note that Ang II and the activation of AT1 receptors are major stimuli for the activation of NADPH oxidase (Chrissobolis & Faraci, 2008; Mehta & Griendling, 2007), which has been implicated in promoting vascular abnormalities during aging (Mayhan et al., 2008; Park et al., 2007;

Rodriguez-Manas et al., 2009). In relation to the role of the renin–angiotensin system with vascular aging, it is of interest that klotho can inhibit Ang II-induced superoxide formation in endothelial cells (Rakugi et al., 2007).

CONCLUSIONS

Aging produces diverse effects on blood vessels throughout the circulation. These changes are particularly prominent in the brain, where aging produces structural changes in the vessel wall, reductions in resting blood flow, and impairment of vasodilator responses. Impairment of the normal function of the blood–brain barrier also occurs with increasing age. Both oxidative stress and local inflammation appear to be key mechanisms that promote vascular disease during aging. Activation of the renin–angiotensin system plays a key role in producing vascular abnormalities by promoting oxidative stress and inflammation. In contrast, molecules including superoxide dismutases and peroxisome proliferator-activated receptor γ protect vascular cells by limiting these changes. A better understanding of the mechanisms that promote vascular dysfunction or protect against these changes may result in targeted approaches that could prevent or delay the progression of cerebrovascular disease that occurs with normal aging.

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Chapter | 20 |

Pulmonary Function in Aging Humans

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CHAPTER CONTENTS

Introduction 421
  Cross-sectional versus Longitudinal Experimental Designs 423
  Sampling Considerations 424
Sensory Feedback 425
  Chemoreceptor Function 425
  Secondary Receptors of the Respiratory System 426
  Summary 426
Central Integration 426
Controlled Variables 426
  Morphological Considerations 426
  Mechanical Considerations 427
    Changes in Collagen and Elastin Composition 427
    Elastic Recoil of Lung Tissue and the Chest Wall 429
    Static Work of Breathing 431
    Airway Resistance, Dynamic Airway Collapse, and the Closing Volume 432
    Respiratory Muscle Function 433
    Summary 434
  Static Lung Volumes and Capacities 434
  Dynamic Lung Volumes and Ventilatory Flows 435
  Alveolar Ventilation and Perfusion 436
Regulated Variables 438
  Arterial Oxygen Partial Pressure 438
  Arterial Carbon Dioxide Partial Pressure 439
  Summary 439
Concluding Remarks 439
References 440

INTRODUCTION

In healthy, young individuals, the respiratory system is not considered to impose either structural or functional limitations upon homeostasis relative to the demands encountered during daily life, including exercise (Stickland et al., 2008). This remains true right through to maximal exercise for most people (Dempsey, 1986) and across a wide range of ages (Johnson et al., 1994) in habitually active people. However, for those with thoracopulmonary dysfunction, inadequacies of ventilation and gas exchange can limit exercise tolerance. As people age, but especially those choosing to adopt a sedentary lifestyle, a wide range of physiological functions decline (Masoro, 1995; Groeller, 2008), with sedentary individuals inexorably progressing toward disease states at rates that exceed those seen in their physically active counterparts (Blair et al., 1996; Booth et al., 2000; Chakravarthy, 2008). Indeed, such people become increasingly more intolerant of work, exercise, and daily stress. In this chapter, the impact of aging upon the respiratory system and how these changes may affect the regulation of blood gases are explored. To facilitate comprehension for all readers, Table 20.1 provides definitions for the more complex terms used but not defined within this chapter.

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Table 20.1 Definitions of essential terms

TERM	DEFINITION
Alveolar (physiological) shunt	Alveoli that are perfused but not ventilated
Closing volume	The air volume trapped behind collapsed airways
Compliance	A measure of the ease with which tissues can be stretched
Dead space (anatomical and alveolar)	Ventilated lung regions that do not participate in gas exchange: conducting airways (anatomical) or underperfused alveoli (alveolar)
Diffusing capacity	The volume of oxygen that can be transferred from the alveoli to the blood for a given partial pressure gradient
Dynamic airway collapse	The closure of downstream airways during forced expiration due to pleural pressure exceeding airway pressure
Elastance	A measure of the ability of a stretched tissue to recoil to its original shape
Elastic load	Breathing load applied by restricting the tidal volume that one can inhale
Flow-resistive load	Breathing load applied by reducing the diameter of external airways
Flow-resistive work	Work performed to overcome frictional forces within the airways or due to the movement of tissues against other tissues
Hyperpnea	Increased minute ventilation in proportion to the metabolic demand for oxygen
Hyperventilation	Increased minute ventilation that is out of proportion to the metabolic demand and associated with a reduced arterial partial pressure of carbon dioxide
Maximal aerobic power	Maximal or peak oxygen consumption
Maximal voluntary ventilation	The maximal airflow one can generate through the lungs in 12–15 s
Owles point	The point beyond which the relationship between minute ventilation and oxygen consumption changes from being linear to being curvilinear
Pressure–volume relaxation curve	A relationship obtained by measuring respiratory pressures when one relaxes against an occluded airway, across a range of lung volumes
Resistance	The respiratory pressure required to generate flow through airways; it is dependent upon airway diameter
Static work of breathing	The respiratory work associated with expanding the lungs and chest wall
Transpulmonary pressure	The pressure gradient across the lung: alveolar pressure minus pleural pressure
Transrespiratory pressure	The pressure gradient between the alveoli and the body surface
Transthoracic pressure	The pressure gradient across the chest wall: pleural pressure minus body surface pressure
Venous admixture	Blood returning from the lungs that is deoxygenated relative to that which may be expected; about 2% of the right cardiac output
Ventilatory equivalent	The minute ventilation required to support each additional liter of oxygen consumed

The *milieu intérieur* of all animals must be regulated to levels conducive with life (Bernard, 1865; Cannon, 1929). Not surprisingly, evolution has selected in favor of beings capable of controlling a wide range of anatomical structures and physiological functions that support homeostasis across a broad range of internal and external stresses. These homeostatic processes include, among others, the physiological regulation of mean arterial and central venous blood pressures, blood gas partial pressures, plasma volume and osmolality, blood glucose concentration, and mean body temperature. While the respiratory system fulfills various important functions, its principal role centers on the regulation of arterial oxygen and carbon dioxide partial pressures.

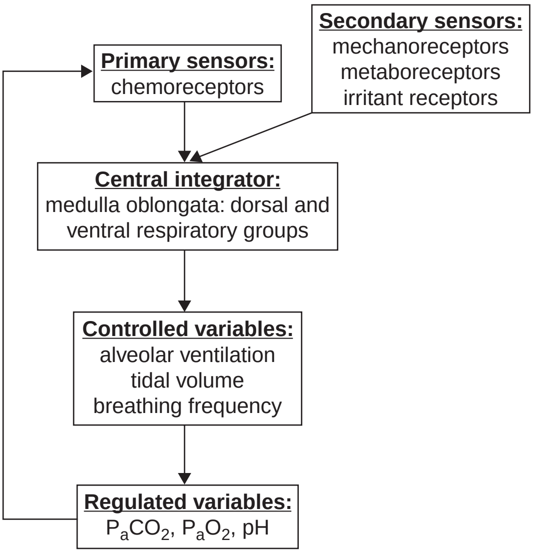

Figure 20.1 A systemic overview of pulmonary regulation.

For consistency with contemporary literature concerning physiological regulation, the word control (modulation) will be reserved to describe how the functioning of anatomical structures is changed via the autonomic nervous system to achieve homeostasis (Werner et al., 2008). For instance, tidal volume, breathing frequency, and alveolar ventilation are controlled such that oxygen delivery to the alveoli matches the metabolic demand for oxygen while simultaneously ensuring that carbon dioxide removal keeps pace with its production. These controlled variables subserve blood gas regulation and routinely change over very wide ranges to ensure that the arterial partial pressure of oxygen is maintained above, while carbon dioxide partial pressure is held below, critical levels. While changes in alveolar ventilation over the range 5–200 L min^{-1} are well tolerated, wide and protracted changes in blood gas concentration are not conducive to life, and these blood gas partial pressures are therefore regulated within narrow (physiological) ranges. Variables that dictate the status of the *milieu intérieur* are known as regulated variables.

The essential link between these regulated variables and the structures of the central nervous system (medulla oblongata) that control tidal volume, breathing frequency, and alveolar ventilation is provided via sensory feedback loops (Figure 20.1). The primary sensors for this regulatory system are the central and peripheral chemoreceptors that monitor blood gas concentrations, with feedback eliciting centrally mediated and proportional changes to the controlled variables. Accordingly, this review is placed within this integrated regulatory context, and Figure 20.1 provides the mechanistic framework around which the ensuing discussion is developed, commencing with the affects of aging upon relevant sensory functions. Also for consistency with contemporary practice, structural and functional variations that can be demonstrated to depend upon the age of an individual, and might be expected to be evident in most older people, will be described as age-dependent changes. Those variations accompanying aging in some individuals, but the mechanism of which lacks an age dependency, will be referred to as age-related or age-associated changes.

Before embarking upon this discussion, however, it is necessary to reinforce several methodological points that have, over the years, challenged our ability to appreciate adequately the impact of aging upon pulmonary function. These points relate to choices made by investigators with regard to experimental design and the selection of population samples.

Cross-sectional versus Longitudinal Experimental Designs

The validity of applying outcomes from any experiment to large population samples, by definition, relies upon assumptions made by scientists concerning matters such as experimental design and subject selection. Perhaps the most fundamental decision of all, with respect to aging research, is the use of cross-sectional or longitudinal experimental designs (Masoro, 2001). Both designs have strengths and weaknesses. In the former, subjects are selected to provide a simultaneous snapshot of a wide range of ages within one experiment, even though, by definition, the older individuals contain a higher representation of those who possess superior survival traits. The implicit assumption from this design is that younger individuals will eventually attain the characteristics of their more senior counterparts upon reaching those ages. The validity of this assumption will now be briefly explored. However, for more complete discussions of these design issues as they apply to pulmonary function, readers are directed to Glindmeyer and colleagues (1982), Ware and associates (1990), and Knudson (1991).

Stature and lung volume are intrinsically linked (Hutchinson, 1844; Glindmeyer et al., 1982), with differences in height accounting for much of the variability in static and dynamic lung volumes (Ferris et al., 1965; Boren et al., 1966; Roberts et al., 1991). However, environmental factors (e.g., nutrition) independently and powerfully affect stature. Thus, the interaction of these relationships can represent a significant limitation when cross-sectional experimental designs are used to investigate pulmonary function (cohort effects: Glindmeyer et al., 1982; Knudson, 1991; Zeleznik, 2003).

To illustrate this limitation, let us compare average males born 60 years apart (1920 versus 1980), look at their height at the age of 20 years and its reduction with aging, and compare their heights in the year 2000 (80 years versus 20 years). Reasonable stature approximations at 20 years would be 172 and 174 cm (respectively), with this difference principally attributable to environment influences. Let us assume that, from 20 to 50 years, height is well maintained and then decreases by 0.8 cm to 60 years and by 1.2 cm decade^{-1} thereafter; both reasonable assumptions. If we compared these individuals simultaneously in 2000, we might expect their heights to be 168.8 cm (80-year-old) and 174.0 cm (20-year-old). Indeed, the person born in 1980 would contract to 172 cm when he reaches 70 years, and this equals the maximum height of our person born in 1920. Thus, at no other time in the first 70 years of their lives did these individuals ever share a common stature. Taking this exercise one step further, both individuals would experience longitudinal height reductions from 20 to 80 years of ~0.05 cm year^{-1}, as dictated by these assumptions. However, simultaneous (cross-sectional) height comparisons made in 2000 show a decline in stature that is almost twofold greater (~0.09 cm year^{-1}). If one now uses pulmonary function predictions based upon these cross-sectional data, then errors will occur. Furthermore, much of the age-dependent stature reduction is associated with changes in the intervertebral spaces (Edge et al., 1964) in combination with a loss of bone mass (osteopenia) from the vertebrae, and these changes also affect lung volumes.

These elementary computations show that the primary assumption of the cross-sectional design is not robust, and, with regard to stature, it can be flawed, leading to an overestimation of this age-dependent change. In fact, if we use the prediction equation of Knudson and others (1976) to estimate the vital capacity of these individuals at various ages, we would see that, at 20 years, these volumes would be 5.14 and 5.27 l (respectively), while at 80 years, they would still present with a vital capacity difference of 130 ml, attributable entirely to the 2-cm difference in stature being maintained throughout those 60 years, and showing an annual decline of 320 ml decade^{-1}. However, a cross-sectional comparison of vital capacity for these individuals in 2000 (3.19 versus 5.27 l) would overestimate this change: 350 ml decade^{-1}.

Sampling Considerations

Within any population, there exists considerable intersubject variability. When scientists wish to infer population trends from experimental observations, as is the case with aging research, then choices concerning subject selection should ensure that this variability is reflected within the sample chosen. However, the inclusion of individuals possessing some characteristics, while perhaps more faithfully reproducing societal variations, may confound data interpretation concerning the nature of the aging process. While there is no doubt that aging is associated with broad decrements in physiological function (Masoro, 1995; Groeller, 2008), and an increasing incidence of medical complications, some lifestyle choices are known to accelerate these changes (Blair et al., 1996; Booth et al., 2000; Chakravarthy, 2008). The selection of such individuals, even without clinical evidence of dysfunction, may result in the masking of true aging. One such example involves smoking.

Smokers experience greater age-related decrements across a broader range of pulmonary functions than do nonsmokers (Knudson, 1981; Ware et al., 1990). Indeed, nonsmokers and asymptomatic individuals with a history of superior levels of habitual physical activity display considerable resistance to these changes (Booth & Lees, 2006), with peak function being well maintained for longer (Leblanc et al., 1970; Knudson, 1981). Thus, adverse changes in pulmonary function in older smokers exaggerate age-dependent trends, leading to spurious data interpretation. Yet for many years, researchers included smokers within their population samples (e.g., Frank et al., 1957; Cotes et al., 1966; Black & Hyatt, 1969; Burr et al., 1985; Fowler et al., 1987; Vollmer et al., 1988; Johnson et al., 1991a), though sometimes for good reason, and this practice continues within some contemporary research (DeLorey & Babb, 1999; Babb & Rodarte, 2000).

In an extension of this discussion, some would argue that we should seek to identify age-dependent changes in pulmonary function only in those without disease. However, some disease states are clearly age-dependent (primary aging) and should not be dismissed (Masoro, 2001), while others are merely associated with aging (secondary aging), and the inclusion of such individuals can skew experimental outcomes. This same argument has recently been extended to the recruitment of control subjects into a wide range of physiological and clinical experiments. It has been recommended that control samples be selected to ensure that their structural and functional status more closely matches the physically active people from whom we evolved (Holloszy & Kohrt, 1995; Booth & Lees, 2006). The logic here is that a more sedentary lifestyle is associated with an acceleration of physiological deterioration (Vandervoort & McComas, 1986; Kalu & Masoro, 1988; Fleg & Lakatta, 1988), such that the effects of aging and habitual inactivity may be additive. Indeed, given the very strong causal link between exercise habits, or the lack thereof, and a wide range of diseases (Blair et al., 1996; Booth et al., 2000; Chakravarthy, 2008), one can no longer assume an asymptomatic status to be indicative of a disease-free state.

From the previous discussion, it is evident that an extensive distillation of the exclusively age-dependent changes in pulmonary function is beyond the scope and confines of this chapter. However, in the text that follows, an attempt has been made to review the relevant literature with some sensitivity to this need.

SENSORY FEEDBACK

Within any regulatory loop (Figure 20.1), the decision of where to commence viewing parts of the loop can often be quite arbitrary. In this instance, it seems logical to commence with a discussion of the chemical and mechanical sensors that initiate deviations from the inherent ventilatory rhythm and volume excursions dictated by the medulla oblongata.

Chemoreceptor Function

There is a significant body of evidence indicating that the response to changes in blood gas partial pressures, via chemoreceptor feedback, appears to deteriorate with aging (Kronenberg & Drage, 1973; Hirshman et al., 1975; Altose et al., 1977; Peterson et al., 1981; García-Río et al., 2007). That is, the sensitivity of the ventilatory response to hypoxic and hypercapnic challenges appears to be attenuated within older individuals. However, most early experiments were performed by relying upon the magnitude of the ventilatory response as the primary index of chemoreceptor sensitivity. While these observations may indeed be correct, it is also possible that the ventilatory responses could have been affected by age-dependent changes in pulmonary function, such that pulmonary mechanical or neuromuscular changes were being measured, and not altered chemoreceptor or central integrator sensitivity. In attempts to isolate this possibility, subsequent researchers have used the inspiratory mouth-occlusion pressure technique (Whitelaw et al., 1975), in which the airway is transiently occluded during inspiration, and the inspiratory pressure 100 ms after occlusion is then taken as the index of central ventilatory drive. In addition, mean inspiratory flow has been investigated (Milic-Emili & Grunstein, 1976), and this index is derived by normalizing tidal volumes to the corresponding inspiratory duration.

Peterson and colleagues (1981) incorporated all three measurement approaches in their evaluation of hypoxia and hypercapnia in aged individuals (65–79 years). They confirmed previous evidence, with the ventilatory responses of their older subjects being about half that observed in their younger counterparts (see also Brischetto et al., 1984), and these differences persisted even when data were normalized for body size. These observations demonstrated that it was possible that chemoreceptor sensitivity decreased with aging, but one could not exclude the possibility that mechanical or neuromuscular factors were involved. However, both mean inspiratory flows and occlusion pressures confirmed these ventilatory observations. Since the inspiratory duty cycle (inspiratory time divided by total cycle duration) did not differ between the two groups, it was deemed unlikely that these age-dependent changes reflected altered medullary control of ventilatory timing (von Euler, 1977). Furthermore, when occlusion pressures were normalized to maximal inspiratory pressure, the age difference persisted, excluding the possibility that muscle weakness influenced these data. Accordingly, the authors concluded that these observations were more consistent with reduced central inspiratory muscle drive with advancing age. They speculated that equivalent hypoxic and hypercapnic responses were unlikely to reflect altered chemoreceptor function, but may perhaps have resulted from altered medullary function.

These observations are most convincing and have most recently been verified by García-Río et al. (2007), who also demonstrated that responses did not change further beyond 75 years. However, not all researchers provided supporting evidence. For instance, Rubin and associates (1982) failed to observe age-dependent differences in either the ventilatory (also see Patrick & Howard, 1972; Chapman & Cherniack, 1987) or the occlusion pressure responses to hypercapnia. They did, however, observe a significant reduction in mean inspiratory flow, and when they simultaneously evaluated data from four different studies (Patrick & Howard, 1972; Kronenberg & Drage, 1973; Altose et al., 1977; Rubin et al., 1982), they found a statistically significant reduction in the ventilatory responses of the elderly to hypercapnia.

On balance, one may interpret these observations to show a blunting of the hypercapnic and hypoxic responses. There also appear to be age-dependent differences in the interaction of these stimuli, with older individuals being less sensitive to challenges provided by combined hypercapnic and hypoxic gas mixtures (Chapman & Cherniack, 1987). Moreover, Poulin and colleagues (1997) found that ventilatory responses to a hypercapnic challenge were not affected by aging when applied under normoxic or hyperoxic states, but when applied during hypoxia, the ventilatory responses were dampened.

One obvious mechanism for such observations is a deterioration of the chemoreceptors themselves. Indeed, two rat studies, Conde et al. (2006) and Dymecka et al. (2006), have described such changes. The latter provided morphological evidence that the carotid bodies of aged rats had atrophied. The former group measured neurotransmitter turnover within the chemoreceptors, and carotid sinus nerve sensory activity during hypoxia and hypercapnia. Their observations included a lower volume of chemoreceptive tissue, along with slower turnover times and reduced neurotransmitter release in their older animals.

Secondary Receptors of the Respiratory System

The respiratory system is inextricably integrated within several regulatory systems and, as such, it obtains feedback not just from the chemoreceptors, but from various mechanical and metabolic sensors distributed throughout the body. Indeed, during exercise, feedback from these secondary receptors dominates ventilatory control. It is therefore appropriate that we briefly review the affect of aging upon some pulmonary mechanoreceptor feedback.

Tack and others (1981) investigated changes in the perception of ventilatory elastic loads. These loads were applied by switching an open-circuit airway into a closed circuit, at the end of which was one of 13 rigid containers, each with different dimensions. Thus, the volume of air within each closed circuit was changed, thereby imparting an elastic load. They found that elderly individuals possessed a reduced ability to distinguish among changes in these elastic loads. In a subsequent study, the same group applied a range of flow-resistive loads (Tack et al., 1982) and found that aging reduced the ability to perceive differences among these resistors (tubes of varying diameter). Rubin and associates (1982) also reported a reduced responsiveness to resistive loading. In this experiment, there were no significant differences in the inspiratory occlusion pressure, but mean inspiratory flow was significantly lower in the aged. Thus, not only were these older subjects less aware of such changes (Tack et al., 1982), they also demonstrated less powerful centrally mediated pulmonary reactions (Rubin et al., 1982). Furthermore, Tack and others (1983) also tested the ability of younger and older subjects to reproduce a fixed tidal volume while breathing against flow-resistive and elastic loads. They found that their older subjects were less able to achieve the target volumes as loading increased. These observations are qualitatively similar to age-dependent reductions in other mechanoreceptor functions (Kokmen et al., 1978).

Summary

The ventilatory response to hypoxic and hypercapnic challenges appears to be less sensitive in most, but not all, older individuals. It is possible that chemoreceptor sensitivity decreases with aging, but the available human evidence is more consistent with a reduced inspiratory muscle drive accompanying altered central nervous system function. However, animal studies have provided evidence of the carotid bodies becoming atrophied in older rats. Thus, it is possible that age-dependent reductions in ventilatory sensitivity can accompany changes to both chemoreceptor and medullary functions. In addition, the respiratory system obtains feedback from mechanical and metabolic sensors. Aging clearly affects mechanoreceptor feedback, with elderly individuals being less able to distinguish changes in elastic and resistive respiratory loads.

CENTRAL INTEGRATION

Feedback from the peripheral and central chemo-, metabo-, and mechanosensitive structures is evaluated within the dorsal and ventral groups of the medulla oblongata (Figure 20.1). This central integration results in a modulation of effector function (Werner et al., 2008), and it is quite possible that central processing is affected by aging. However, the complexity of mammalian regulatory systems makes an unequivocal quantification of such changes almost impossible to perform.

Nevertheless, Peterson and associates (1981) and Tack and colleagues (1981, 1982) have speculated that their observations do not reflect reduced sensory discrimination and feedback, but a gradual deterioration of the central integration and processing of this information. Clearly, evidence from Conde and others (2006) and Dymecka and associates (2006) concerning chemoreceptor function in aging rats, while not contesting the possibility of reduced central function, demonstrates that sensory feedback may also be impaired.

CONTROLLED VARIABLES

To facilitate optimal alveolar gas exchange (Figure 20.1), air and blood are pumped through the lungs such that the volume and timing of both cycles are adjusted to maximize metabolic efficiency (Otis, 1954, 1964). Indeed, the adaptations that accompany repeated performance of most neuromuscular tasks (e.g., rowing, running, cycling, swimming) result in greater efficiency. However, before we can explore age-dependent changes in the function of the ventilatory pump, we must first determine whether the morphological and mechanical properties of the ventilatory pump have changed during the aging process, since changes in either of these characteristics will affect ventilation and its metabolic cost, and also alveolar gas exchanges.

Morphological Considerations

Ventilation delivers air to the alveoli (alveolar ventilation) for diffusive gas exchange within the blood. In young adults, approximately 57% of the lung volume is made up of the alveoli (Weibel & Gomez, 1962). The larger airways represent ≈10% of this volume, with the smaller bronchioles and alveolar ducts taking a further 27% (Weibel & Gomez, 1962). These structures provide pathways for the oscillating, convective delivery of oxygen to, and the removal of carbon dioxide from, the alveoli. The remaining lung volume (6%) is made up of pulmonary blood vessels and other tissues. As a consequence of this configuration, the surface area of adult lungs is about 60–80 m^2 (Weibel & Gomez, 1962; Weibel, 1963), and natural selection has ensured that this surface area corresponds to that of the pulmonary capillaries.

This state differs somewhat, however, from the morphological pattern evident within both adolescents and the elderly, such that neither postnatal growth nor degenerative pulmonary changes associated with senescence follow linear functions (Thurlbeck & Angus, 1975). It appears that, while the airways increase in size, the alveoli increase in number during the first 8 years (Dunnill, 1962; Thurlbeck & Angus, 1975) and then expand in size over the next 10–15 years to attain the mature state (Thurlbeck & Angus, 1975). Indeed, the development of pulmonary function ends at 20–25 years (Knudson, 1981). At the other age extreme, autopsy evidence from a 74-year-old has shown a reduction in the relative lung volume occupied by the alveoli, declining from 57 to 52% (Weibel & Gomez, 1962). While these data were collected from only one elderly specimen, subsequent postmortem experiments by Ryan and associates (1965) and Thurlbeck (1967a) verified this observation. Moreover, the alveoli appear to become progressively enlarged with advancing age (Sobin et al., 1988), with some evidence of alveolar fusion (Pump, 1971), not unlike that seen with emphysema (Pierce & Ebert, 1958a; Anderson et al., 1964).

As a consequence, one often sees reference within the older literature to the phrase "senile lung." As one might anticipate, these changes are often accompanied by a significant reduction in the area–volume ratio of elderly lungs, with the possibility of a loss in the number of alveoli (Thurlbeck, 1991; Janssens et al., 1999). Furthermore, there is a reduction in the alveolar surface area, which displays a gradual decline beyond 30 years and can approach a 30% reduction by the ninth decade (Thurlbeck, 1967b). These morphological changes have potentially significant implications for gas exchange (Hamer, 1962; Muiesan et al., 1971).

These age-dependent structural changes appear also to be reflected within the shape of aged lungs. For instance, Anderson and colleagues (1964) found that, beyond 60 years, the lungs take on a more spherical shape, as reflected by an enlargement of the anteroposterior diameter relative to lung height. The latter was found to remain stable beyond the sixth decade, while the former continued to increase through to the eighth decade. Thus, the ratio of these dimensions changed from 0.75 in specimens <40 years of age to 0.90 in lungs obtained from 80- to 90-year-old individuals. This characteristic had previously been described as though the thorax had taken on a barrel shape (Pierce & Ebert, 1958a).

With respect to airway diameter, Niewoehner & Kleinerman (1974), also using postmortem specimens, demonstrated that while the diameters of the larger airways did not appear to change with aging, those of the bronchioles increased to a peak at about the fourth decade and thereafter declined. This change will adversely affect flow resistance within these airways (Niewoehner & Kleinerman, 1974).

Coupled with these changes in the lung tissue are morphological variations in the pulmonary vasculature. Thurlbeck & Angus (1975) suggested that changes within the aged lung did not represent just altered alveolar shape, but an absolute reduction in the tissue of each alveolus. Though unable to measure pulmonary capillaries, they interpreted an observed reduction in alveolar parenchymal tissue to also reflect a loss of pulmonary capillaries.

Aging is accompanied by increased wall thicknesses of the pulmonary arteries (Semmens, 1970), and Heath (1964) has presented microscopic evidence of fibrosis within the inner layers of the pulmonary arteries. However, Butler & Kleinerman (1970) did not find a change in capillary density with aging. Nevertheless, these changes will reduce vessel compliance and increase pulmonary vascular resistance and arterial pressure (Emirgil et al., 1967; Ehrsam et al., 1983; Lam et al., 2009) and thereby affect pulmonary capillary perfusion. Accordingly, Krumholz (1966) described a reduced capillary blood volume in older individuals, and others have reported small reductions in pulmonary perfusion (Krumholz, 1966; Georges et al., 1978; Crapo et al., 1982), but these seem to occur only in the very aged (Knudson, 1991).

In summary, the primary morphological characteristic of aging lungs is a reduction in the volume occupied by the alveoli, which gradually become enlarged and fewer in number. This change is accompanied by reductions in the area–volume ratio, the alveolar surface area, and the diameter of the bronchioles. Furthermore, the pulmonary vasculature changes, experiencing increases in wall thicknesses, reduced vessel compliance, and increased vascular resistance and arterial pressure.

Mechanical Considerations

Changes in Collagen and Elastin Composition

Connective tissue contains three fibrous substances: collagenous fibers, elastic fibers, and reticular fibers. All three are contained within the lung, but our focus is upon the first two and how aging may affect their composition and pulmonary function.

The collagenous fibers of the lungs are made from the protein collagen, and all significant pulmonary structures contain these collagens. Indeed, these are the most important proteins within the lungs, and they give the lungs tensile strength (a resilience to being stretched). The elastic fibers, which are about 50% fewer in number than the collagenous fibers (Pierce & Ebert, 1965), contain the protein elastin and possess the characteristic of elasticity or elastance, the ability to be distorted by stretching and then to return to an original shape. This is easy to visualize with an elastic band. However, just like the band, the elastic fibers of the lung will, over time, lose some of their inherent elasticity (recoil). Since the elastic fibers can also be stretched quite easily, they are said to be very compliant (an ability to be stretched without breaking). Indeed, elastin can be extended to ≈140% of its resting length before fiber failure (Starcher, 1986). Conversely, the collagenous fibers have a low compliance but, if stretched, are highly elastic. However, these fibers will fail when stretched beyond 2% of their resting length (Starcher, 1986). Because of the helical configuration of the collagenous fibers, the lungs can expand without actually stretching these fibers, but once uncoiled, further lung expansion is resisted (Mead, 1961; Pierce & Ebert, 1965). Thus, both the collagens and elastin are responsible for the elastic recoil of the lungs, and this affects the work of breathing.

In terms of their tissue contribution by mass, the collagens comprise about 15% (Laurent, 1986) and elastin about 30% (Starcher, 1986) of the dry mass of the lungs. The vast network of these fibers throughout the lungs, and their intimate association with each other, means that forces encountered by the lungs are distributed across the bulk of the lung mass (Pierce & Ebert, 1965). Indeed, alveolar expansion is transmitted through these collagenous and elastic fibers to the airways and blood vessels, thereby ensuring that not only airflow, but also local perfusion, more closely matches local ventilation. In fact, the elastic recoil of the lung is dictated by the presence of elastic and collagenous fibers within the parenchyma, and in particular at the mouths of alveoli and the alveolar ducts (Pierce & Ebert, 1965). This recoil causes the tendency of the alveoli to return toward zero volume, while simultaneously expanding the airways.

Some structures, for example airways, alveoli, and many blood vessels, are both compliant and elastic, while others do not possess either quality (bone), and still others have a relatively low compliance but are highly elastic (tendons). Like all living structures, these collagenous and elastic fibers are continually being remodeled by fibroblasts, so during the course of aging, and in various disease states, the integrity of the collagens and elastin is dictated by functional and pathological changes that may affect protein turnover. Changes in the composition of collagens and elastin within the lungs can affect the flow-resistive and elastic mechanics of ventilation and the static and dynamic lung volumes.

The properties of the collagenous and elastic fibers appear to change with advancing age. For example, Sobin and others (1988) showed a clear increase in the width of both collagen and elastin fibers in the alveolar walls of recently deceased older individuals (16–83 years). Furthermore, these fibers tended to become straighter in older specimens, though intersubject variability was quite large. The contemporary view of many is that aging humans experience a loss of elastin and an increase in collagen within parenchymal tissue. Nevertheless, the literature does not provide an unequivocal position.

A number of investigators have furnished evidence for a loss of elastin and an increase in collagen across species. For instance, Wright (1961) reported a gradual decline for aging humans in both the thickness of individual elastic fibers and their total number. This loss was ubiquitous, evident even in adults ages 50–60, but was most pronounced in individuals >80 years of age. This observation is somewhat consistent with those of Sugihara and colleagues (1971), who observed that lung parenchymal tissue experienced an increase in its resting length and a reduction in its compliance with advancing age, with the former causing the latter. Similarly, Pump (1974) described age-dependent reductions in the size and number of interalveolar fenestrae. However, Berend and associates (1980) were unable to verify this on excised specimens. More recently, D'Errico and others (1989), using postmortem histochemical analyses, found a reduction in elastic fibers within the alveolar wall, but not within the airways. Similarly, Foster & Curtiss (1990) have shown a dramatic (75–80%) reduction in the precursor of elastin (tropoelastin mRNA) in the lungs of aging rats. More recently, Huang and colleagues (2007) demonstrated similar changes in the parenchymal tissue of mice, along with an elevation in collagenous fiber content. However, they found that these changes were not simultaneous, with the reduction in elastin preceding the collagen elevation.

Conversely, Pierce & Ebert (1965) found that aging was associated with an increase in elastin within some parts of the lungs, but not within the parenchyma. This was confirmed through electron microscopy by Adamson (1968). However, Rickert & Forbes (1976) reported that the total amount of connective tissue in the lung varied independently of age, but there was a decrease in collagen present within the tissue. This observation was contradicted by more recent data provide by Takubo and associates (1999), who investigated the affects of aging on the extracellular matrix of mouse lungs. They found that, while the total collagen content was significantly higher, the elastin content did not change significantly with age. Previously, Berend and others (1980) observed that the characteristics of the collagenous fibers did not appear to change with aging. Similarly, Lang and colleagues (1994) reported that in nonsmokers, there was not a significant correlation between age and the collagen content of alveolar tissue, when expressed relative to either the surface area of the wall tissue or the volume of the distended lung. Thus, as alveolar surface area per unit lung volume declines with age (Ryan et al., 1965; Thurlbeck, 1967a; Sobin et al., 1988), the main collagenous framework of the lungs appears to be maintained.

From this discussion, it is clear that a consensus within the literature still does not exist for this topic. While the view of some is that advancing age is accompanied by a loss of elastin and increase in collagen from the parenchymal tissue, one struggles to find an unequivocal voice on this topic.

Elastic Recoil of Lung Tissue and the Chest Wall

It is evident that some structural changes accompanying aging can have a direct impact on the elastance of the lungs and chest wall. Our first interest with respect to these mechanical changes is the well-established tendency for the lungs to become more compliant (Frank et al., 1957; Pierce & Ebert, 1958b; Turner et al., 1968; Gibson et al., 1976; Knudson et al., 1977; Colebatch et al., 1979; Chaunchaiyakul et al., 2004). While in this section the focus is upon the mechanical properties of the lungs and chest wall, such changes will also affect the elastic and flow-resistive work of breathing and the static and dynamic lung volumes.

The classical work of Turner and associates (1968) is perhaps the most widely cited of this research (e.g., Levitzky, 1984; Knudson, 1991; Sprung et al., 2006), with their data showing that, at a lung volume of approximately 60% of the total lung capacity, the static recoil (transpulmonary) pressure of the lungs decreases at a rate of approximately 0.1 kPa decade^{-1} (1 cm H_2O decade^{-1}). Unfortunately, closer inspection of this seminal research reveals that the sample used for this experiment was less than ideal. There was a bias toward the younger ages (61% <40 years), with 20% falling within the range 40–50 years and 19% >50 years. Only two subjects were >60 years (both were 61 years old), and since it is now recognized that pulmonary function changes in a nonlinear manner beyond the fourth decade (Glindmeyer et al., 1982; Knudson, 1991), these limitations are not insignificant. However, of greater importance is the fact that only three of the eight individuals >50 years were nonsmokers, while three were heavy smokers (>9000 lifetime packs).

These limitations were addressed to varying degrees in subsequent research by Gibson and others (1976), Knudson and colleagues (1977), and Chaunchaiyakul and associates (2004). Of these studies, we will focus upon only that of Knudson and others (1977), for it contained the largest sample, covered the greatest age range, and included both genders. This group studied 51 healthy subjects across three age groups (25–35, 36–64, and 65–75 years), screened from an original pool of >3000 (Knudson et al., 1976) and satisfying rigorous selection criteria (no cardiopulmonary disease, never smoking, negative for α_1-antitrypsin deficiency, could forcibly exhale 75% of vital capacity in 1 s, normal chest X-ray and physical examination). In agreement with Turner and colleagues (1968), this investigation also reported that lung elastic recoil decreased as age progressed, resulting in a leftward displacement of the transpulmonary pressure–volume relaxation curve, as shown in Figure 20.2. This effect is more pronounced at higher lung volumes (Turner et al., 1968; Knudson et al., 1977) and may be a function of the progressive increase in alveolar size at high lung volumes as people age (Knudson, 1991). However,

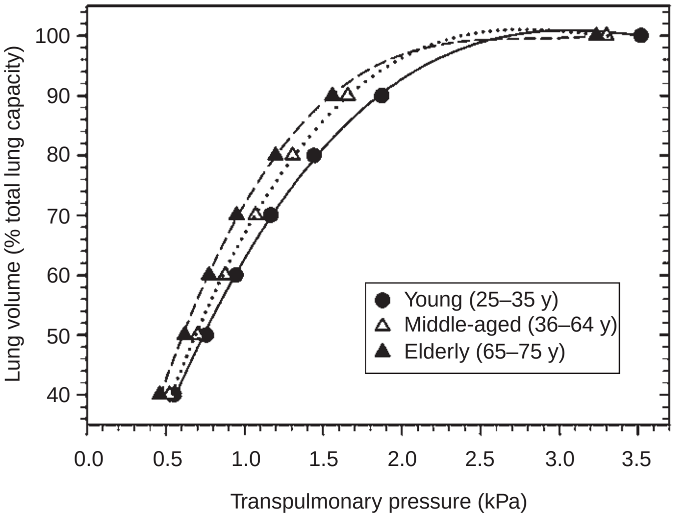

Figure 20.2 Age-dependent changes in static lung-tissue elastic recoil drawn using data extracted from Knudson and associates (1977). Least-squares, best-fit regression curves were applied to each data set.

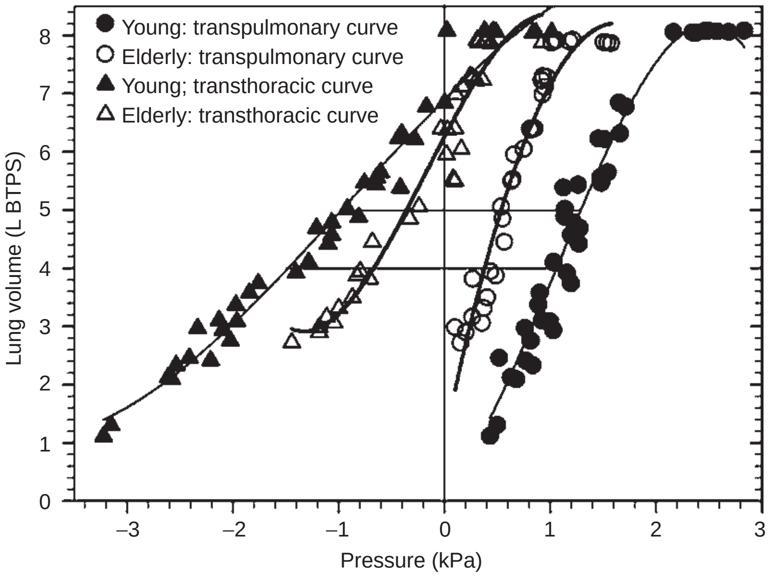

Figure 20.3 Age-dependent changes in the static pressure–volume relaxation curves of the lung tissue (transpulmonary) and chest wall (transthoracic). Lung volumes include residual volume. Representative data from one young and one elderly subject from Chaunchaiyakul et al. (2004) are shown, used by permission of the American Physiological Society. Horizontal lines illustrate volume (1 L) and pressure changes during tidal breathing, and thus define the dimensions of lung-tissue and chest-wall static work. Least-squares, best-fit regression curves were applied to each data set.

the data of Knudson and colleagues (1977) can quite confidently be claimed to be more reflective of true aging and without bias introduced through sampling limitations. Indeed, these observations, which have been closely supported by changes in excised lungs (Berend et al., 1980), show that at 60% of total lung capacity, static lung recoil pressure declined at 0.03 kPa decade^{-1}. This is about 65% less than originally reported by Turner and associates (1968). Such a small change might be expected to have minimal physiological significance in resting states, but, as will become apparent below, this does not hold during exercise, particularly when considered with the chest wall.

Gender differences in lung recoil changes were apparent in some (Bode et al., 1976) but not other studies (Colebatch et al., 1979). However, such differences may reflect a gender-related stature divergence and its affect on the number of alveoli within each lung (Angus & Thurlbeck, 1972), and therefore total pulmonary recoil pressure (Knudson et al., 1977). Thus, when normalized for stature-dependent lung-volume differences, the between-gender variations in lung-tissue recoil were largely removed, in both young and aging samples (Gibson et al., 1976; Knudson et al., 1977). However, this view is contested by Berend and associates (1980), who found that the excised lungs of men and women displayed roughly equivalent recoil. They suggested that, despite the best efforts of the researchers, the gender differences may have been a simple artifact associated with a failure to inflate the lungs completely at total lung capacity. Regardless of the explanation, one may reasonably conclude that gender-dependent variations in lung-tissue elasticity do not appear to exist, either in young adults or across the age spectrum (Knudson et al., 1977; Berend et al., 1980).

Our second area of interest in pulmonary mechanics, though less thoroughly investigated, centers upon the chest wall, which may undergo more significant modifications with aging. Indeed, the rib cage tends to get stiffer (Thomas et al., 1986), and this is associated with a gradual calcification of the intrathoracic joints (Edge et al., 1964; Estenne et al., 1985) and reduced intervertebral spaces (Edge et al., 1964). As a consequence, inspiration results in proportionately greater abdominal than rib cage displacement relative to that observed in younger individuals (Rizzato & Marazzini, 1970), and this becomes more evident during exercise (Teramoto et al., 1995).

The affects of aging on chest-wall mechanics have been investigated by just a few groups (Pierce & Ebert, 1958b; Mittman et al., 1965; Rizzato & Marazzini, 1970; Estenne et al., 1985; Chaunchaiyakul et al., 2004). The consensus from these experiments is that, with advancing age, the chest wall becomes less compliant (Mittman et al., 1965; Chaunchaiyakul et al., 2004), and this includes both its rib cage and its abdomen–diaphragm components (Estenne et al., 1985). In addition, there is a rightward displacement of the thoracic pressure–volume relaxation curve, as illustrated in Figure 20.3. These changes have a significant impact upon the work of breathing.

Static Work of Breathing

The combined influences of changes in the mechanical attributes of the lung tissue and chest wall are expressed within changes in the static work of breathing, since ventilation results in the expansion of the lungs and chest wall, and since static work is a function of the instantaneous volume of each structure. Indeed, with advancing age, the relative importance of each compartment may vary among individuals, depending upon just how each has been affected by aging.

There are separate reports that aging humans experience a reduction in lung-tissue (Frank et al., 1957; Turner et al., 1968; Gibson & Pride, 1976; Galetke et al., 2007) and an elevation in chest-wall static work (Mittman et al., 1965). However, only one group has simultaneously investigated both of these static work components during aging (Chaunchaiyakul et al., 2004). From this research, and in agreement with earlier observations on the separate static work components, it is evident that the pressure–volume relaxation curves are displaced, with that for the lung tissue moving leftward and that for the chest wall moving to the right. These changes have significant implications for the static work of breathing, and in Figure 20.3, variations in transpulmonary and transthoracic pressure over a 1-L tidal volume are defined (horizontal lines). The areas within these lines and between each pressure–volume relaxation curve and the zero pressure axis define the lung-tissue and chest-wall static work (respectively; Taylor & Morrison, 1999). Though not illustrated, the static work for the entire system is similarly derived using the transrespiratory pressure–volume relaxation curve, which is simply the sum of the other two curves, and total respiratory static work equals the lung-tissue plus the chest-wall fractions.

Figure 20.4 shows differences in chest-wall and lung-tissue static work during rest and while exercising at 60% of the heart-rate reserve, for three groups of subjects ages 20–83 years (Chaunchaiyakul et al., 2004). These data were normalized to a 1-L tidal volume since the absolute static work reflects variations in breathing pattern. At rest, no significant differences in total static work of breathing were found among these age groups. However, static work performed on both the lung tissue and chest wall changed significantly with advancing age. In the former compartment, static work fell by 41% (middle-aged) and 46% (elderly). These observations are entirely consistent with changes in lung-tissue elastic recoil. Chest-wall static work, which quantifies elastic energy stored within the chest wall, became significantly less negative with age (Figure 20.4), decreasing by ≈75% (middle-aged) and 110% (elderly).

A similar pattern for static work, normalized to tidal volume, was evident during exercise, which was set at an equivalent relative intensity for all subjects (Figure 20.4). Because of the rightward displacement of the chest-wall pressure–volume curve (Figure 20.3), more transthoracic pressure–volume points fell to the right of the zero pressure axis as the lungs filled. That is, while chest-wall static work is normally negative at rest in younger adults, during exercise, tidal excursions crossed the zero pressure axis, producing positive transthoracic pressures (Taylor & Morrison, 1999). This means that less energy was now being stored during inspiration and that a progressively greater fraction of the positive work that is performed on the chest wall must now be executed by the respiratory muscles. In fact, ≈85% more positive chest-wall static work was evident for the middle-aged subjects, while the elderly group performed ≈175% more positive work, relative to their younger counterparts (Figure 20.4). Similarly, some negative transpulmonary pressures were produced, reflecting energy storage at the end of inspiration that can be regained during exhalation. As a consequence of these altered mechanical changes, the nature of respiratory muscle work appears to have changed with aging (Chaunchaiyakul et al., 2004).

Positive static work must be performed by the inspiratory muscles, and each age group contained individuals in whom this type of work was performed on the chest wall. However, the older subjects experienced a much greater tendency to shift to performing more inspiratory muscle work when exercising, and this change reflected a modification in the manner in

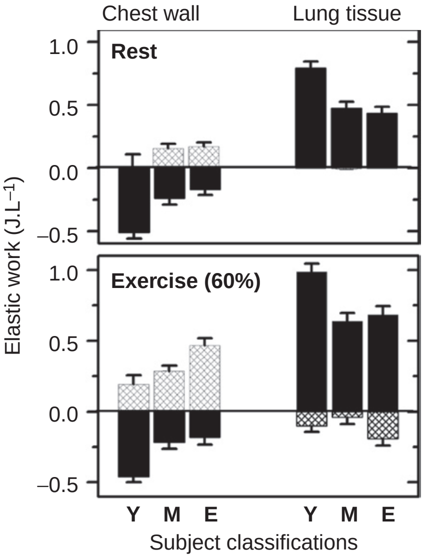

Figure 20.4 Chest-wall and lung-tissue static work of breathing in young (Y; 20–30 years, $N = 20$), middle-aged (M; 40–50 years, $N = 19$), and elderly (E; >60 years, $N = 19$) healthy adults during rest (top) and steady-state exercise at 60% of the cardiac reserve (bottom). Data were normalized to a 1-L tidal volume (from Chaunchaiyakul et al., 2004, and used by permission of the American Physiological Society).

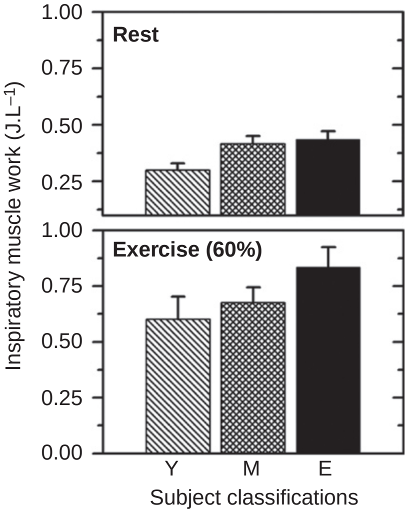

Figure 20.5 Inspiratory muscle work in young (Y; 20–30 years, $N = 20$), middle-aged (M; 40–50 years, $N = 19$), and elderly (E; >60 years, $N = 19$) healthy adults during rest (top) and steady-state exercise at 60% of the cardiac reserve (bottom). Data were normalized to a 1-L tidal volume (from Chaunchaiyakul et al., 2004, and used by permission of the American Physiological Society).

which energy was being stored within the chest wall over the ventilatory cycle. A simple way to envisage energy storage is to evaluate the ratio of positive-to-negative chest-wall static work (ignoring signs). At rest, the ratio for the young adults was 0.04, while that for the elderly group was 1.00, indicating equal positive and negative components. During exercise, the ratios were greater for each group, but for the younger individuals they remained stable across three different work rates (0.42–0.49). In the elderly subjects, positive chest-wall static work increased to become two- to threefold greater than negative work (2.57–3.44). Thus, positive chest-wall work, which assists expiration, became a major static work component during exercise in the elderly subjects.

Static inspiratory muscle work is the sum of the positive and negative static work components derived for both the lung and the chest wall (Taylor & Morrison, 1999; Figure 20.5). Each age group had similar inspiratory muscle work at rest, and this increased during exercise. While the form of static work changed from the young to the elderly, this change did not significantly alter inspiratory muscle work during exercise. Instead, it modified the ventilatory phase in which this work was performed. Thus, with advancing age, a parallel displacement of the chest-wall pressure–volume curve resulted in a shift from energy being stored primarily during expiration to an equivalent or greater energy storage during inspiration, and this was evident at rest and during exercise. These changes represent a redistribution of the tissues on which, and the ventilatory cycle phase during which, this work was performed. It is believed that these observations represent a physiological consequence of aging in asymptomatic individuals, which was largely independent of age-related changes in the ventilatory pattern (Chaunchaiyakul et al., 2004).

Airway Resistance, Dynamic Airway Collapse, and the Closing Volume

The mean internal diameter of the bronchioles, but not the large airways, decreases quite significantly in older adults (Niewoehner & Kleinerman, 1974). Since it is the medium-sized bronchi that offer the greatest resistance to airflow (Macklem & Mead, 1967), such changes have an impact on ventilation, particularly during heavy breathing (e.g., exercise) and forced expiratory maneuvers. Indeed, it is through the relationship defined by Poiseuille's Law that the net result of these age-dependent changes in the morphological and mechanical properties of the adult lung will dictate airflow. That is, for a given driving pressure, flow is largely dictated by the fourth power of the airway diameter, such that halving the airway diameter increases flow resistance 16-fold.

In young asymptomatic adults, there is considerable tolerance to changes in airway dimensions. However, in all individuals, there is a critical size below which even slight bronchiolar diameter reductions will adversely affect airflow. Not surprisingly, age-dependent changes in these dimensions, which significantly increase airway resistance in the elderly during spontaneous breathing (Frank et al., 1957; Mead et al., 1967), mean that some people are closer to this critical diameter, and for these older individuals, there is the possibility that even minor subsequent airway narrowing will significantly obstruct airflow (Niewoehner & Kleinerman, 1974).

The flow-resistive work of breathing is determined by the extent to which respiratory muscle force must be generated to overcome the frictional forces associated with airflow and tissues movements (Otis et al., 1950; Otis, 1964). Thus, flow-resistive pulmonary work is a function of instantaneous airflow and, because of age-dependent changes in airway dimensions, it has been established that this form of pulmonary work is elevated with aging (Otis, 1964), particularly during exercise (Aaron et al., 1990). This is perhaps best illustrated through changes in the metabolic cost of breathing, as reflected in that portion of the whole-body oxygen consumption that may be assigned to the respiratory muscles (Aaron et al., 1992a).

Hyperpnea initially increases proportionately with external work (the ventilatory equivalent), but it eventually reaches a point beyond which hyperventilation commences (Owles point or the ventilatory turnpoint; Owles, 1930), and minute ventilation now increases out of proportion to the metabolic demand for oxygen. This typically occurs at approximately 60–65% of one's maximal aerobic power. In young, healthy adults working below this point, the respiratory muscles add ≈10% to the whole-body oxygen consumption (Aaron et al., 1992b). This contribution increases to ≈15% during moderate-to-heavy exercise and to approximately 40% when approaching peak aerobic power (Aaron et al., 1992b). When older individuals were contrasted with younger subjects at an equivalent peak aerobic power, it was found that respiratory muscle oxygen consumption was significantly elevated at the same minute ventilation, with this deviation being evident across ventilatory flows from ≈80 L min^{-1} through to peak ventilation (Aaron et al., 1990). Thus, greater respiratory muscle work was required from this aging sample, and this was presumably due to a greater airway resistance (Frank et al., 1957; Mead et al., 1967) and inspiratory muscle work observed in older individuals (Chaunchaiyakul et al., 2004).

In all people, the terminal airways of the (gravity-dependent) alveoli will eventually collapse during exhalation, and this will result in air being trapped behind these airways (Milic-Emili et al., 1966; Burger & Macklem, 1968). This is most evident when the lungs approach residual volume, and this trapped air is called the pulmonary closing volume, and its addition to the residual volume constitutes the closing capacity. However, with aging, the point at which airway collapse occurs moves progressively upstream, resulting in a gradual elevation in the closing volume (Anthonisen et al., 1969; Knudson et al., 1977). In young adulthood, the closing volume is ≈10% of the vital capacity, doubling beyond 40 years (Anthonisen et al., 1969; Leblanc et al., 1970). Moreover, with advancing age, further increments in the closing volume mean not only that it can exceed the functional residual capacity (Holland et al., 1968; Leblanc et al., 1970), but it can even impede tidal volume excursions (Knudson, 1991), particularly during exercise (Johnson et al., 1991b). This has a considerable impact upon the distribution of ventilation and pulmonary gas exchange.

Several mechanisms explain this increased closing volume in the aged. First, there are age-dependent changes in lung-tissue elastic recoil (Wright, 1961; Mead et al., 1967; Babb & Rodarte, 2000). During inspiration, alveolar expansion is transferred to the airways by the collagenous and elastic fibers within the parenchyma (Pierce & Ebert, 1965). As these fibers lose their inherent elasticity with aging (Huang et al., 2007), their capacity to expand the airways diminishes. This effect is most evident at high lung volumes (Turner et al., 1968; Knudson et al., 1977). However, dynamic airway collapse occurs over the last 25–40% of forced expiration, and, over this volume range, the impact of changes in parenchymal elasticity on airway dimensions is much less important (Knudson, 1991). Therefore, a second mechanism may perhaps be more likely to explain this collapse: a loss of the natural recoil of the airways (Knudson et al., 1977). The airways are now less able to resist collapsing. Moreover, the narrower bronchial airways of the elderly are closer to their critical diameters (Niewoehner & Kleinerman, 1974), thereby increasing the physiological significance of further narrowing. Third, it has been suggested that older individuals experience a greater transpulmonary pressure at the point of airway collapse (Holland et al., 1968; Islam, 1980). Since transpulmonary pressure is the difference between the alveolar and the intrapleural pressures, and since airway flutter occurs at transpulmonary pressures close to zero (Mead et al., 1967), with closure generally occurring with negative pressures (intrapleural greater than alveolar pressure), this age-dependent change equates with closure commencing not just at a greater absolute lung volume, but also at higher alveolar pressures. The latter is presumably a function of elevated downstream airway resistance.

Respiratory Muscle Function

It is widely recognized that skeletal muscle function declines with aging, and this occurs through the frank loss of muscle mass (sarcopenia; Baumgartner et al., 1998; Doherty, 2003) and reduced neuromuscular structures and functions (Booth et al., 1994). These changes are also reflected within respiratory muscle function (Chen & Kuo, 1989; Johnson et al., 1991b; Tolep & Kelsen, 1993; Enright et al., 1994; Britto et al., 2009), although this is not universally observed (McElvaney et al., 1989). However, there appears to be a more pronounced reduction in the cross-sectional area of the expiratory (intercostal) muscle fibers beyond 50 years, while that of the inspiratory muscles appears to be well maintained (Mizuno, 1991), even though inspiratory muscle strength appears to be reduced (Britto et al., 2009).

These changes do not necessarily have adverse physiological consequences for most elderly people (Johnson & Dempsey, 1991), particularly within unstressed states. Nevertheless, habitual endurance training has beneficial affects upon respiratory muscle function, maximal voluntary ventilation, peak exercise ventilation, and the static lung volumes (Leith & Bradley, 1976; Clanton et al., 1987). Indeed, respiratory muscle training has been shown to be beneficial for some ventilatory functions in both young healthy and aged individuals (Belman & Gaesser, 1988; O'Kroy & Coast 1993; Nicks et al., 2009). However, ventilatory function during exercise appears not to be improved (Belman & Gaesser, 1988), principally because exercise in healthy individuals is not limited by the respiratory system (Stickland et al., 2008). Not surprisingly, for older, habitually active individuals, the age-dependent deterioration in pulmonary function is slower than observed in their sedentary counterparts

(Hagberg et al., 1988). No doubt functional changes evident within the sedentary are more reflective of lifestyle choices than of aging per se.

Summary

The properties of both the collagenous and the elastic fibers within the lung tissue change with advancing age. Unfortunately, while some suggest there is a loss of elastin and an increase in collagen from the parenchymal tissue, an unequivocal consensus on this topic is not available. Nevertheless, it is clear that older lungs have greater compliance and a reduced elastic recoil. At the same time, the chest wall becomes stiffer, and these combined influences modify the mechanical attributes of the lungs and chest wall, and the work of breathing. Indeed, aged lungs can be inflated significantly more easily, while more inspiratory muscle work must be performed to expand the chest wall. These differences become more pronounced during exercise, and change both the stage of the ventilatory cycle and the tissues on which this work must be performed. Moreover, morphological changes increase airway resistance, particularly during exercise, and the metabolic cost of breathing is elevated. Older airways also collapse more readily during more forceful expiratory efforts, and this adversely influences ventilation and gas exchange. Finally, respiratory muscle function declines with aging, particularly the expiratory muscles. Fortunately, these changes do not always have adverse consequences for the elderly, except during exercise.

Static Lung Volumes and Capacities

The intimate relationship between stature and vital capacity was first recognized and described by Hutchinson (1844). However, over the past 50 years, researchers have become acutely aware of the additional interaction of age-dependent changes in the morphological and mechanical properties of thoracopulmonary structures with this stature dependency, not just with respect to vital capacity, but also with regard to several other volume compartments. Indeed, one commonly sees reference to the vital capacity, in both its forced and its slowly expired forms, being reduced by about 20–30 ml year^{-1} (Levitzky, 1984; Oskvig, 1999; Sprung et al., 2006), implying that age-dependent decrements in lung volumes, capacities, and peak flows follow linear functions. However, we now know this generalization to be an oversimplification, as many pulmonary functions change quite gradually over the first 4 decades after the attainment of a physiological peak, particularly within healthy nonsmokers, and then decline more rapidly thereafter (Knudson, 1981, 1991; Glindmeyer et al., 1982).

The total lung capacity shows considerable resilience to aging, decreasing only slightly with senescence (Figure 20.6; Knudson, 1991; Sparrow & Weiss, 1995), if at all. This lung volume marks the point at which the strength of the inspiratory muscles is countered by the elastance of the chest wall resisting expansion, once the lung volume has reached

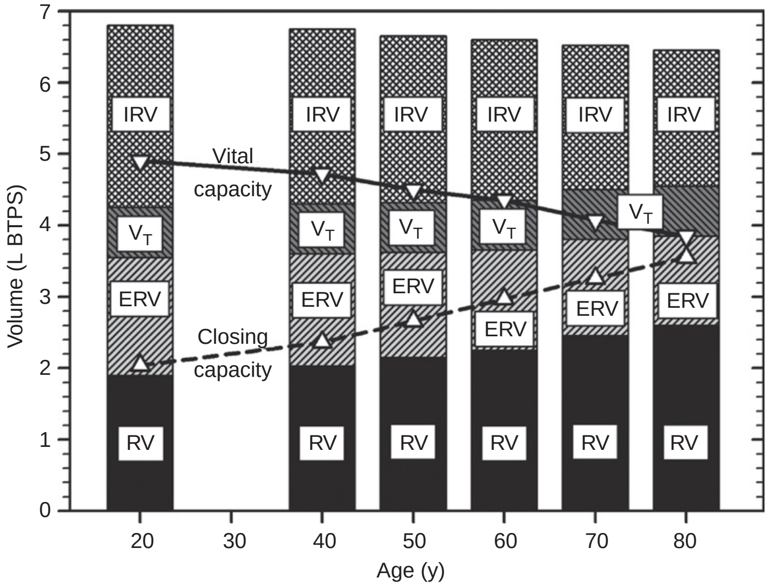

Figure 20.6 Age-dependent changes in the static lung volumes and capacities. Abbreviations: RV, residual volume; ERV, expiratory reserve volume; V_T, tidal volume (assumed to remain constant); IRV, inspiratory reserve volume. Sources: Edelman and colleagues (1968), Holland and associates (1968), Knudson (1981, 1991), Sparrow & Weiss (1995).

60–70% of the vital capacity, and it defines the maximal volume of air that can voluntarily be inhaled. Paradoxically, one finds that the age-dependent reductions in skeletal muscle strength and chest wall compliance described above are not accompanied by a corresponding fall in total lung capacity. This observation can first be explained by an elevated lung compliance, enabling greater lung expansion for the same muscular force, thereby largely cancelling the greater chest wall stiffness, and perhaps also by an elevation in the residual volume.

The balance of maximal expiratory effort with the recoil of the lungs and chest wall determines the residual volume (Leith & Mead, 1967): the volume of air remaining within the lungs following a maximal voluntary exhalation. With advances in age, weaker respiratory muscles, an elevated pulmonary compliance, and reduced chest wall compliance conspire to expand this volume (Figure 20.6). Furthermore, because of age-dependent narrowing of the bronchioles (Niewoehner & Kleinerman, 1974), it becomes increasingly harder to exhale forcibly, with dynamic airway collapse ensuring that more air remains trapped within the lungs at the point of terminating a maximal expiratory effort. It is therefore routinely reported that the residual volume rises with age (Frank et al., 1957; Boren et al., 1966; Edelman et al., 1968).

Perhaps the most widely studied lung volume is that which represents the difference between the total lung capacity and the residual volume. Hutchinson (1846) recognized the significance of this volume within the pattern of breathing encountered during daily activities, naming it the vital capacity. It is universally reported that this lung volume decreases with aging (Frank et al., 1957; Boren et al., 1966; Rizzato & Marazzini, 1970; Enright et al., 1993). However, this reduction is not linear since, beyond about 40 years, its rate of decline gradually gathers momentum (Figure 20.6; Schoenberg et al., 1978; Ware et al., 1990; Knudson, 1991). Because total lung capacity remains somewhat stable, or decreases only slightly, this change in vital capacity is almost entirely due to a progressive elevation in the residual volume (Knudson, 1991). Thus, the nonlinearity within the vital capacity reduction must be a function of that which occurs within the residual volume, and it must therefore be evident in all volumes, capacities, and peak flows (Knudson, 1981; Glindmeyer et al., 1982), with the possible exception of the total lung capacity. However, the use of nonlinear functions for age is rarely evident within prediction equations used to evaluate pulmonary function (Ferris et al., 1965; Boren et al., 1966; Withers et al., 1988; Roberts et al., 1991), since such terms, while being physiologically more precise, often fail to enhance predictive precision (Enright et al., 1993). Therefore, an appropriate statistical simplification has led many to assume falsely that pulmonary functions decline in a linear manner with advancing age. Moreover, according to Glindmeyer and others (1982), data derived from the many cross-sectional aging studies are unlikely to reveal this nonlinear pattern, which becomes apparent only when the same individuals are repeatedly evaluated over time (longitudinal experimental designs).

The net effect of the age-dependent increase in the compliance of the lungs and decrease in chest wall compliance will be reflected within the functional residual capacity: the summation of the residual volume and the expiratory reserve volume. In fact, by definition, this volume is dictated by the balance between the inherent tendency of the lungs to deflate and that of the chest wall to expand (Otis et al., 1950). Since the residual volume increases while the expiratory reserve volume declines, the trend for the functional residual capacity to show an age-dependent elevation (Figure 20.6; Turner et al., 1968; Roberts et al., 1991) is not always observed (Boren et al., 1966; Edelman et al., 1968).

In summary, the lung volumes and capacities are variously influenced by aging. While the total lung capacity is resilient, the residual volume and functional residual capacity increase, and the vital capacity and the expiratory reserve volume decrease with aging. Each of these changes occurs in a nonlinear manner.

Dynamic Lung Volumes and Ventilatory Flows

Some of the more useful diagnostic pulmonary function tests, at least for those with obstructive pulmonary disorders, are those that evaluate expiratory flows and volumes during maximal ventilatory maneuvers. Under these conditions, the morphological and mechanical properties of the airways will dictate expiratory flows within the effort-independent region for both healthy and diseased lungs (Hyatt et al., 1958; Holland et al., 1968). Indeed, this independence means that such forced expiratory flow–volume curves are highly reproducible. Therefore, since a number of the age-dependent pulmonary function changes resemble those seen in patients with obstructive diseases (Pierce & Ebert, 1958a; Anderson et al., 1964; Ito & Barnes, 2009), these tests are also sensitive to aging, particularly at the lower lung volumes and within individuals in whom the zone of dynamic airway collapse has migrated significantly upstream.

One test quantifies the exhaled volume over the first second of a forced expiratory effort ($FEV_{1.0}$). Knudson (1991) calculated the age-dependent annual decrement in this volume to be 28 ml $year^{-1}$ beyond 25 years using data from 14 cross-sectional studies. However, Burrows and colleagues (1986) found that when individuals were studied longitudinally, this rate of decline was not actually observed until subjects were really quite aged. Therefore, the nonlinear aging described above for the static lung volumes is also evident for peak expiratory flows and dynamic lung volumes (Knudson, 1981; Glindmeyer et al., 1982; Sherrill et al., 1989). That is, functional degradation is minimal within middle-aged people, but becomes more apparent with advancing age (Dontas et al., 1984; Knudson, 1991). Thus, age-dependent changes cannot be adequately modeled as a linear function of age. Instead, a much better relationship can be obtained when age and height squared are incorporated into prediction equations (Knudson, 1991).

Furthermore, it is universally agreed that the forced vital capacity, maximal exhaled volume in 1 s, and maximal expiratory flow over the middle part of the vital capacity all experience reductions with advances in age (Gibson et al., 1976; Glindmeyer et al., 1982; Johnson et al., 1991; Ostrowski et al., 2005). These decreases are most likely to be related to the occurrence of dynamic airway collapse at progressively larger lung volumes in the aged.

If these observations are now combined with those for the static lung volumes, then one can visualize the impact of aging by comparing the maximal flow–volume loops of young and elderly adults (Figure 20.7). Since the residual volume increases with age (Frank et al., 1957; Boren et al., 1966; Edelman et al., 1968), the loop for the aged individual is displaced leftward. The inspiratory phase typically reveals lower peak flows, but has a shape very similar to that of the younger individual, since this limb is effort dependent and since the respiratory muscles have become weaker. The total lung capacity for this individual is lower, reflecting data from Figure 20.6, but this is not always observed. During expiration, the maximal flow is reduced, as are flows at equivalent absolute lung volumes. Also evident is a slight concavity of the effort-independent limb at the lower lung volumes, reflecting a more pronounced dynamic airway collapse. This trend is consistent with that seen in people with mild airway obstruction (Fowler et al., 1987).

In summary, the forced vital capacity, maximal exhaled volume in 1 s, and maximal expiratory flow each display age-dependent reductions, and these are related to greater dynamic airway collapse seen in the aged.

Alveolar Ventilation and Perfusion

From the discussion above, it is apparent that age-dependent pulmonary changes conspire to reduce both alveolar ventilation and its distribution within the lungs, relative to that obtained when these individuals were young adults. For instance, the number of alveoli, the size of the air–alveoli interface, and the area–volume ratio of the lungs each decline with advancing age (Ryan et al., 1965; Thurlbeck, 1967b; Pump, 1971; Sobin et al., 1988). Changes in parenchymal elasticity can result in a gradual elevation in the closing volume, even during resting tidal excursions (Holland et al., 1968; Leblanc et al., 1970; Knudson et al., 1977; Knudson, 1991), while chest-wall stiffening will elevate the work of breathing, particularly when metabolic demands are greater (Frank et al., 1957; Aaron et al., 1990; Chauncharyakul et al., 2004).

During slow tidal breathing, the mechanical configuration of the lungs dictates that, in an upright

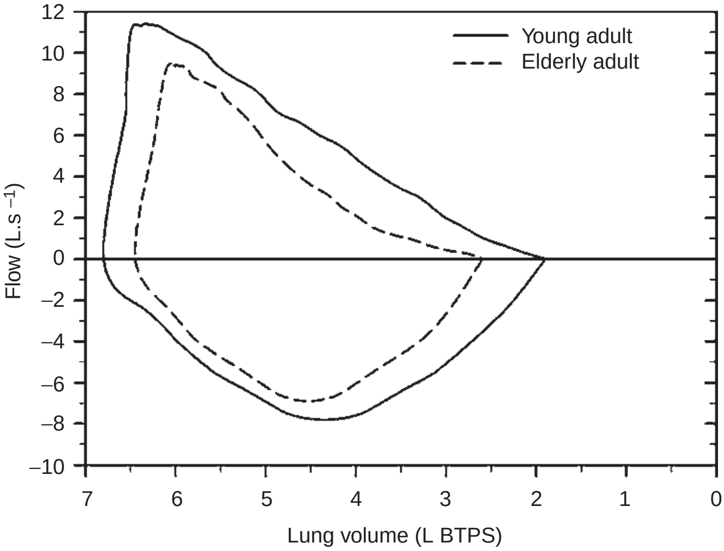

Figure 20.7 Schematic representation of age-dependent changes in the maximal flow–volume loop using static lung volume data from Figure 20.6 and the observations of Gibson and associates (1976), Knudson and associates (1977, 1983), and Fowler and colleagues (1987).

posture, the basal (dependent) alveoli receive >50% more ventilation than do the apical regions (West & Dollery, 1960; Milic-Emili et al., 1966; Holland et al., 1968). Thus, the smaller basal alveoli, which are compressed because of the tissue mass above, are more easily inflated. This apparent paradox can largely be explained by alveoli from different regions operating over various ranges of their individual pressure–volume curves, with enlarged alveoli having a lower compliance. Similarly, in healthy young adults, the distribution of right ventricular output favors capillaries in the basal alveoli (West & Dollery, 1960) because of variations in vascular transmural pressure gradients, and this state is advantageous for gas exchange.

When people breathe at a low functional residual capacity, however, the distribution of air to the basal alveoli is reduced (Milic-Emili et al., 1966), while perfusion remains stable, and gas exchange can be impaired, resulting in a lower arterial oxygen saturation (Nunn et al., 1965). Since the closing volume increases with aging and can exceed 50% of the total lung capacity (Holland et al., 1968; Figure 20.6), one may expect to see a simultaneous reduction in the basal distribution of inspired air. Indeed, Greifenstein and associates (1952), Edelman and colleagues (1968), and Leblanc and others (1970) have all described altered alveolar distribution of ventilation in older subjects, with subjects as young as 65 years experiencing closure of dependent airways during seated spontaneous breathing. This has more recently been confirmed by Krieg and associates (2007), who observed the ventilation of resting older (upright) individuals to be distributed more toward alveoli in the middle rather than the basal regions of the lungs.

As a consequence, aging modifies the distribution of ventilation, with some alveoli becoming unventilated, the so-called alveolar (physiological) shunts, and others becoming underventilated. For instance, Craig and others (1971) reported that such alveoli increased from approximately 5 to 15% over the ages 20–70 years, while Edelman and colleagues (1968) found that their elderly men (>60 years) required an approximately 30% greater minute ventilation at rest to achieve the same degree of alveolar gas mixing as demonstrated by younger adults. This change is attributable to greater airway closure with aging and in particular within dependent regions. Slower, deeper breathing improves the uniformity of air distribution, but more forced ventilatory efforts, such as those encountered during pulmonary function testing and strenuous exercise, exacerbate this state (Edelman et al., 1968).

The dead space also reveals an age-dependent increase (Fowler, 1950; Tenney & Miller, 1956), as does the dead space-to-tidal volume ratio (Sorbini et al., 1968). However, given the morphological changes previously described, this is more likely to be evident within the perfusion-dependent alveolar (physiological) component, rather than within the anatomical dead space (the dimension-dependent conducting airways). Therefore, in parallel with these ventilatory modifications, aging adults must also experience a decrement in pulmonary perfusion via a reduction in either total perfusion or its distribution within the lungs.

There is abundant evidence to show that the pulmonary vasculature experiences age-dependent alterations, some of which may be associated with changes in the parenchymal elastin and collagen composition. For example, the pulmonary capillary blood volume declines in some aged individuals (Krumholz, 1966; Georges et al., 1978; Crapo et al., 1982), but this seems to be neither pronounced nor gradual, occurring primarily in the very aged (Knudson, 1991). Aging also appears to affect the number of underperfused alveoli (alveolar dead space; Fowler, 1950; Tenney & Miller, 1956; Raine & Bishop, 1963; Brischetto et al., 1984). However, as in healthy young adults, the distribution of pulmonary blood flow still favors basal capillaries (Holland et al., 1968; Kronenberg et al., 1972), although there is some evidence showing an increase in apical blood flow (Bachofen et al., 1973; Kronenberg et al., 1973). More recently, a greater variation of blood flow distribution within the lungs of older individuals has been described (Levin et al., 2007). While 60% of this variability could not be explained, age accounted for 23%, and the combination of age and stature could explain 40% of this heterogeneity. Furthermore, older individuals seem to display an age-dependent elevation in pulmonary artery resistance, and this is particularly evident under exercise stress (Emirgil et al., 1967; Ehrsam et al., 1983). As a consequence of these changes and a lower cardiac compliance, pulmonary vascular pressures are elevated (Arbab-Zadeh et al., 2004).

These modifications to both ventilation and perfusion affect alveolar shunting and dead space, both of which reduce gas exchange with aging (Raine & Bishop, 1963; Craig et al., 1971; Wahba, 1983), and contribute to the venous admixture and to differences in alveolar to arterial partial pressures. As a consequence, arterial oxygen partial pressure declines with age (Craig et al., 1971; Delclaux et al., 1994), while the alveolar–arterial oxygen gradient widens. These alterations are also reflected in a decline in diffusing capacity (Donevan et al., 1959; Hamer, 1962; Crapo & Morris, 1981; Guenard & Marthan, 1996; Viegi et al., 2001).

These functional changes seem to correlate well with the reduction in alveolar surface area (Thurlbeck & Angus, 1975), but may also be related to inequalities of alveolar ventilation and perfusion (Guenard & Marthan, 1996) and therefore to changes in the alveolar ventilation-to-perfusion ratio (Raine & Bishop 1963; Holland et al., 1968; Sorbini et al., 1968; Cardus et al., 1997), and to variables that may alter gas exchange between the alveoli and the red cells.

Indeed, Knudson (1991) suggests that factors affecting oxygen transfer have a considerable impact on changes in pulmonary gas exchange observed with advancing age, and Cardus and associates (1997) reported that the inequality of the alveolar ventilation-to-perfusion ratio increased at about 5% per decade from that observed in young adulthood.

The transfer of oxygen from the alveoli to the red cells is dependent upon factors that influence both its diffusive and its convective delivery (mass flow). Two general variables represent possible convective delivery limitations: pulmonary ventilation and pulmonary blood flow. The former dictates the alveolar oxygen partial pressure and thereby establishes the partial pressure gradient necessary for diffusion. The latter, through variations in the circulating hemoglobin content of the blood and changes in pulmonary blood volume and flow, also affects this partial pressure gradient. However, pulmonary blood flow must occur within ventilated alveoli, and ideally at a rate commensurate with ventilation. While changes in these variables may well contribute to reductions in gas exchange, collectively, they do not provide a complete explanation for the decline in diffusing capacity with aging (Knudson, 1991). For this, we must turn our attention to changes imparting a diffusive delivery limitation.

Modifications to the thickness of the alveolar–capillary walls or to the surface areas for gas exchange (alveoli, capillaries, red blood cells) represent possible sites of limitation for diffusive oxygen delivery. Certainly, there is ample morphological evidence showing a reduced total alveolar surface area, concomitant with an enlargement of individual alveoli with aging (Ryan et al., 1965; Thurlbeck, 1967a,b; Sobin et al., 1988). Thus, the average diffusive distance for oxygen from alveolar air to the alveolar membrane is enlarged, contributing significantly to a diffusion limitation (Scheid & Piper, 1980), while the membranes themselves have a smaller total surface area. However, while some groups have reported quite small reductions in pulmonary perfusion (Krumholz, 1966; Georges et al., 1978; Crapo et al., 1982), Butler & Kleinerman (1970) found no evidence to show a change in pulmonary capillary density with aging. It would seem therefore that morphological changes to the lungs, but not necessarily to the pulmonary vascular beds, can account for age-dependent reductions in diffusing capacity (Knudson, 1991).

In summary, older individuals tend to have a reduced basal distribution of inspired air, with some alveoli becoming underventilated or even unventilated. In parallel, aging adults experience reduced pulmonary perfusion, but this still favors the basal capillaries. These modifications combine to reduce gas exchange with aging, and arterial oxygen partial pressure declines with age, while the alveolar–arterial oxygen gradient widens.

REGULATED VARIABLES

The ventilatory and cardiac pumps are controlled such that the partial pressures of oxygen and carbon dioxide of the blood, and also arterial pH, are regulated within ranges that are conducive to optimal function and to life (homeostasis; Figure 20.1). However, because of changes in physical activity, environmental conditions, and health, homeostasis is frequently disturbed, and these regulated variables move outside of their normal regulatory boundaries. One subtle, but very clear, disturbance is associated with aging.

Arterial Oxygen Partial Pressure

For young, healthy adults, arterial oxygen partial pressure is normally regulated around 12.7 kPa (95 mm Hg). However, in the absence of simultaneous changes in arterial carbon dioxide or pH, humans can tolerate a considerable decline in oxygen partial pressure (<6.5 kPa) before the peripheral chemoreceptors become fully activated (Loeschcke & Gertz, 1958). An ascent to altitudes in excess of 3000 m will, because of a reduction in atmospheric pressure, precipitate such a fall (hypobaric hypoxia). Indeed, at the summit of Mt. Everest, where barometric pressure plummets to about 33.5 kPa, the diffusive delivery limitation accompanying this pressure change means that arterial blood becomes so hypoxic (<4 kPa) that all but the most hardly of individuals are incapable of performing any useful work without the aid of supplementary oxygen.

However, arterial oxygen partial pressure in people resting at sea level also declines with age (Craig et al., 1971; Delclaux et al., 1994). The morphological and functional changes that explain this trend have already been described, and most report this reduction to occur at a relatively constant rate (Sorbini et al., 1968; Knudson, 1981; Gunnarsson et al., 1996). Indeed, the consensus favors a linear decline in arterial oxygen partial pressure with age, as reflected within the following prediction: oxygen partial pressure (kPa) $= 13.345 - 0.043 \times$ age (Murray, 1976). Accordingly, it may not be unreasonable to expect to observe some elderly individuals with arterial oxygen partial pressures <10 kPa, and this state may potentially have adverse implications for work and exercise, in much the same manner as does an ascent to altitude. Fortunately, these partial pressures occur along the flatter segment of the oxygen dissociation curve, so that arterial saturation, at least within resting states, remains above 90%, and the physiological consequences of this change are relatively small in unstressed individuals (Janssens, 2005).

For most healthy aged individuals, exercise is limited by cardiovascular rather than pulmonary factors, just as it is in younger adults (Stickland et al., 2008).

Nevertheless, during exercise, several age-dependent differences may be observed. For instance, the elevation in the functional residual capacity normally seen with aging (Figure 20.6; Turner et al., 1968; Roberts et al., 1991) is also evident during exercise (Johnson et al., 1991b), and this can be associated with a significant expiratory flow limitation (dynamic airway collapse; McClaran et al., 1995) and an altered distribution of the inspired air. Indeed, the tidal volume of older individuals will frequently fall within their closing capacity (Johnson et al., 1991b), and the work of breathing increases (Frank et al., 1957; Aaron et al., 1990; Chaunchaiyakul et al., 2004), although this is offset to some extent by a greater bronchodilation response during exercise (Johnson et al., 1991b). Moreover, the alveoli become progressively enlarged, and even fused, with advancing age (Pump, 1971; Sobin et al., 1988), and there is a significant reduction in the alveolar surface area (Thurlbeck, 1967b). These changes can impact negatively upon gas exchange, and the surface area reduction will have an adverse and possibly a proportional effect upon maximal exercise (Massaro & Massaro, 2002).

As a consequence, older individuals generally require greater minute ventilation than their younger counterparts when completing the same absolute work (Davies, 1972; Robinson et al., 1976; Brischetto et al., 1984; McConnell & Davies, 1992), but not at rest (Krumpe et al., 1985). This is achieved first by elevating the tidal volume. Older subjects typically display a more protracted tidal volume response, which continues to increase until almost 60% of the vital capacity (Johnson & Dempsey, 1991). This is no doubt related to pulmonary mechanical changes, by which an elevated airway resistance makes it more efficient to increase ventilation via the tidal volume than through increments in breathing frequency, as is observed in patients with obstructive pulmonary disorders. However, unless adequate care has been taken with subject sampling, and comparisons are made between individuals of different ages but equivalent levels of endurance, this breathing pattern is not always apparent (de Vries & Adams, 1972).

The greater minute ventilation in the aged means that the ventilatory equivalents for both oxygen and carbon dioxide are elevated (Heath et al., 1981; Poulin et al., 1994; Teramoto et al., 1995). While some portion of these changes may reflect adverse gas exchange, it is also possible that older individuals are mechanically less efficient at most forms of work, including breathing, particularly those who choose a sedentary lifestyle. Therefore, some of the additional ventilation must also reflect a corresponding elevation in whole-body metabolic demands (McConnell & Davies, 1992), which decreases after endurance training, as does the ventilatory equivalent for oxygen (Yerg et al., 1985). Furthermore, while these ventilatory increases are significant, they are really quite small at light–moderate exercise intensities, particularly when subjects are matched for endurance fitness (Jones et al., 1985).

During high-intensity exercise (>85% intensity), some well-trained, young endurance athletes may experience exercise-induced arterial hypoxemia at sea level: arterial oxygen partial pressure reduction >1.4 kPa or a desaturation by >5% (Dempsey & Wagner, 1999). This convective impediment is not commonly seen. However, it may represent one of the few pulmonary limits to strenuous exercise in healthy individuals (Stickland et al., 2008), and some have also reported its existence in older athletes (Prefaut et al., 1994). On the basis of the age-dependent decline in arterial oxygen partial pressure, one may perhaps expect an greater relative incidence of arterial hypoxemia in older, endurance-trained people. However, this appears not to be the case (Johnson et al., 1994; Miller & Dempsey, 2004). Instead, increments in work from rest through to near-maximal exercise have little affect upon the partial pressure of arterial oxygen in either young or older people (Johnson & Dempsey, 1991; Johnson et al., 1994).

Arterial Carbon Dioxide Partial Pressure

Young and healthy adults regulate the partial pressure of arterial carbon dioxide at approximately 5.3 kPa (40 mm Hg). There is very little, if any, evidence in resting, asymptomatic subjects that aging has any significant impact upon this state (Brischetto et al., 1984; Gunnarsson et al., 1996; Janssens, 2005), since the alveolar ventilation-to-perfusion relationship is still sufficient to ensure adequate carbon dioxide removal. This state also applies during exercise (Johnson et al., 1994).

Summary

During exercise, particularly in habitually sedentary older people, there may be a significant expiratory flow limitation, with the tidal volume falling inside the closing capacity. In combination with changes in gas exchange, one finds that older individuals generally require greater minute ventilation when performing the same absolute work.

CONCLUDING REMARKS

Aging does not challenge the respiratory system to the point at which either the ventilatory or the gas exchange mechanisms may fail (Rossi et al., 1996). Indeed, most changes occur very gradually and without significant adverse health implications, even for the most elderly of people (Knudson, 1981). This is particularly true for individuals who sustain life-long habitual exercise behaviors (Dempsey et al., 1990). However, the capacity to withstand stress declines with advancing age (Campbell & Lefrak, 1978), and transient homeostatic disturbances are therefore less easily defended. Indeed, when challenged sufficiently, and particularly during ill health (Janssens, 2005), elderly individuals are at greater risk of acute respiratory failure (Rossi et al., 1996; Sevransky & Haponik, 2003).

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Chapter 21

Calorie Restriction in Nonhuman and Human Primates

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CHAPTER CONTENTS

Introduction **447**
Long-Term Effects of Calorie Restriction in Monkeys **448**
  The Study of Health Span vs Life Span 448
    Nonhuman Primate CR Studies 448
    The NIA Study of CR in Rhesus Monkeys 449
    The University of Wisconsin Study of CR in Rhesus Monkeys 450
    What we have Learned from Nonhuman Primate Studies of CR 452
Long-Term Effects of Calorie Restriction in Humans **452**
  Food Restriction During World Wars I and II 452
  Calorie Restriction in Okinawa 452
  Calorie Restriction in Biosphere 2 455
  Calorie Restriction in CALERIE Phase 1 455
    Washington University CALERIE Phase 1 Study 455
    Pennington Center CALERIE Phase 1 Study 456
    Tufts University CALERIE Phase 1 Study 456
  Calorie Restriction in Members of the Calorie Restriction Society 457
Conclusions **457**
References **458**

INTRODUCTION

Calorie restriction (CR) offers a powerful way to explore the process of aging and mechanisms of aging retardation. CR is the only environmental intervention that consistently and strongly increases maximum life span and retards a broad array of indicators of biological aging in many of the laboratory rodents studied so far (Masoro, 2005; Weindruch & Walford, 1988). The effect of CR on longevity is not limited to rodents, as it increases the life span of a variety of invertebrates, e.g., yeast, *Caenorhabditis elegans,* and *Drosophila* (Min & Tatar, 2006), as well as dogs (Kealy et al., 2002). The actions of CR in rodents are thought to depend on chronic CR without malnutrition (i.e., undernutrition with adequate intake of all the essential micronutrients). Although there has long been an emphasis on rodent models in which CR is known to retard aging, there is great interest in studying aging retardation in nonmammalian species under CR-like conditions (Koubova & Guarente, 2003; Piper & Bartke, 2008). Short-lived species are well suited to the investigation of the underlying mechanisms of CR because of their relative simplicity, low cost, and short time required to complete longevity studies. However, the differences in life span, metabolism, and physiology are extensive among nonmammalian systems. Likewise, among mammals, feeding, behavioral, metabolic, physiologic, and pathological differences are not insignificant. Nonhuman primates, and in particular rhesus monkeys (*Macaca mulatta*), provide an extremely valuable model in which to

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determine the ability of CR to extend average and maximum life span in a primate species. In addition, this model offers an ideal opportunity to test candidate mechanisms for aging retardation that have been identified in short-lived species.

A large amount of money and research effort has been, and continues to be, devoted to the study of CR in yeast, worms, insects, rodents, and rhesus monkeys. Presumably this expenditure of funds and research effort is motivated by the belief that the data obtained in these model organisms showing that CR promotes healthy aging and increases life span has relevance to humans. However, while findings in rats, mice, and perhaps also yeast, worms, and flies can suggest possible mechanisms that are relevant to humans, the only way to determine whether CR in humans results in the same metabolic and physiological adaptations as CR in rodents and monkeys is to conduct studies on healthy nonobese individuals. Such studies are difficult to perform in free-living people and there is, therefore, little information available on the effects of CR, particularly long-term CR, in humans. This situation is starting to change and, while research on CR in humans is still at an early stage, scientific data on the health effects of CR with adequate nutrition in humans has accumulated and is presented in this chapter.

LONG-TERM EFFECTS OF CALORIE RESTRICTION IN MONKEYS

Nonhuman primates are humans' closest phylogenetic relative, sharing ~90% of the human genome (King et al., 1988; Sibley & Ahlquist, 1987; Sibley et al., 1990). The rhesus monkey, an Old World primate of either Indian or Chinese origin, has ~93% sequence identity with the human genome (Gibbs et al., 2007) and is among the most commonly used and extensively characterized biomedical models. Because of their evolutionary proximity to humans, information garnered from this model is easily translatable to human medicine (Bontrop, 2001; King et al., 1988; Sibley & Ahlquist, 1987; Sibley et al., 1990). Further validating the use of a rhesus monkey model are the major similarities between rhesus monkeys and humans that extend to almost all aspects of anatomy, physiology, neurology, endocrinology, immunology, and behavior (Colman & Kemnitz, 1998; Colman & Binkley, 2002). In addition, rhesus monkeys also develop and age in ways similar to those of humans and undergo many of the same age-related changes in anatomy, physiology, and behavior while exhibiting some degree of time compression (i.e., median life span in laboratory-housed rhesus monkeys is ~26 years of age, and maximum life span under standard husbandry is ~40 years of age (Colman & Anderson, 2010). Another important and often overlooked benefit of the rhesus monkey model is their relatively large body size. For example, because of their size, repeated blood samples of reasonably large volume are possible, and many standard human clinical techniques are applicable. These features all combine to make the rhesus monkey an invaluable model for biomedical research and of particular utility for translational aging research.

The Study of Health Span vs Life Span

Underlying the importance of research on the biology of aging is the fact that many nations face the demographic reality of a rapidly aging population and the looming health-care challenges that this will bring. In the United States alone, the older population (65 years of age and older) numbered 38.9 million in 2008 and is projected to increase to 40 million in 2010 and to 55 million by 2020, while the population of those 85 years of age and older is projected to increase from 4.2 million in 2000 to 5.7 million in 2010 and then to 6.6 million in 2020 (www.aoa.gov). Without a concomitant increase in health span, or the number of years lived in a healthy, vital state, the impact of this increasing longevity at every level of society, from the individual to the national, will be dramatic. While CR has been shown repeatedly to increase maximum life span in many different model species, it is essential that we look beyond the effects of CR on life span to its effects on health span. We would argue that any intervention that yields an extension of life span without an extension of health span is not successful. Focus is therefore shifting from the study of maximal life span alone to the inclusion of a time course for the development of age-related diseases and conditions. In one study examining this issue (Black et al., 2003), CR F344 rats had a significant increase in life span and health span; however, the period of terminal weight loss, an indicator similar to frailty in humans, was not different between CR and ad libitum-fed animals. So, while CR increased health span and life span, in this study it did not decrease the period of failing health. If humans respond similarly to CR, people will live longer without a change in the quantity of health-care resources utilized.

Nonhuman Primate CR Studies

We have published data showing a positive effect of CR on health span in a primate species; however, there is still a lack of evidence in the scientific literature of a positive effect of CR on life span in a primate species. There are two ongoing studies that are directly testing the effects of CR on aging in rhesus monkeys in an attempt to fill this void. The first

Table 21.1 Key differences between NIA and WNPRC studies of CR in rhesus monkeys

	NIA	**WNPRC**
Start date	1987	1989
Total number of animals	120	76
Animal ages		
Males	1–23 years	6–14 years
Females	1–21 years	8–12 years
Animal origin	Asian and Indian	Indian
Diet	Same for all animals, nonpurified, supplemented 40% by vitamins and minerals	Semipurified, CR diet supplemented 30% by vitamins and minerals
Diet composition	15% protein, 5% fat, 7% fiber, 62% carbohydrate	15% protein, 10% fat, 5% fiber, 65% carbohydrate
Restriction regimen	30% less than age- and weight-matched animals; allotments based on National Research Council guidelines	30% less than each animal's own individual baseline intake level
Feeding schedule	Twice daily	Once daily
Housing	Individual during day, paired at night	Individual
Food intake measurement	One week per year	Daily
Body weight measurement	Quarterly under anesthesia	Weekly while awake
Blood sampling	Under anesthesia	Awake, under minimal manual restraint, samples collected within ~3 min of initiation of restraint

study is based at the National Institute on Aging (NIA; National Institutes of Health, Bethesda, MD, USA) and was initiated in 1987 (Lane et al., 2001a; Mattison et al., 2007). The second study is based at the Wisconsin National Primate Research Center (WNPRC) at the University of Wisconsin (Madison, WI, USA) and began in 1989 (Kemnitz et al., 1993; Ramsey et al., 2000). A total of ~196 rhesus monkeys, both males and females, are being used in the two primate CR studies (see Table 21.1 for study details). Both trials have shown that long-term, moderate (~30%) CR can be safely initiated and maintained in a primate species. Two additional studies of CR in nonhuman primates have been performed but are no longer ongoing. The first of these was a long-term study by Hansen and colleagues at the University of Maryland that focused specifically on obesity and diabetes (Bodkin et al., 2003). In this study, animals were weight-clamped, or maintained at a certain body weight, for ~9 years and were found to have improved glucoregulatory parameters compared to non-weight-clamped animals (Hansen et al., 1995). The second of these studies (Cefalu et al., 1997) was a 4-year investigation by Cefalu and colleagues at Wake Forest University designed to evaluate the effects of CR on the development of atherosclerosis in cynomolgus macaques (*Macaca fascicularis;* a close relative of the rhesus monkey). Details of the study design and data from the two ongoing studies are presented below.

The NIA Study of CR in Rhesus Monkeys

The NIA study began in 1987 with a group of 30 male rhesus monkeys and was expanded to 60 males in 1988. In 1992, the NIA added 60 female rhesus monkeys to this study. NIA monkeys ranged in age from 1 to 23 years at study initiation and, accordingly, the animal's age at the onset needs to be carefully considered when evaluating results from this study. Male monkeys were juvenile (1–2 years of age, $n = 20$), adolescent (3–5 years of age, $n = 20$), and old (16–23 years of age, $n = 20$). Female monkeys were added as three cohorts as well: juvenile (1–3 years of age, $n = 20$), adult (6–14 years of age, $n = 20$), and old (16–21 years of age, $n = 20$). Approximately half of each cohort was assigned to the control group while the remaining half was assigned to the CR group (Mattison et al., 2005). Since many of the animals in the NIA study were still growing when they entered the study, ad libitum food intake levels were based upon National Research Council guidelines (Ingram et al., 1990) as opposed to individual intake levels. Food allotments for the CR animals were reduced by 10% per month over a 3-month period to reach the desired level of 30% fewer calories than age- and weight-matched controls (Mattison et al., 2003). All animals are fed twice daily with the same nutritionally fortified diet containing 15% protein, 5% fat, and 7% fiber. The diet is enriched by 40% in essential vitamins and minerals such that all animals receive at least the minimum recommended dosage of vitamins and minerals.

Not surprisingly, among the earliest and most consistent effects of CR in monkeys was altered body composition and in particular decreased body fat. In the NIA study, both males and females in the CR group weighed less and were generally smaller with lower body fat and lean body mass than age-matched controls (Lane et al., 1999, 1992). In addition, CR animals had less trunk fat and a reduced trunk fat-to-leg fat ratio (Lane et al., 1999). Bone mineral content and bone mineral density were lower in male rhesus monkeys after 11 years of CR; however, this difference can be accounted for by the smaller body mass and lean body mass of the CR animals (Black et al., 2001). Contrary to results in the males, Lane and colleagues (2001b) found that females on CR had similar bone mass compared to age-matched controls.

In the NIA study, the effects of CR on energy metabolism were determined by measuring food energy and intake amount along with energy loses determined by measurement of 24-h energy expenditure, fecal energy density, and the amount of energy lost in feces. This study found that overall energy balance and the individual components used to calculate energy balance were not altered by CR (Lane et al., 1995b). However, in the youngest group of males daytime activity was lower than in age-matched controls (Moscrip et al., 2000), and utilizing all animals, rectal body temperature was reduced by ~0.5°C compared to age-matched controls after 6 years of CR (Lane et al., 1996). In a study of short-term CR (1 month at 30% CR) in young monkeys (2.5 years of age) both absolute and lean mass-adjusted 24-h energy expenditures were reduced by ~24% in CR compared to control animals. Concomitant with this decreased energy expenditure was an ~1.0°C drop in body temperature (Lane et al., 1996).

Given the reliably altered body composition with the imposition of a CR regimen, it is not surprising that glucoregulatory control is consistently improved in CR compared to control animals. While during the first 2 to 3 years of the NIA study, glucose and insulin levels were not altered by CR, after 3 to 4 years of CR reductions in fasting glucose levels became apparent, and after 6 years of study, CR animals showed significant improvements in several parameters of glucose metabolism as measured by an intravenous glucose tolerance test (Lane et al., 1995a).

Life-span studies are both difficult and expensive to complete, particularly in longer-lived species such as primates. Therefore, an appropriate biological tool to gauge the effectiveness of an intervention must be identified. The identification of such biomarkers is challenging. The androgenic steroid dehydroepiandrosterone (DHEA) and its sulfated form DHEAS have been used as such biomarkers. Measurement of these steroids in the NIA population has produced conflicting results. Originally, 2–3 years of CR was reported to have no effect on the age-related decrease in DHEA (Roth et al., 1993), then 3–6 years of CR was found to attenuate significantly the postmaturational decline in DHEAS (Lane et al., 1997), and most recently (Downs et al., 2008) CR failed to prevent the age-related decline in DHEAS and further dampened DHEAS rhythms compared to controls.

There is evidence from rodent studies that CR retards age-associated immune disease (Masoro, 2005). Messaoudi and colleagues have been studying the effect of CR on immune senescence in rhesus monkeys. They found that within the adolescent group, CR can delay T cell senescence as measured by higher numbers of circulating naïve T cells, lower numbers of inflammatory cytokine-secreting memory T cells, and higher proliferative capacity (Messaoudi et al., 2006). A more recent study of the juvenile and old onset groups suggested that CR started early in life led to advanced T cell senescence, while adult-onset CR improved T cell function (Messaoudi et al., 2008).

The University of Wisconsin Study of CR in Rhesus Monkeys

The University of Wisconsin study began in 1989 with a group of 30 adult (8–12 years of age) male rhesus monkeys (Kemnitz et al., 1993; Ramsey et al., 2000). In 1994, this study was expanded to include an additional 30 adult male (6–14 years of age) and 30 adult female (8–12 years of age) rhesus monkeys (Kemnitz et al., 1993; Ramsey et al., 2000). Following a baseline period of food intake assessment for each cohort, the animals were evenly randomized based on age, body weight, and baseline food intake levels to either the control or the CR group. Food allotments for each CR animal were then reduced by 10% per month for 3 months from each animal's own individual baseline level to achieve the goal of an individualized 30% CR. All animals are fed their individualized allotment of food in the morning. Each day in the late afternoon, all food is removed from each animal's cage and quantified, and a 100-calorie piece of food enrichment (e.g., apple, banana, grapes) is given. All animals receive a semipurified diet containing 15% protein and 10% fat (calculated as % dry weight). The diet given to the CR animals is enriched by ~30% in vitamins and minerals to account for the ~30% reduction in food intake in this group.

By design, animals assigned to the CR group receive ~70% of their ad libitum food allotment (Kemnitz et al., 1993; Ramsey et al., 2000). Correspondingly, the CR animals at the WNPRC weigh ~30% less than their age- and sex-matched control counterparts. It is also not surprising that the majority of this weight difference is accounted for by a decrease in fat mass (Colman et al., 1998, 1999). It is important to note, however, that CR animals had reductions not only in total body fat, but also in fat located specifically in the abdominal region (Colman et al., 1999). This is important because of the well-known correlation between abdominal fat mass and risk for metabolic syndrome. Another important component of body composition is skeletal muscle mass. Sarcopenia, or the loss of skeletal muscle with advancing age, is a serious health concern leading to physical disability, reduced quality of life, and increased health-care expenditures. We have shown that, similar to humans, rhesus monkeys lose significant skeletal muscle mass with advancing age (Colman et al., 2005). When adult (average starting age 16 ± 0.5 years, range 15–20 years) male rhesus monkeys are followed longitudinally, the average upper leg muscle loss is 15.0 ± 4.6% after 3 years of study and 22.6 ± 5.7% after 6 years of study (McKiernan et al., 2009). Furthermore, comparing control and CR animals, body-weight-adjusted skeletal muscle mass declined more rapidly in the control group (Colman et al., 2008), leading to the conclusion that CR opposes the development of sarcopenia.

Energy expenditure has long been considered to play an important role in the aging process. Energy expenditure data from the WNPRC study has provided varying results. In one study, body-mass-adjusted energy expenditure, determined by indirect respiration calorimetry, initially decreased; however, this decrease was transient (Ramsey et al., 2000). In a later study, in males, 24-h energy expenditure decreased by ~1.6% annually with most of the difference attributable to decreasing physical activity with advancing age. This rate of change was not altered by CR. CR animals had ~10% lower 24-h energy expenditure compared to controls, with most of the difference attributable to lower nighttime energy expenditure in the CR animals. However, this difference was ameliorated following adjustment for fat-free mass and fat mass (Raman et al., 2007).

As in the NIA study, glucoregulatory function was consistently improved by CR in the WNPRC study. Specifically, changes in indexes of glucose metabolism as measured by minimal modeling of data from intravenous glucose tolerance testing were apparent within 0.5 to 1 year after successfully establishing the 30% CR (Gresl et al., 2001, 2003; Kemnitz et al., 1993). Furthermore, in the WNPRC study, glucose homeostasis was maintained and diabetes completely prevented by CR. Most strikingly, even animals that had compromised metabolic function prior to initiation of the CR diet showed no impairment of glucose homeostasis (Colman et al., 2009) years later.

Metabonomics is a systems biology approach that quantifies the dynamic multiparametric metabolic response to a given stimulus. Investigators with the WNPRC study performed the first ^{1}H NMR metabonomic analysis of phenotypic changes associated with age and long-term CR in rhesus macaques (Rezzi et al., 2009). The results revealed attenuation of aging-dependent alterations in lipoprotein and energy metabolism by CR, noted by increased HDL and decreased VLDL levels. Importantly, removing the effect of aging, CR animals showed metabolic trajectories that correlated with higher insulin sensitivity. The plasma profiles of insulin-sensitive animals were marked by higher levels of gluconate and acetate, suggesting a CR-modulated increase in metabolic flux through the pentose–phosphate pathway, a metabolic shift that would be expected to provide additional reducing power for biosynthesis and response to oxidative stress.

Brain atrophy is prevalent with human aging and is a common feature of many of the diseases that affect the brain. Therefore, the effect of CR on brain atrophy is of great interest. In a 2009 study (Colman et al., 2009), the regional effects of age and diet on gray matter volume were determined. Animals that had been subjected to long-term CR showed preservation of gray matter volume in subcortical regions, including the caudate, putamen, and left insula. Furthermore, CR significantly modified the aging effect in the midcingulate cortex, lateral temporal cortex bilaterally, and right dorsolateral frontal lobe. Therefore, CR reduced age-associated brain atrophy in key regions related to motor function and aspects of executive function.

Two critical indicators of the success of a CR paradigm are delays in mortality and in the onset of age-associated disease. Thus, mortality and the occurrence of age-associated conditions (e.g., diabetes, cancer, cardiovascular disease) were explored in the WNPRC study (Colman et al., 2009). To assess the overall incidence of age-associated disease, the age at which animals experienced their first age-associated diagnosis was determined. The effect of CR in reducing disease onset was profound. An animal in the control group was three times more likely to have an age-related disease diagnosis than an animal in the CR group. Therefore, animals on CR display a clear increase in health span. Additionally, CR conferred a decreased risk of dying from an age-related disease. Specifically, at any point in time, the control animals had three times the rate of death from an age-related cause compared to animals under CR.

What We Have Learned from Nonhuman Primate Studies of CR

As described above, there are several important differences in study design between the two ongoing long-term studies of CR in nonhuman primates (see Table 21.1). It is important to realize, however, that even given these crucial differences, the most basic response to CR in rhesus monkeys is consistent across the two studies. Both groups have described similar positive effects on body composition and glucoregulatory function. This speaks highly for the overriding effects of CR regardless of study design. Although the UW study has published regarding positive effects of CR on morbidity and preliminary survival data, such data are not yet available from the NIA study. There are many advantages to using a nonhuman primate model over other model organisms, but one potential drawback is the relatively long life span of rhesus monkeys compared to other models. While significantly shorter than human life spans, rhesus monkey life-span studies take considerable time to complete. As such, both the UW and the NIA studies are still ongoing, with the anticipation of having ultimate life-span data by 2025.

LONG-TERM EFFECTS OF CALORIE RESTRICTION IN HUMANS

Data from animal studies show that CR without malnutrition, i.e., with an adequate intake of essential nutrients, promotes health and longevity (Masoro, 2005; Weindruch & Walford, 1988). It seems highly unlikely that data on the effects of severe (i.e., 25 to 30%) CR on aging will ever be obtained from randomized clinical studies on humans because of an inability to obtain compliance and funding. However, data on the effects of relatively short-term CR in humans has accumulated from epidemiological and observational studies of unintentional induced CR and from randomized studies lasting 6 to 12 months (Table 21.2). A larger scale, multiple-center randomized study of the effects of 2 years of 25% CR, CALERIE 2, is currently in progress and should provide additional information. The only data on the effects of long-term CR on humans are coming from research on a group of the Calorie Restriction Society members who have been practicing severe CR for an average of 12 years (7 to 25 years).

Food Restriction during World Wars I and II

Food restriction during the First and Second World Wars in some European countries, such as Denmark and Norway, was associated with a sharp decrease in mortality (Hindhede, 1921; Strom & Jensen, 1951). Full records exist of the nutritional conditions in these two European countries during the war. In Denmark, CR due to war-imposed food regulation started at the beginning of 1917 and became very severe by October 1917, but CR was not associated with malnutrition, because the Danish Health Service enforced an adequate consumption of vegetables, whole rye bread enriched with wheat bran, barley porridge, and milk. Comparison of the death rates before and during the war showed that the mortality rate dropped by ~34% in the period 1917–1918 (Hindhede, 1921). A similar situation occurred in Norway during the Second World War. Comparison of the food diaries of working-class families in Oslo before and during the war showed that calorie intake dropped by ~20% during the war. In 1936–1937 average calorie intake was 3470 kcal/day, while in 1942–1945 calorie intake was reduced to 2850 kcal/day (Strom & Jensen, 1951). Malnutrition was avoided because of an increased consumption of fresh vegetables, potatoes, fish, and whole cereals (Strom, 1948). Starting in 1941, mortality from cardiovascular disease declined sharply in both men and women of all ages, reaching the nadir in 1943–1945 when mortality was reduced by ~30% compared to the prewar level in adults younger than 59. However, after the end of the war mortality began to increase rapidly again toward the prewar level (Strom & Jensen, 1951).

Calorie Restriction in Okinawa

Another natural experiment on the effects of CR without malnutrition on morbidity and mortality took place in Okinawa, one of Japan's southern prefectures. Until 1960 the reported daily calorie intake of inhabitants of Okinawa Island was 1785 kcal per day, ~15 and ~40% less than the average calorie intake of a mainland Japanese (2068 kcal/day) and U.S. (2980 kcal/day) resident, respectively (Bureau of Agricultural Economics, US Department of Agriculture 1949. Consumption of food in the United States, 1909–1948, Misc. Pub 691; U.S. Department of the Office of the Civil Administrator of the Ryukyu Islands, 1949). Although low in energy content, the traditional Okinawan diet was nutritionally adequate, with a high intake of nutrient-dense foods such as fresh vegetables and fruits, sweet potatoes, soy, and fish. Consistent with a long-term adaptation to CR, the body mass index (BMI) of adult Okinawans was ~21 kg until the 1960s. In this older cohort of Okinawans (age 65+) mortality from coronary heart disease; cancer of the prostate, colon, and breast; and lymphoma was markedly lower than in the average mainland Japanese and U.S. population (Kagawa, 1978). In 1995 the age-adjusted mortality rate for coronary heart disease was 193 deaths per 100,000 people in U.S. men and only 33 deaths per 100,000 people in Okinawan men. For breast cancer the age-adjusted mortality rate per 100,000 persons

Table 21.2 Effects of long-term calorie restriction in human subjects

TYPE OF STUDY	REFS	INITIAL WEIGHT STATUS[a]	NUMBER OF CR SUBJECTS	DEGREE OF CR (%)	DURATION OF CR	FINDINGS IN CALORIE-RESTRICTED GROUP
Longitudinal	Walford et al., 2002	Lean	8	~22[b]	18 months	↓ BMI, ↓ BP, ↓ insulin, ↓ glucose, ↓ LDL-c, ↓ HDL-c, ↓ TG, ↓ uric acid, ↓ T3, ↓ WBC
RCT	Racette et al., 2006	Lean and overweight	19	20	12 months	↓ Body fat, ↓ VAT, ↓ SAT, ↓ FFM
RCT	Weiss et al., 2006	Lean and overweight	18	20	12 months	↑ ISI, ↓ insulin, ↓ glucose, ↑ adiponectin
RCT	Villareal et al., 2006	Lean and overweight	18	20	12 months	↓ Spine and hip BMD, ↑ CTX, ↓ leptin
RCT	Fontana et al., 2007	Lean and overweight	18	20	12 months	↓ Total cholesterol and LDL-c, ↓ TG, ↓ CRP, ↓ HOMA-IR
RCT	Weiss et al., 2008	Lean and overweight	18	20	12 months	↓ T3, ↔ TSH, ↔ T4
RCT	Hofer et al., 2008	Lean and overweight	18	20	12 months	↓ DNA and RNA oxidation in white blood cells
RCT	Riodan et al., 2008	Lean and overweight	18	20	12 months	Improved left ventricular diastolic function
RCT	Weiss et al., 2007	Lean and overweight	18	20	12 months	↓ Muscle mass and absolute physical work capacity
RCT	Fontana et al., 2008	Lean and overweight	18	20	12 months	↔ IGF-1, ↔ IGF-1/IGFBP-3
RCT	Heibronn et al., 2006	Lean and overweight	12	25	6 months	↓ Body fat, ↓ insulin, ↓ BT, ↓ 24-h EE, ↓ T3, ↔ DHEAS, ↓ DNA damage marker
RCT	Redman et al., 2007 Larson-Meyers et al., 2006	Lean and overweight	12	25	6 months	↓ % body fat, ↓ VAT, ↓ SAT, ↓ FCS, ↓ AIRg
RCT	Lefevre et al., 2009	Lean and overweight	12	25	6 months	↓ TG, ↑ HDL-cholesterol, ↓ factor VIIc, ↔ CRP, ↔ fibrinogen, ↔ BP, ↔ BA-FMD
RCT	Redman et al., 2008	Lean and overweight	12	25	6 months	↔ Spine and hip BMD, ↑ CTX, ↓ bsALP
RCT	Martin et al., 2007 Williamson et al., 2008	Lean and overweight	12	25	6 months	No change in cognitive function, no eating disorders
RCT	Civitarese et al., 2007 Allard et al., 2008	Lean and overweight	12	25	6 months	↑ PGC-1 and SIRT1 mRNA
RCT	Das et al., 2007 Pittas et al., 2005	Lean and overweight	32	30	6 months	↓ BMI, ↓ insulin, ↓ HOMA-IR, ↓ CRP, ↑ ISI

(*Continued*)

Table 21.2 (Continued)

TYPE OF STUDY		INITIAL WEIGHT STATUS[a]	NUMBER OF CR SUBJECTS	DEGREE OF CR (%)	DURATION OF CR	FINDINGS IN CALORIE-RESTRICTED GROUP
RCT	Pittas et al., 2006	Lean and overweight	32	30	12 months	↓ Body fat, ↓ RMR, ↓total cholesterol and LDL-c, ↓ TG, ↑ HDL-c
RCT	Ahmed et al., 2009	Lean and overweight	32	30	12 months	↑ Delayed-type hypersensitivity response, ↑ proliferative response of T cells, ↑ PGE2
Cross-sectional	Fontana et al., 2004	Lean	18	∼30	∼7 years	↓ Body fat, ↓ LDL-c, ↑ HDL-c, ↓ TG, ↓ glucose and insulin, ↓ BP, ↓ CRP, ↓ PDGF, ↓ carotid artery IMT
Cross-sectional	Fontana et al., 2006	Lean	28	∼30	∼7 years	↓ T3, ↔ TSH, ↔ T4
Cross-sectional	Meyer et al., 2006	Lean	25	∼30	∼7 years	Improved left ventricular diastolic function, ↓ TNF-α, ↓ TGF-β
Cross-sectional	Fontana et al., 2008	Lean	28	∼30	∼7 years	↔ IGF-1, ↔ IGF-1/IGFBP-3, ↓ insulin, ↓ CRP

Abbreviations: CR, calorie restriction; RCT, randomized controlled trial; BMI, body mass index; BP, blood pressure; T3, triiodothyronine; HR, resting heart rate; REE, resting energy expenditure LDL-c, LDL-cholesterol; HDL-c, HDL-cholesterol; TG, triglycerides; WBC, white blood cell count; CRP, C-reactive protein; PDGF, platelet-derived growth factor; IMT, intima-media thickness; TSH, thyrotropin; T4, thyroxin; TNF-α, tumor necrosis factor-α; TGF-β, transforming growth factor-β; IGF-1, insulin-like growth factor-1; IGFBP-3, insulin-like growth factor binding protein 3; VAT, visceral adipose tissue; SAT, subcutaneous adipose tissue; FFM, free-fat mass; ISI, insulin sensitivity index; BMD, bone mineral density; CTX, C-telopeptide of type I collagen; FCS, fat cell size; HOMA-IR, homeostasis model assessment of insulin resistance; BT, body temperature; EE, energy expenditure; DHEAS, dehydroepiandrosterone sulfate; AIRg, acute insulin response to glucose; BA-FMD, brachial artery flow-dependent vasodilation; bsALP, bone-specific alkaline phosphates; PGC-1, peroxisome proliferator-activated receptor γ, coactivator 1α; SIRT1, sirtuin 1; PGE2, prostaglandin E2.

[a] Based on body mass index.

[b] Weight loss was caused by reduced energy intake and increased energy expenditure (∼70–80 h of work per week).

was 33 in U.S. women and 6 in Okinawan women. For prostate cancer the age-adjusted mortality rate per 100,000 persons was 28 in U.S. men and 4 in Okinawan men (Willcox et al., 2007). In the year 2000, life expectancy at age 65 was 24.1 years for Okinawan women and 18.5 years for Okinawan men, compared to only 19.3 years for U.S. women and 16.2 years for U.S. men of the same birth cohort (Centers for Disease Control and Prevention, 2009; Japan Ministry of Health, Labor, and Welfare, 2000). As a consequence, Okinawa has one of the highest numbers of centenarians in the world (~50 per 100,000 inhabitants) or about four to five times the average for most developed countries (Japan Ministry of Health, Labor, and Welfare, 2000). However, after the 1960s the average BMI of Okinawans steadily increased (in 1998 average BMI was 25.6), probably because of the Westernization of dietary habits with increased calorie intake. As a consequence, morbidity and mortality from chronic disease have increased dramatically in younger generations (Kagawa, 1978).

The Okinawan centenarians have been cited as proof that CR may increase maximal life span in humans. However, although the number of centenarians is higher in Okinawa than elsewhere, the oldest people in Okinawa are no older than the oldest people eating ad libitum in other parts of the world. We cannot exclude that maternal CR may be responsible for maladaptive intrauterine changes resulting in permanent alterations (Ozanne & Constancia, 2007; Tarry-Adkins et al., 2008*)* that might partially undermine the antiaging effects of CR on maximal life span in the Okinawan centenarians.

Calorie Restriction in Biosphere 2

In September 1991 four men and four women participated in a 2-year ecological experiment that took place in "Biosphere 2," a completely closed self-sustaining ecological system near Tucson, Arizona. Soon after the complete closure of this ecological system, the crew members experienced a forced decrease in calorie intake for 18 months, because of an unanticipated decrease in food availability (Walford et al., 2002). During the first 6 months, the participants consumed ~30% fewer calories (from ~2500 to ~1784 kcal/day), rising then to ~2000 kcal/day for the remaining 12 months, while sustaining high levels of physical activity (~70–80 h of work per week) required by their daily duties. This combination of semivegetarian CR diet and increased physical activity resulted in a decrease in BMI in men from 23.7 ± 1.8 to 19.3 ± 0.9 and a decrease in BMI in women from 21.2 ± 1.5 to 18.5 ± 1.2. Blood pressure dropped from ~110/78 to ~90/56 mm Hg and returned to preentry levels after they resumed an "ad libitum" diet. Total cholesterol dropped from 190 to 120 mg/dl, while HDL-cholesterol decreased from 54 to 34 mg/dl, probably because of the high-carbohydrate (72–80% of calories) and low-fat (9–13% of calories) content of this diet. Moreover, the eight biospherians showed many of the same adaptations previously reported in calorie-restricted rodents, including marked reductions in fasting glucose and insulin, a decrease in serum T3 concentration, and a rise in total and free cortisol concentrations (Walford et al., 2002). Interestingly, in contrast to the hormonal adaptations that occur in CR rodents, 18 months of CR in these volunteers did not result in reductions in serum concentration of IGF-1 and testosterone (Walford et al., 2002).

Calorie Restriction in CALERIE Phase 1

Three clinical trials of the effects of 6 or 12 months of CR in nonobese humans were recently completed (CALERIE, phase 1) at Washington University in St. Louis, at the Pennington Center in Baton Rouge, and at Tufts University in Boston. CALERIE phase 1 studies were conducted to evaluate whether CR is feasible, tolerable, and safe in free-living individuals. In phase 1 each clinical center designed its own protocol to assess these methodological issues. The volunteers in CALERIE phase 1 were initially overweight, and their BMIs dropped to the upper limit of normality by the end of the study. Thus, the findings of these studies may be more relevant to the effects of weight loss than of chronic severe CR. CALERIE phase 2, a multicenter study of the effect of 25% CR of 2 years' duration in 21- to 47-year-old healthy women and in 21- to 50-year-old healthy men with BMI values in the 22 to 28 range, is in progress.

Washington University CALERIE Phase 1 Study

At Washington University in St. Louis, Missouri, 48 nonobese (BMI between 23.5 and 29.9) men and women, ages 50–60 years, were randomized to one of three 1-year-long interventions: (1) a 20% reduction in calorie intake (CR; $n = 19$), (2) a 20% increase in energy expenditure (endurance exercise) without changes in energy intake (EX; $n = 19$), and (3) healthy lifestyle ($n = 10$) (Racette et al., 2006). Participants were assigned in a 2:2:1 allocation scheme across the three groups. Forty-six volunteers completed the study (1 CR and 1 EX participant dropped out). Based on the doubly labeled water measurements the CR group achieved 11.5 ± 2.1% CR, whereas based on heart-rate monitor data the EX group achieved 58.7 ± 6.7% of their prescribed exercise (Racette et al., 2006). At baseline, the mean BMI of the study participants was 27.3 ± 0.3. After 1 year, BMI values were 24.4 ± 0.6 in the CR group, 25.0 ± 0.5 in the EX group, and 27.4 ± 0.5 in the healthy lifestyle group. Total fat mass went from 33.1 ± 1.1 to 27.7 ± 1.2% in the CR group and from 31.7 ± 1.0 to 26.7 ± 1.0% in the EX group, with no change in the healthy lifestyle group. Visceral fat mass was reduced by 39% in the EX group and by 37% in CR group (Racette et al., 2006). As expected, serum leptin concentration decreased, and serum adiponectin concentration increased, significantly in the EX and CR groups (Villareal et al., 2006; Weiss et al., 2006). The CR- and exercise-induced weight losses resulted in similar improvements in insulin sensitivity, fasting insulin, insulin area under the curve, LDL-cholesterol, and the total cholesterol–HDL ratio, but only CR reduced the serum concentration of C-reactive protein, a marker of inflammation (Fontana et al., 2007; Weiss et al., 2006). Serum triiodothyronine concentration was significantly reduced in the CR group, but not in the EX group, even though the reductions in body weight and fat mass were similar in the two groups (Weiss et al., 2008). In this study, both CR- and exercise-induced weight loss resulted in a significant reduction in oxidative damage to DNA and RNA measured in white blood cells and in improvements in diastolic left ventricular function (Hofer et al., 2008; Riordan et al., 2008). CR decreased bone mass, muscle size and strength, and maximal aerobic capacity in proportion to the reduction in body weight (Villareal et al., 2006; Weiss et al., 2007). Interestingly, in disagreement with the hormonal adaptations that occur in CR rodents, CR in these volunteers did not result in reductions in serum concentration of IGF-1 or estradiol or in an increased serum cortisol concentration (Fontana et al., 2008; Villareal et al., 2006; Weiss et al., 2006).

Pennington Center CALERIE Phase 1 Study

At the Pennington Center in Baton Rouge, Louisiana, 48 overweight (BMI 25–30) volunteers (men <50 years of age and women <45 years of age) were randomized to one of four 6-month interventions: (1) 25% CR with adequate nutrition (CR; AHA Step 1 diet), (2) 12.5% CR + 12.5% exercise-induced increase in energy expenditure (CR + EX), (3) liquid calorie diet until 15% weight loss was achieved and then clamped at the new lower weight (very low calorie diet), and (4) control. Forty-six volunteers completed the study (1 from the control group and 1 from the very low calorie diet dropped out). At baseline, the mean BMI of the study participants was 27.8 ± 0.7, and body fat was 24.8 ± 3.1% in men and 37.6 ± 4.1% in women (Heilbronn et al., 2006; Redman et al., 2007). After 6 months, total fat mass was reduced by 24 ± 3% in the CR group, by 25 ± 3% in the CR + EX group, and by 32 ± 3% in the very low calorie diet group, with no change in the control group. Visceral fat mass, subcutaneous fat cell size, and intrahepatic lipid content were also significantly reduced in the three intervention groups (Larson-Meyer et al., 2006; Redman et al., 2007). At 6 months, the insulin sensitivity was significantly improved in the CR + EX and very low calorie diet groups and tended to increase in the CR group (Larson-Meyer et al., 2006). Fasting insulin and serum concentrations of HDL-cholesterol and triglycerides significantly improved in the three intervention groups. Surprisingly, serum LDL-cholesterol concentration and diastolic blood pressure were reduced in the CR + EX group but not in the CR alone or very low calorie diet group (Lefevre et al., 2009). Systolic blood pressure, fibrinogen, homocysteine, glucose concentrations, and endothelial function (measured with brachial artery flow-mediated dilation) were unchanged in the intervention groups, while serum C-reactive protein was significantly lower only in the CR + EX and control groups (Heilbronn et al., 2006; Lefevre et al., 2009). In all three intervention groups, serum T3 concentration and 24-h energy expenditure were significantly reduced from baseline, whereas serum DHEAS was unchanged (Heilbronn et al., 2006). Probably because of the short duration of this study and the relatively young age of the study subjects, CR did not result in a reduction in bone mass, although markers of bone turnover increased significantly in all three intervention groups (Redman et al., 2008). CR was not associated with change in cognitive test performance or increased eating disorder symptoms (Martin et al., 2007; Williamson et al., 2008). The authors of this study reported an increase in mitochondrial DNA content (without changes in the activity of key mitochondrial enzymes) in skeletal muscle biopsies and a reduction in DNA damage (measured with the comet assay) in white blood cells, without change in plasma concentrations of protein carbonyls, a marker of protein oxidation (Civitarese et al., 2007; Heilbronn et al., 2006). Gene expression studies in both skeletal muscle biopsies and cells cultured with 10% serum from CR volunteers revealed an upregulation of PGC-1 mRNA and SIRT-1 mRNA levels (Allard et al., 2008; Civitarese et al., 2007).

Tufts University CALERIE Phase 1 Study

At Tufts University School of Medicine in Boston, Massachusetts, 46 overweight (BMI 27.9 ± 1.5; range 25–29.9) men and women ages 24–42 years, with a fasting plasma glucose level of <100 mg/dl were randomized for 1 year to one of four interventions: (1) 10% CR/high-fiber diet ($n = 6$), (2) 10% CR low glycemic load/high-fiber diet ($n = 6$), (3) 30% CR/high-fiber diet ($n = 17$), and (4) 30% CR low glycemic load/high-fiber diet ($n = 17$). Participants were assigned in a 1:1:3:3 allocation scheme across the four groups. Data on the effects of both 10 and 30% CR were reported in some (Ahmed et al., 2009; Das et al., 2007), but not all articles (Pittas et al., 2005, 2006) published by the Tufts University research group so far. Moreover, in some articles they reported only the data at 6 months (Ahmed et al., 2009; Pittas et al., 2005, 2006) and in others the data at 6 and 12 months (Das et al., 2007). At 6 months, the 10% CR group lost 6.97 ± 6.4 kg and the 30% CR group 10.20 ± 3.9 kg, but the difference in weight loss between the 10% CR and the 30% CR group did not reach statistical significance (Pittas et al., 2005, 2006). The 30% CR resulted in significant weight loss and improvement in insulin sensitivity (measured by oral glucose tolerance test and a frequently sampled intravenous glucose tolerance test), fasting insulin concentration, first-phase acute insulin secretion, and lipid profile independent of the low- or high-glycemic index of the diet (Das et al., 2007; Pittas et al., 2006). At 6 months, plasma CRP concentration was reduced in the 30% low-glycemic CR group, but not in the 30% high-glycemic CR group (Pittas et al., 2006). At 6 months, 30% CR significantly improved ex vivo and in vivo measures of T-cell-mediated function, including delayed-type hypersensitivity response and proliferative response of T cells to T cell mitogens and prostaglandin E2 production (Ahmed et al., 2009).

Calorie Restriction in Members of the Calorie Restriction Society

Data from studies conducted in members of the Calorie Restriction Society, who follow a regimen of self-imposed CR with adequate nutrition in the belief that CR will extend their health span and life span, have been published (Fontana et al., 2004, 2006, 2008; Meyer et al., 2006). The CR group is composed of very lean (BMI 19.7 ± 1.8) adult men and women (51.6 ± 12.7 years of age; range 35–82 years), who have been eating ~1800 kcal/day for an average of 12 years, which is ~30% fewer calories than age- and sex-matched subjects eating Western diets rich in energy-dense foods. The CR society members eat a diet rich in nutrient-dense foods, including a wide variety of vegetables, fruits, whole grains, nuts, egg whites, fish, low-fat dairy products, and lean meat, which supplies more than 100% of the Recommended Daily Intake for all essential nutrients (Fontana et al., 2004). In a subgroup of these individuals on whom data were obtained by their physicians prior to starting CR, the CR resulted in a reduction of BMI from 23.7 (pre-CR) to a currently stable BMI of 19.7 and a total body fat of ~10% (Holloszy & Fontana, 2007). Moreover, a review of the medical records available for this subgroup of CR volunteers showed that CR caused profound reductions in the major cardiometabolic risk factors for coronary heart disease, including lowering of total cholesterol, LDL-cholesterol, and triglycerides and a large increase in HDL-cholesterol concentrations, lower fasting glycemia and HOMA-IR index, and a remarkable lowering effect on systolic and diastolic blood pressure (Fontana et al., 2004; Holloszy & Fontana, 2007). The CR-mediated anti-inflammatory effect in the CR Society members is also remarkable, as reflected in almost undetectable levels of C-reactive protein and low levels of circulating tumor necrosis factor-α (Fontana et al., 2004; Meyer et al., 2006). Probably as a consequence of these adaptations, carotid artery intima-media thickness is significantly lower in the CR group than in the age- and sex-matched control group (Fontana et al., 2004). Compared with control subjects consuming a Western diet, the CR Society members also show some of the same hormonal alterations that have been reported in CR rodents, including low serum T3 concentration. Serum triiodothyronine concentration was ~30% lower in individuals practicing long-term CR than in endurance athletes, even though percentage body fat was low and similar in these two groups (Fontana et al., 2006). However, major differences in the effects of CR exist between rodents and humans. For example, the published data from animal studies show that CR decreases serum IGF-1 concentration by ~30–40% in rodents, but does not reduce total IGF-1 or IGF-1/IGFBP-3 ratio levels in healthy humans, unless protein intake is also markedly reduced (Fontana et al., 2008; Sonntag et al., 1999). These results suggest that the high protein intake (~1.7 g/kg/day or ~24% of calories from proteins) of the CR Society members might be responsible for the different effects of CR in mice and humans.

Currently, the only direct evidence that CR may influence the rate of aging in humans is that CR Society members have better left ventricular diastolic function, a marker of intrinsic aging of the heart (Kitzman et al., 1991), than healthy age- and gender-matched controls eating Western diets (Meyer et al., 2006). Factors that could be responsible for this antiaging effect of CR on diastolic function could be a reversal and slower accumulation of connective tissue and myocardial fibrosis due to the lower levels of inflammation, TGF-β1, and blood pressure in the CR group (Meyer et al., 2006).

More studies are needed to address the potential detrimental effects of long-term severe CR in humans. Extreme CR (especially if associated with inadequate micronutrient intake) can cause detrimental health effects including increased risk of bone fractures and susceptibility to infections, low muscle mass and strength, anemia, amenorrhea, and infertility (Fontana & Klein, 2007). Finally, more studies are warranted to determine the optimal calorie intake (and relative macro- and micronutrient composition) needed to promote health, based on age, sex, genetic background, and energy expenditure.

CONCLUSIONS

Data are accumulating on the beneficial effects of CR without malnutrition in nonhuman and human primates. We know that in both nonhuman and human primates CR with adequate nutrition protects against obesity, type 2 diabetes, and cardiovascular diseases, which are leading causes of morbidity and mortality. Cancer incidence and mortality seem also to be reduced in CR monkeys and studies of CR humans reveal a consistent reduction in metabolic factors associated with increased cancer risk. Nonetheless, nothing is known about the long-term effects of CR on the age-associated increased risk of bone fractures, immune deficiencies, and dementia or on the rate of aging in nonhuman and human primates. More studies are needed to elucidate the molecular mechanisms underlying the beneficial effects of CR in nonhuman and human primates and to characterize new markers of aging/longevity that can assist scientists and clinicians in predicting mortality and morbidity.

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Chapter 22

Age-Related Changes in Thermoreception and Thermoregulation

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CHAPTER CONTENTS

Introduction **463**
Thermoreception **464**
- Anatomy and Physiology of Skin Thermoreception 464
- Anatomy and Physiology of Deep Body Thermoreception 465
- Anatomy and Physiology of CNS Thermoreception 465
- Age-Related Changes in Thermoreception 466

Thermogenesis, Heat Gain, and Heat Retention **466**
- Obligatory Thermogenesis 468
  - Basal Metabolic Rate 468
  - Diet-Induced Thermogenesis 468
  - Baseline Physical Activity and Posture 468
- Facultative Thermogenesis 469
  - Physical Activity 469
  - Shivering Thermogenesis 469
  - Humoral Thermogenesis I: “Classical Neuronal” Nonshivering Thermogenesis 469
  - Humoral Thermogenesis II: “Nonclassical” Nonshivering Thermogenesis 469
- Gain and Retention of Heat 469
  - Behavioral Measures for Heat Gain and Retention 469
  - Capacitive and Insulative Properties of the Body 470
  - Autonomic Heat Retention by Peripheral Vasoconstriction 470

Heat Loss and Reduction of Heat Gain **470**
- Peripheral Blood Flow 471
- Evaporative Heat Loss 472

Central Thermoregulatory Control **472**
- Thermoregulatory Control 472
- Central Control of the Circadian Rhythm in Temperature 473

Conclusion **473**
References **474**

INTRODUCTION

This chapter focuses on age-related changes in temperature sensitivity and thermoregulation in humans. Core temperature is the net result of heat production and heat loss. Deviations from the optimal temperature occur for two main reasons: physical activity generates heat and environmental heat or cold affects body temperature as well. These changes need to be sensed, processed, and counteracted if necessary. Thus, the thermoregulatory system can be conceptualized as containing three parts: thermosensitive afferent pathways, neuronal integration and control systems, and descending effector pathways altering heat gain or loss. The functional anatomy and physiological mechanisms of these compartments and their alterations with aging are covered in separate sections: thermoreception; thermogenesis, heat gain, and heat retention; heat loss and reduction of heat gain; and central thermoregulatory control including circadian rhythms.

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For a more elaborate discussion of age-related changes in circadian thermoregulatory processes the reader is referred to a review (Van Someren et al., 2002). The effect of age on fever regulation is beyond the scope of this chapter (for a review see Norman & Yoshikawa, 1996). An important discrimination can be made concerning primary and secondary age-related changes. Primary age-related alterations are those that are present even in the very fit and healthy elderly or after correction for such secondary alterations. Secondary age-related changes are those that cannot be attributed to aging per se, but to factors for which the elderly are at a higher "risk." Examples are a sedentary lifestyle, a lower fitness level, and a various disease states. Of note, most people of age 70 years or more have one or more ongoing disease processes. These may give reversible or irreversible secondary contributions to the changes in thermoregulation that occur with aging.

Humans need to regulate their body temperature to survive. Most of the deaths due to hypothermia or hyperthermia occur in elderly subjects (Bai et al., 1995; Brody, 1994; Collins & Exton-Smith, 1983). The reason the elderly especially are at such an increased risk of thermoregulatory deficit is not a new question, yet still important and not solved. Is it because their capability to sense temperature is compromised, because their thermoregulatory capacities are limited, or because their physiology is less tolerant to extreme temperatures? This chapter discusses what is known about the changes in thermoreception and thermoregulation with increasing age.

THERMORECEPTION

Homeostatic regulation requires the accurate readout of internal and external temperatures and reporting to the integration and control systems to allow for adequate regulatory measures. The readout or sensing part of the system is referred to as thermoreception, which may or may not be associated with an actual subjective conscious experience of warmth, cold, or thermal comfort. Also, the inputs that thermosensitive structures relay to the brain are not all exclusively used for thermoregulatory purposes, but may also affect other processes, notably sleep and vigilance (Van Someren, 2004, 2006). Temperature-sensitive structures are present in the skin, deep body, and central nervous system (CNS). The sensation of warm and cold mainly depends on the activity of cutaneous thermoreceptors, the physiological thermoregulatory responses mainly depend on core temperature, and the emotional experience of thermal comfort or discomfort depends on the total thermoregulatory state, including the input from core and skin thermoreceptors (Bulcao et al., 2000; Hensel, 1981). Subjective comfort is dependent on, but not equal to, thermal sensation. Sensations rely mainly on skin thermoreceptors and occur quickly, whereas comfort is a slower process depending on integration of skin and internal thermoreceptors as well as the sensations resulting from thermoregulatory actions.

Anatomy and Physiology of Skin Thermoreception

The thermosensitivity of the *skin* is determined by the cutaneous nerve endings—mostly without clear corpuscles—of neurons located in the dorsal root ganglia. The firing rate of the afferent fibers depends on both the static temperature and the rate of change of the temperature of the skin. Cold receptors increase their firing rate with a decreasing or static low temperature. They are located at a depth of ±0.16 mm at the endings of thin myelinated A∂ fibers. Warmth receptors increase their firing rate with increasing or static elevated temperature and are located at a depth of ±0.45 mm at the endings of the slower unmyelinated C fibers. Even small skin temperature changes can have profound effects since they induce simultaneous and opposite changes in the most sensitive range of both cold and warmth receptors. The strength of the response to changes in temperature depends on the baseline temperature. A warm skin can detect even a small increase in temperature, whereas the detection of cooling needs quite a large decrease in temperature. At low baseline levels, the reverse is true (Bushnell et al., 1983; Handwerker et al., 1982; Kenshalo, 1977). Figure 22.1 shows a schematic overview of the range of temperatures that affect thermoreceptive fibers.

The thermosensitive fibers ascending from the skin reach the spinal cord via the dorsal root ganglion and terminate on second-order neurons in lamina I of the dorsal horn. From the dorsal horn, the thermosensitive afferents are projected mainly via the contralateral anterolateral spinothalamic tract (Schwark et al., 1997), but projections via the ipsilateral dorsolateral spinocervical tract have also been demonstrated (cf. Hensel, 1981). Thermosensitive nerve endings in the face, originating in the trigeminal ganglion cells, innervate second-order neurons in the trigeminal nucleus in the medulla oblongata. Ascending secondary fibers join the spinal ascending fibers to terminate on third-order neurons in the ventrobasal thalamic relay nuclei, which project to the somatosensory cerebral cortex where the skin temperature distribution is topographically represented. Much like the out-of-proportion "homunculus" representing tactile sensations (Penfield & Boldrey, 1937), the temperature of the face and extremities is also disproportionally represented in the brain. Indeed the fingers and lips are very sensitive to mild warming (Green, 1984; Meh & Denislic,

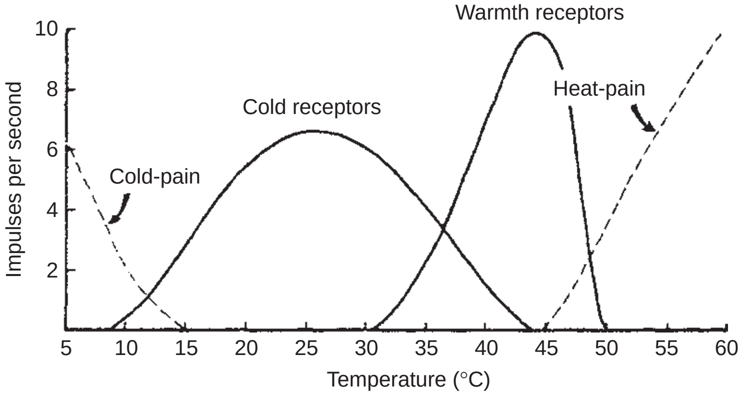

Figure 22.1 Discharge frequency of fibers transmitting cold pain, cold, warmth, and heat pain, plotted against the temperature applied to the skin (from Guyton, 1991, used by permission).

1994). Ascending secondary fibers are also relayed to the midbrain raphe nuclei, the reticular formation, and the hypothalamus (Burstein et al., 1987, 1990; Burstein & Giesler, 1989; Cliffer et al., 1991).

Anatomy and Physiology of Deep Body Thermoreception

The anatomy and physiology of thermoreception in the *deep body* is much less understood. Carotid baroreceptors and chemoreceptors may be sensitive to the temperature of the blood. Intra-abdominal temperature may affect thermoregulatory centers via the splanchnic nerve (cf. Hensel, 1981). Vagal afferents convey information of thermosensitive nerve endings from most internal organs via the cervical and thoracic branches to neurons in the nucleus of the solitary tract (Berthoud & Neuhuber, 2000).

Anatomy and Physiology of CNS Thermoreception

At all levels of the neural axis, from the spinal cord to the cerebral cortex, so-called “thermosensitive neurons” are found (reviewed in Boulant, 1981; Hensel, 1981; Van Someren, 2000). Thermosensitive neurons are defined as neurons whose evoked or spontaneous firing rate depends on local and/or peripheral (cutaneous) temperature more than would be predicted from the normal temperature dependence that is present in all biochemical processes (known as “Q10”: the ratio of activities at temperatures 10 °C apart). Neurons that increase their firing rate with warming are called “warm-sensitive neurons,” and neurons that increase their firing rate with cooling are called “cold-sensitive neurons.” An example is shown in Figure 22.2. Warm-sensitive neurons account for about 30% of the neurons in thermosensitive brain structures and

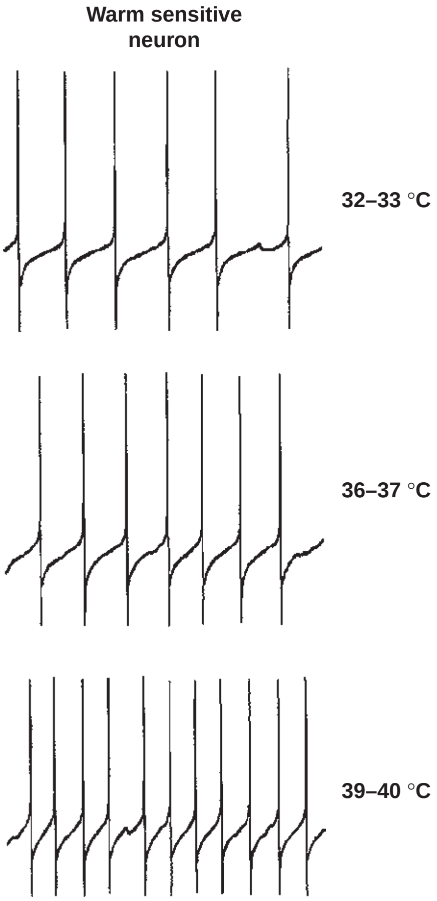

Figure 22.2 Effect of local temperature changes on the firing rate of a warm-sensitive neuron recorded in a suprachiasm atic nucleus (SCN) tissue slice. From the top to the bottom, the peak–trough vertical axes cover ±120, 115, and 100 mV, respectively. The vertical axes cover ±600 ms (from Burgoon & Boulant, 2001, used by permission).

cold-sensitive neurons for about 5–10%. A detailed account of their representation in the central nervous system is found in a paper by Van Someren (2000). In summary, these neurons have been demonstrated in the midbrain reticular formation including the raphe nuclei and locus coeruleus; in hypothalamic areas including the posterior hypothalamus, preoptic area and anterior hypothalamus (POAH); in parts of the basal forebrain including the horizontal limb of the diagonal band of Broca; in thalamic nuclei including the ventrobasal complex and midline reuniens and the reticular nuclei; and in parts of the cerebral cortex including, but not limited to, the somatosensory cortex.

There is considerable integration of thermal signals at all levels of the neural axis (early studies, e.g., Jessen, 1976; Jessen et al., 1968; Simon, 1972; Simon et al., 1965; reviewed in Hensel, 1981; Van Someren, 2000). For example, about two-thirds of the thermosensitive neurons in the preoptic anterior hypothalamic area also respond to thermal stimulation of the spinal cord and skin. When skin temperature is high, the preoptic anterior hypothalamic area neuron's firing rate is high, relatively independent of changes in local brain temperature, indicating the predominant impact of skin temperature on preoptic anterior hypothalamic area warm-sensitive neurons (Boulant & Hardy, 1974). At a lower level of the neuraxis, thermosensitive neurons in the midbrain reticular formation are sensitive to ascending thermal information originating in the skin, but not to thermal stimulation of the preoptic anterior hypothalamic area. Almost all locally thermosensitive neurons in the spinal cord also respond to thermal stimulation of the skin, whereas temperature-insensitive neurons in the spinal cord do not respond to skin temperature changes.

Age-Related Changes in Thermoreception

The number of studies on the effect of age per se on thermal senses is quite limited. The free nerve endings associated with thermal sensations appear to remain intact in elderly humans (Montagna & Carlisle, 1979), as do the conduction velocity and number of the smaller diameter afferents subserving thermal sensations (A∂ and C) and the neocortical primary sensory areas (Brody, 1992; Raz et al., 1992). Still, thermal perception is attenuated. As shown in Figure 22.3, Meh & Denislic (1994) found a marked decrease in perception sensitivity, especially in the distal parts, in agreement with earlier work of Kenshalo (1986), who noted that the elderly show mainly a decreased sensitivity to warm—not cold—stimuli applied to the plantar side of the feet, but not to other locations. It should be noted that the between-subject thermosensitivity variability also increased with age, indicating diminished sensitivity in some but intact sensitivity in other elderly subjects; elderly insomniacs, for example, are more prone to show a diminished discriminative perceptual capacity than good sleepers are (Raymann & Van Someren, 2008).

The most pronounced secondary age-related cause of a loss of thermoreceptive capacities may be diabetes, for which risk strongly increases with age. A marked loss of sensitivity, especially of the feet, has even been found in diabetics without symptoms or signs of a clinical neuropathy (Jensen et al., 1991).

It is poorly understood why the threshold for sensing changes in temperature increases with age, if the thermosensitive nerve endings, ascending fibers, and primary cortical projection areas appear to remain intact. One proposed mechanism is that the properties of the skin important for thermal conductivity, e.g., the density of collagen fibers and elastic tissue, change during the course of life (cf. Ballester & Harchelroad, 1999; Collins & Exton-Smith, 1983; Heft et al., 1996). Another hypothesis is that the age-related reduction in vascular supply to skin tissues is involved: the functionality of cold receptors is highly dependent on oxygen supply (Iggo & Paintal, 1977).

There is no age-related change in what people regard as a comfortable temperature on average. However, older rodents and humans show a much less precise operation of provided instruments to control variable environmental temperatures compared to young subjects. They thus tolerate larger *deviations* from this average before discomfort is felt and action is undertaken (Collins et al., 1977, 1981; Frank et al., 2000; Kaji et al., 2000; Tochihara, 2000), indicating a decreased subjective thermal perception.

In summary, the results indicate a loss of thermal perception in the absence of macroscopic neuroanatomical changes, especially for cold stimuli applied at the lower extremities. Structural changes in the skin may be involved.

THERMOGENESIS, HEAT GAIN, AND HEAT RETENTION

When the sensory system described above reports a deviation from the allowed temperature range, controller systems need to initiate countermeasures aimed at either gain and preservation or loss of heat. This section discusses obligatory and facultative heat generation, as well as measures for retention of body heat and for gain of environmental heat.

Obligatory thermogenesis refers to the continuously generated heat produced by intrinsic metabolic processes or vital behavior. On top of obligatory thermogenesis, facultative thermogenesis occurs with physical activity, shivering thermogenesis, and

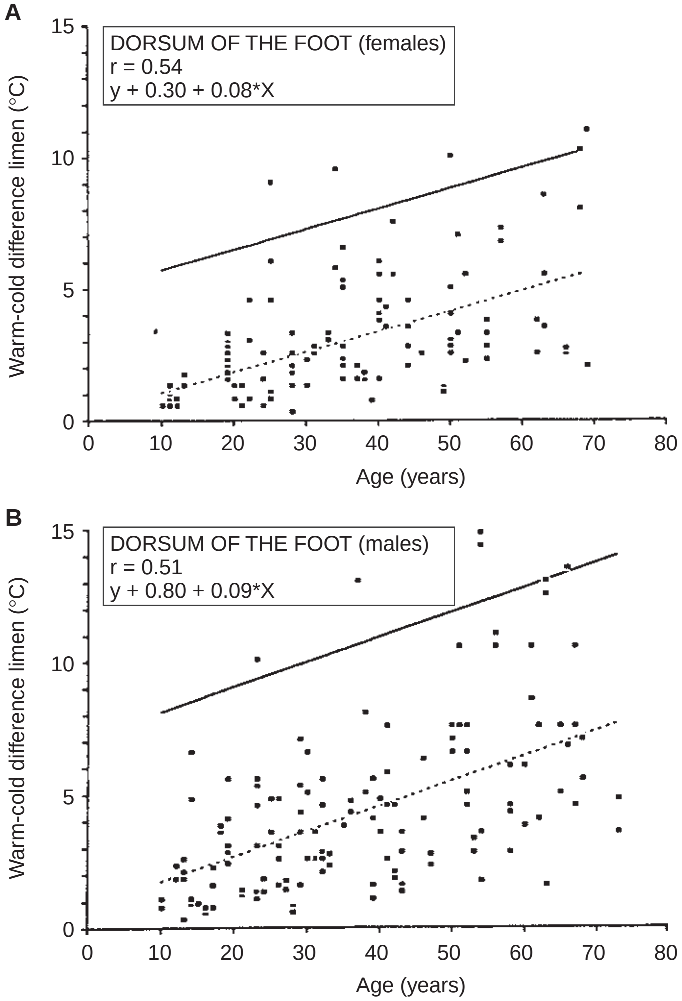

Figure 22.3 The interval of temperatures applied to the skin necessary to elicit a temperature sensation (limen) increases with age. Results of temperature sensitivity assessed at the dorsum of the foot in (A) 67 females ages 10–69 years and (B) 83 males ages 10–73 years. Regression equations clearly indicate that the unresponsive range increases with aging (from Meh & Denislic, 1994, used by permission).

humoral thermogenesis. Humoral thermogenesis can be subdivided into (1) the "classical" nonshivering thermogenesis, i.e., the sympathetic, norepinephrine-induced mitochondrial heat production in brown adipose tissue, and (2) hormonal thermogenesis, associated with epinephrine, glucagon, thyroid, growth hormone (GH), and adrenocorticotropic hormone (ACTH). Another subdivision of thermogenesis often made is behavioral versus autonomic thermogenesis. Both occur in obligatory as well as facultative ways.

Heat can also be gained and preserved, in humans and most laboratory rodents mostly through behavioral thermoregulation such as creating a microclimate by means of warm clothing and bedding; the intake of hot drinks; seeking a sunny, warm, dry, wind-sheltered environment; and taking positional measures such as curling up, huddling, and cuddling. In addition there is the autonomic thermoregulatory possibility of constriction of the skin vasculature that prevents heat exchange from the warm blood, via the skin, to the cool environment.

If exposed to cold, the body temperature drops further and takes longer to recover in older animals and humans, and even more so in the frail elderly, putting them at risk of hypothermia. The mechanisms for the generation, gain, and preservation of heat, as well as their circadian and age-related modulations are discussed below.

Obligatory Thermogenesis

Basal Metabolic Rate

At rest the human metabolic rate provides a continuous internal source of heating, accounting for about 60–75% of the total daily energy expenditure (Poehlman & Horton, 1990). The relative thermogenic contribution of the various body parts has been estimated as follows: skin and muscles 18%; brain 16%; liver, lungs, heart, kidneys, and other internal organs 56%. During physical activity muscles form the most important source of heat generation. The basal metabolic rate augmented by the heat generated by physical activity and digestion of food is referred to as the total energy expenditure. Catecholamines and sympathomimetic agents such as ephedrine, caffeine, and theophylline increase the resting metabolic rate in humans.

Age-related changes: in humans, the basal metabolic rate declines with age, especially after the age of about 30 to 50 years (McDonald & Horwitz, 1999; Poehlman et al., 1993). Elia and colleagues (2000) suggested that the basal metabolic rate decline accounts for 44% of the decrease in *total* energy expenditure of about 0.69 MJ/day per decade for men and 0.43 MJ/day per decade for women. The decline in basal metabolic rate is strongly related to the relative loss of fat-free, heat-producing tissue (Kenney & Buskirk, 1995; Poehlman et al., 1993) and to the decrease in fitness level present in many elderly people (Poehlman & Horton, 1990). Increased plasma norepinephrine concentrations, likely to increase the basal metabolic rate, are found in highly fit, physically active but not in sedentary elderly persons (Poehlman & Horton, 1990). A decreased basal metabolic rate is likely to underlie the age-related decrease in core temperature (Falk et al., 1994), which is more pronounced in elderly men (cf. Florez-Duquet & McDonald, 1998).

In summary, basal metabolic rate decreases with age, for the most part and reversibly secondary to a decreased fitness level.

Diet-Induced Thermogenesis

Digesting food increases metabolic demand and increases core temperature (Kräuchi & Wirz-Justice, 1994), more pronounced and longer so for ingested protein than for ingested carbohydrates and fat.

Age-related changes: food intake decreases with age (cf. Elia et al., 2000; Poehlman & Horton, 1990). The thermal effect of food may be reduced in the elderly as well, possibly as a consequence of an attenuated sympathetic response to meal ingestion (McDonald & Horwitz, 1999). Other studies found no effect of age (cf. Elia et al., 2000). The variability in results may be related to a secondary effect of aging, since the thermal effect of food is decreased in subjects with a high percentage of body fat and a low level of fitness and spontaneous physical activity (Poehlman et al., 1991; Tataranni et al., 1995; Witt et al., 1993).

In summary, food-intake and the thermal effect of food both decrease with aging, the latter especially in nonlean, unfit elderly people.

Baseline Physical Activity and Posture

Minimal, obligatory physical activity results in heat production from muscular activity. Even only changing the body posture from a supine to an upright position increases the core temperature by 0.1 to 0.5 °C, more so for standing than for sitting. Compared to conditions of continuous sleep and bed rest, changes in core temperature have been noted due to (1) mere wakefulness, (2) an upright posture, and (3) the activity level. Being *awake* rather than asleep, but still in a supine posture and without any activity, elevates core temperature by 0.06 to 0.31 °C (Barrett et al., 1993; Macchi et al., 1995; Van Dongen et al., 1996). If awake, changing the body *posture* from supine to upright increases the core temperature by 0.1 to 0.5 °C, whereas the mean skin temperature drops by about 5 °C (Minors & Waterhouse, 1989; Tikuisis & Ducharme, 1996; Van Dongen et al., 1996). Even a change from supine to semisupine (10°) increases core temperature (Aizawa & Cabanac, 2002). The effect of standing is stronger than the effect of sitting. Considering the additional temperature increase due to essential *activity*, Levine et al. (1999) estimated the energy expenditure for standing and walking compared to sitting to increase by 11 and 106%, respectively. There is an ISO norm (No. 8996) on the relation between metabolism and several activities. It should be noted that all these findings concern short-term laboratory findings and that prolonged bed rest for several days, as may occur in frail, ill elderly people, is in contrast associated with an increase in core temperature, probably because of dehydration (Aizawa & Cabanac, 2002).

Age-related changes: a sedentary lifestyle lowers heat production (Collins & Exton-Smith, 1983). Elia et al. (2000) estimated that 46% of the age-related decline in total energy expenditure is due to decreased physical activity. Gander et al. (1986) found no age differences in the daytime temperature increase due to natural home activity compared to laboratory bed rest. In contrast, Monk & Buysse (1989) reported that in fact the reduced baseline physical activity level is a major factor in the decreased diurnal circadian rhythm in temperature.

In summary, the contribution of posture and activity to energy expenditure is attenuated especially in the sedentary elderly and may contribute to a lower daytime temperature.

Facultative Thermogenesis

In a cold environment, action needs to be taken to prevent core temperature from dropping. Thermogenesis is initiated only if the body cools below a critical threshold, below which the intensity of the action increases with a certain gain, also referred to as "sensitivity," with further cooling.

Physical Activity

Physical activity increases metabolic heat production and temperature.

Age-related changes: moderate physical activity increases the core temperature of the elderly less than it does in young people. This finding cannot be attributed to a secondary age-related decrease in fitness level (Falk et al., 1994).

In summary, the thermogenic contribution of physical activity is attenuated even in the fit elderly.

Shivering Thermogenesis

Shivering thermogenesis, the production of heat by skeletal muscle tremor, is more dependent on core than on skin temperature (Bulcao et al., 2000). Shivering can increase the metabolic rate up to a factor 5 and is in addition to peripheral vasoconstriction (discussed below) a second major autonomous cold-protective response in humans (cf. Bell et al., 1992; MacKenzie, 1996; Young & Lee, 1997).

Age-related changes: in the elderly shivering commences at a lower core temperature threshold (Frank et al., 1997). Heat production may also be less because of both a smaller muscle mass and a decreased level of contraction compared to young people. The effect of age may be more prominent in males than in females.

In summary, the lower temperature threshold and decreased efficiency of shivering may contribute to a lower core body temperature during exposure to cold especially in elderly men.

Humoral Thermogenesis I: "Classical Neuronal" Nonshivering Thermogenesis

Nonshivering thermogenesis is defined as "heat production due to metabolic energy transformation by processes that do not involve contraction of skeletal muscles" (IUPS Thermal Commission, 2001), which mainly involves burning of brown adipose tissue, triggered by sympathetic activity. Although long thought of as being of minor importance in humans (cf. Collins & Exton-Smith, 1983), more recent studies demonstrate substantial amounts of metabolically active brown adipose tissue, more so among women than men (Cypess et al., 2009; Virtanen et al., 2009). Brown adipose tissue is activated with cold exposure, but less so in obese people (van Marken Lichtenbelt et al., 2009).

The mechanism of activation has been described in rats. Norepinephrine release from the sympathetic nervous system is sensed by β_3-adrenergic receptors on brown adipose tissue and induces the expression of mitochondrial uncoupling proteins. Heat can be produced by uncoupling the metabolic chain from oxidative phosphorylation in the inner membranes of mitochondria (cf. Horvath et al., 1999).

Age-related changes: brown adipose tissue decreases with age and is especially low in elderly people with a high body mass index (Cypess et al., 2009). This might be involved in the attenuated cold-induced increase in metabolic rate in old people, which does not appear to be secondary to a decreased fitness level (cf. Falk et al., 1994; Smolander, 2002).

In summary, the importance of brown adipose tissue thermogenesis may not be as negligible in humans as previously thought. Thus, the decrease in the mass of brown adipose tissue in the elderly is a possible factor in the thermoregulatory problems of the elderly.

Humoral Thermogenesis II: "Nonclassical" Nonshivering Thermogenesis

The humoral response to a cold environment is not limited to an increased sympathetic output inducing uncoupling protein in brown adipose tissue. First, sympathetic liberation of norepinephrine and epinephrine from the adrenal medulla into the bloodstream induces glycogenolysis in muscle and liver cells. Furthermore, thyroxine enhances the metabolic rate of most cellular chemical reactions. Other humoral factors increasing the metabolic rate include testosterone, GH, glucagon, insulin, ACTH, and dehydroepiandrosterone (DHEA; cf. Jansky, 1995; Lardy et al., 1995).

Age-related changes: the levels of most humoral factors change with aging. Examples are the strongly reduced secretion of growth hormone during sleep and the decrease in DHEA by about 2% per year (Vermeulen, 1995).

In summary, aging appears to be associated with a decrease in the capacity for nonclassical nonshivering thermogenesis.

Gain and Retention of Heat

Behavioral Measures for Heat Gain and Retention

Humans rely mostly on behavioral measures to prevent hypothermia in a cold environment, because they have only limited autonomic capabilities. Examples of behavioral measures are the creation and application of clothing, bedding, shelter, and heating systems. In contrast to most autonomous thermoregulatory responses, behavioral thermoregulation is not postponed until a drop in core temperature occurs, but is triggered by changes in skin temperature (cf. Cheng et al., 1995; Daanen, 1996; Grahn et al., 1998).

Age-related changes: the elderly regulate their indoor ambient temperature less precisely and tolerate larger deviations from the comfortable average before action is taken (Collins et al., 1977, 1981; Kaji et al., 2000; Tochihara, 2000).

In summary, age attenuates the behavioral response to a cool environment.

Capacitive and Insulative Properties of the Body

The core of the body is isolated from the environment by skin and subcutaneous tissue, with a prominent protective role for subcutaneous fat. There is some evidence that repeated exposure of the skin to cold increases local subcutaneous fat deposits and thus enhances thermal insulation and heat retention (Imamura et al., 2000). Although piloerection does occur in humans exposed to cold, it is of no functional significance, in contrast to the effectiveness in furred animals.

Age-related changes: the age-related decreases in heat production and in total body water content both attenuate the thermal buffering capacity (Ballester & Harchelroad, 1999; Collins & Exton-Smith, 1983) and the decrease in insulating subcutaneous tissue further contributes to that (Richey et al., 1988).

In summary, the decreased "heat reservoir" and insulating subcutaneous tissue make the aged more vulnerable to deviations from the set point and may decrease the stability of temperature.

Autonomic Heat Retention by Peripheral Vasoconstriction

Peripheral vasoconstriction is an important autonomic response to cold exposure, which restricts heat transfer from the core to the environment through the skin. Peripheral vasoconstriction is more dependent on core than on skin temperature (cf. Bulcao et al., 2000; Cheng et al., 1995; Daanen, 1996; Grahn et al., 1998). Cutaneous vasoconstriction is predominantly controlled through the sympathetic part of the autonomic nervous system. Most sympathetic activation promotes vasoconstriction. During cold stress, norepinephrine is released from sympathetic nerve endings and induces vasoconstriction through α-receptors or vasodilation via β-receptors. The skin of the extremities mainly contains α_2 receptors and thus shows strong vasoconstriction (cf. Frank et al., 1997; Kellogg et al., 1989). In contrast, there is poor vasoconstrictive capacity in the face, resulting in poorly attenuated heat loss from this site during cold exposure (cf. MacKenzie, 1996).

When exposed to cold, increased sympathetic output to the adrenal medulla induces it to release more epinephrine as well as some norepinephrine into the bloodstream. As mentioned above norepinephrine is a strong vasoconstrictive agent, as is epinephrine but to a lesser extent. Other powerful vasoconstrictive agents are angiotensin, acting on all arterioles, and vasopressin (cf. Guyton, 1991).

Age-related changes: under thermoneutral ambient conditions the elderly have a lower skin temperature at the extremities (Rasmussen et al., 2001), suggesting enhanced vasoconstriction. The vasoconstrictive response to cold exposure, however, is attenuated in elderly people, more so in men than women, and this may be the most important factor involved in poor cold defense (Florez-Duquet & McDonald, 1998). The age-related loss of vasoconstriction is present at the threshold, gain, and maximum level. Frank and colleagues (2000) demonstrated that the threshold for cold-induced vasoconstriction lies at a lower core temperature in old age and that the maximal vasoconstriction is reduced, possibly because of an attenuated release of norepinephrine. The mechanism underlying the decreased cold-induced vasoconstriction is most likely an increased arterial wall stiffness. A decrease in the smooth muscle α-adrenergic receptor density has also been demonstrated, which is, however, compensated for by an increased sympathetic nervous system activity, leaving the net result unchanged (cf. Florez-Duquet & McDonald, 1998).

In summary, the delayed and slower evolving vasoconstrictive response to a cool environment will contribute to a lower and more variable body temperature in elderly people.

HEAT LOSS AND REDUCTION OF HEAT GAIN

If core body temperature increases because of heat exposure or intense physical activity, heat production should be limited and instead radiation, conduction, convection, and evaporation of heat from the body to the environment should be promoted. Radiation is the emission of heat through infrared electromagnetic waves. Conduction is the transmission of heat to other objects and air by direct contact. Convection aids conduction if the warm air rises up and away from the body or is promoted by air movement (wind). Evaporation of water from the skin and lungs also continuously draws heat from the body, further increasing when sweating occurs. Heat loss is promoted by both behavioral and autonomic responses. Behavioral measures include the intake of fluids to prevent dehydration, decreasing the level of physical activity, and seeking a cool, shady, windy environment. Autonomic measures include a decrease of the sympathetic outflow to the periphery.

Peripheral Blood Flow

An increase in core or skin temperature induces peripheral vasodilation. Cutaneous vasodilation results in increased skin blood flow, which promotes three heat-loss-enhancing mechanisms. First, heat is convected from the internal organs and working muscles to the skin. Second, the resulting increase in skin temperature promotes dry heat loss by convection and radiation to the (cooler) environment. Third, the increase in skin temperature also elevates the skin-to-ambient vapor pressure gradient, which promotes sweat evaporation. At neutral (24–25 °C) ambient temperatures, with a core temperature of about 37 °C and a skin temperature of about 34 °C, the human core temperature is mainly controlled through alterations in skin blood flow and less so by changes in metabolism or evaporative heat loss (cf. Brooks et al., 1997). If heat loss is required, the total perfusion of the skin with warm blood may increase from ≈0.2–0.5 to 7–8 L/min, resulting in an up to eightfold increase in the transfer of heat from the core to the skin (cf. Guyton, 1991). Such elevated skin blood flow can take as much as half of the cardiac output and requires a redistribution of blood flow from other circulations, the splanchnic and renal circulations in particular (Kenney, 2001).

In the extremities—i.e., the palmar and plantar sides of hand and foot, respectively, as well as the nail bed, elbows, lips, cheeks, ears, and nose—this huge increase in skin blood flow is accomplished mostly by the opening of arteriovenous anastomoses, which are shunts between the arteries and the venous plexus. Arteriovenous anastomoses are sympathetically innervated, and both cholinergic and noradrenergic terminals have been found, as well as α-adrenoreceptors (cf. Daanen, 1996).

At rest and in thermoneutrality skin blood flow is controlled by the sympathetic vasoconstrictor system. During warming of the skin, not only a release of the tonic adrenergic vasoconstrictor tone but also an active vasodilator system is activated, accounting for up to 80 to 95% of the increase in peripheral blood flow (cf. Brooks et al., 1997). The neurotransmission mechanism of active vasodilation is not fully understood and may be related to sympathetic sudomotor activity, although acetylcholine is not implicated (Kellogg et al., 1989). It has also been suggested that parasympathetic cholinergic innervation of the vessels induces a sequence of steps leading to nitric oxide release, which relaxes the vascular smooth muscles (McCann et al., 1998).

For vasomotor control, three regions can be distinguished: (1) the extremities, (2) the trunk and proximal limbs, and (3) the face (cf. Hensel, 1981). Modulation of the sympathetic constriction is strongly present in the extremities. Active vasodilation may play a more prominent role in the trunk and proximal limbs. On the forehead there is little vasoconstrictive response to cooling, but a vasodilation in response to warming does occur.

Age-related changes in peripheral blood flow: elderly people consistently show a lower skin blood flow at any core temperature in three experimental conditions: (1) passive whole-body heating, (2) exercise-induced body heating, and (3) local skin heating (cf. Ho et al., 1997; Kenney, 1988; Kenney & Ho, 1995; Kenney et al., 1990; Minson & Kenney, 1997; Minson et al., 1998). An example is shown in Figure 22.4. First, in old age, the threshold for vasodilation with heating is increased (cf. Collins & Exton-Smith, 1983), which is secondary to poor fitness: no increase in threshold is found when fit elderly subjects are compared to fit young subjects (Kenney, 2001). Indeed, regular aerobic exercise in the long term results in a lower core temperature threshold needed to induce the onset of vasodilation (Ho et al., 1997; Thomas et al., 1999). Second, the gain, i.e., the slope of blood flow increase versus core or skin temperature increase, decreases with age (Kenney, 2001; Tochihara, 2000). Third, the maximal skin blood flow declines with age and cannot fully be attributed to changes in fitness level (Havenith et al., 1995; Kenney, 2001; Martin et al., 1995; Rooke et al., 1994).

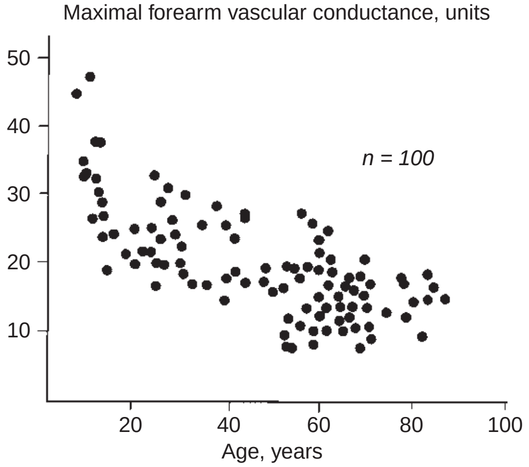

Figure 22.4 Prolonged heating of the skin at 42°C elicits maximal skin blood flow in the heated area. The skin temperature of the left forearm was uniformly clamped at 42°C by spraying a fine mist of water over the surface. Maximal forearm skin vascular conductance is shown as a function of age in 100 healthy subjects ranging in age from 5 to 85 years. Each filled circle represents the maximal vascular conductance for an individual subject. Maximal forearm skin conductance (minimal resistance) decreases fairly linearly across this large age span (from Kenney, 2001, based on data from Martin and colleagues, 1995, used by permission).

Multiple mechanisms contribute to the age-related reduction in vasodilatory capacity. Structural changes in the cutaneous vasculature may limit vessel wall expansion (cf. Collins & Exton-Smith, 1983; Kennaway, 1994; Kenney, 2001; Kenney, 1988; Montagna & Carlisle, 1979). Evans and colleagues (1993) suggested that thermally induced cutaneous blood flow is reduced in older persons at nutritive capillary sites, but not at arteriovenous anastomosis-rich sites. An increased sympathetic (noradrenergic) vasoconstrictor tone is unlikely, and rather a diminished sensitivity of the active vasodilator system may occur (Kenney et al., 1997). A decrease in cardiac output and in the redistribution of the circulation from the splanchnic and renal flows to the skin may contribute as well, the former more so in the unfit elderly (Ho et al., 1997; Minson et al., 1998).

In addition to fitness, two other secondary age-related factors may be involved in the decreased vasodilatory capacity. First, dehydration, which often occurs in the elderly, lowers skin blood flow (cf. Havenith, 2001; Kenney et al., 1990). Second, much like exercise, regular exposure to extreme ambient temperature, i.e., heat or cold, lowers the threshold of core temperature needed to induce the onset of increased skin blood flow (Hensel, 1981). Those living in homes for the elderly may seldom experience such exposures.

In summary, vasodilatory capacity decreases with age because of multiple primary and secondary mechanisms.

Evaporative Heat Loss

An increase in core temperature, and to a lesser extent in skin temperature, induces evaporative heat loss. Sweating is the principal evaporative mechanism used during exercise and in hot environments. Sweat glands are innervated by the cholinergic, so-called "sudomotor" sympathetic fibers, i.e., other fibers than those involved in controlling the skin vasomotor tone (Liguori et al., 2000; Mano, 1998). Sympathetic sudomotor innervation of the hairy skin is activated during heat stress, whereas the sudomotor innervation of the distal glabrous skin is mainly activated during psychogenic stress. Up to 2 L of sweat per hour can be produced, allowing for the removal of 10 times the basal rate of heat production (cf. Guyton, 1991). Evaporative respiratory heat loss, as used by some animals, is of little importance in humans.

Age-related changes: the elderly have an attenuated sudomotor response, i.e., the sweating response to thermal stimulation is markedly decreased. Both the threshold and the maximal capacity are affected (cf. Collins & Exton-Smith, 1983; Hensel, 1981). However, this finding may be secondary to poor fitness. Havenith et al. (1995) applied multiple regression analyses on predictors of poor responses to heat stress and demonstrated that low levels of fitness and physical activity account for most of the attenuated sweating response. Intact sweating capacity in fit elderly men and women has been demonstrated indeed in most studies (Inoue et al., 1999; Pandolf, 1997; Yousef et al., 1984), but not in all (Anderson & Kenney, 1987). The cause of lower sweating ability may be an attenuated response of the gland to stimulation or a structural alteration in the glands or surrounding tissue. No age-related difference in the density of heat-activated sweat glands was found (Anderson & Kenney, 1987). A secondary factor often occurring in aging and negatively affecting the sweating response is dehydration (Havenith, 2001).

In summary, elderly people especially with a low level of fitness have a decreased sweating response.

CENTRAL THERMOREGULATORY CONTROL

Thermoregulatory Control

The primary brain area that integrates central and peripheral thermosensitive inputs, and coordinates thermoregulatory outputs, is the preoptic anterior hypothalamic area. In animal studies, thermode warming or cooling of the preoptic anterior hypothalamic area elicits autonomous and behavioral heat loss or retention responses, respectively (cf. Boulant, 1999; Hensel, 1981). The preoptic anterior hypothalamic area is not the only area of importance. A first organizational principle is that there appears to be a hierarchical organization of regulation of temperature at several levels of the neuraxis (Satinoff, 1978). Spinal reflexes are sufficient for warming of a single hand or foot to cause vasodilation in the other. The representation of thermosensitive cells and ascending projections throughout the neuraxis have already been discussed in detail under Thermoreception, so this section will focus on general thermoregulatory output control centers. A second organizational principle is that units (neurons) in this system are not necessarily involved exclusively in thermoregulatory control, but may sense other deviations, e.g., in osmolality, or serve other functions, e.g., the regulation of sleep and vigilance, as well (Raymann et al., 2008; Raymann & Van Someren, 2007; Van Someren, 2004, 2006).

Structures of importance for thermoregulatory control include, in an ascending sequence, the peripheral sympathetic pathways, the spinal cord, parts of the brain-stem reticular formation, the preoptic anterior and posterior hypothalamic nuclei, the ventrobasal and intralaminar thalamic nuclei, and the somatosensory cortex. Characteristically, these centers can modulate ascending, descending, and reflex transmission (e.g., Arancibia et al., 1996). The strength of vasomotor reflexes induced by changes in local skin temperature, for example, can be modulated by the higher regulatory centers.

With the exception of vasomotor control, which is continuously active in the thermoneutral range, homeotherms, including humans, rely primarily on behavioral thermoregulation and only secondarily on autonomic thermoregulation. The anatomical sites involved in the two types of regulation may differ. It has been demonstrated, for example, that stimulation of the posterior hypothalamus mainly elicits thermoregulatory behavior, whereas stimulation of the preoptic anterior hypothalamic area may in addition elicit autonomic thermoregulation (Hensel, 1981).

For the control of vasomotor tone, efferents from the preoptic anterior hypothalamic area project via the medial forebrain bundle to the vasoconstrictor area of the rostral ventrolateral medulla (Gilbert & Blatteis, 1977; Morrison, 2001; Smith et al., 1998). From this area, a distinct population of sympathetic noradrenergic premotor neurons projects to the spinal cord where they excite the sympathetic vasoconstrictor neurons that give rise to the unmyelinated innervation of the skin blood vessels. Even at rest, there is a continuous slow firing of these neurons, inducing a permanent partial constriction called the vasomotor tone. The vasomotor center receives input not only from the hypothalamus, but also from several cortical areas, the amygdala, the septum, and the hippocampus (cf. Guyton, 1991).

Innervation of brown adipose tissue may originate in the sympathetic premotor neurons in the rostral raphe pallidus (Morrison, 2001).

The primary motor center for shivering is located in the dorsomedial portion of the posterior hypothalamus. It receives inhibitory input from the preoptic anterior hypothalamic area and excitatory input from skin and spinal cord thermoreceptors (cf. Guyton, 1991; Tanaka et al., 2001). The output itself is not rhythmic: the muscle oscillation is probably related to a muscle spindle stretch reflex.

The shivering pathway runs from the posterior hypothalamus caudally through the midbrain tegmentum and pons, close to the rubrospinal tracts, to the cerebrospinal and reticulospinal tracts.

Age-related changes in central thermoregulatory control: age-related changes in the brain areas involved in thermoregulation have remained virtually unexplored. Aged rats have fewer neurons in the anterior hypothalamic area (Hsu & Peng, 1978) and have a smaller body temperature response when injected intracerebroventricularly with prostaglandin E2, norepinephrine, serotonin, dopamine, and carbachol (Ferguson et al., 1985).

In summary, there is only marginal information about age-related changes in the neural substrate of thermoregulatory control, and it is at present not known to what extent these findings are indeed relevant for age-related changes in thermoregulatory control.

Central Control of the Circadian Rhythm in Temperature

The circadian (literally "about a day") rhythm in body temperature is controlled by the hypothalamic suprachiasmatic nucleus, representing the biological clock of the brain. The suprachiasmatic nucleus actually consists of two small (±0.25 mm^2; ±10,000 vasopressin neurons each) nuclei located at the bottom of the anterior hypothalamus just above the optic chiasm and separated by the third ventricle. Projections within the hypothalamus, notably the subparaventricular zone, are involved in the circadian modulation of thermoregulation (Lu et al., 2001). A multisynaptic projection to the melatonin-producing pineal is important for the circadian regulation of temperature. Under control of the suprachiasmatic nucleus, melatonin is secreted only during the night, and in humans induces a strong peripheral vasodilation. This heat-loss-promoting property of melatonin may account for ±40% of the amplitude of the circadian rhythm in the core temperature under resting conditions. Melatonin may act through both POAH melatonin receptors (Krause & Dubocovich, 1990) and receptors in the vasculature (cf. Cagnacci, 1997).

Age-related changes in the circadian temperature rhythm: the circadian amplitude of human body temperature declines from childhood to senescence—estimates range from an about 10 to 50% decrease. Moreover, many studies have confirmed an advanced phase (Czeisler et al., 1992; Duffy et al., 1998) and it appears that especially the early morning rising phase of the rectal rhythm is advanced (Duffy et al., 1998; Monk & Kupfer, 2000). The nocturnal temperature minimum in the elderly indeed remains up to 0.3–0.4 °C higher than the minimum in young adults (Weitzman et al., 1982), especially in poorly sleeping elderly persons (Lack et al., 2008). The age-related changes may result from alterations in the suprachiasmatic nucleus. Thus, the number of neurons expressing the peptide vasopressin and its mRNA declines in old age (Swaab et al., 1985). Vasopressin is strongly implicated in the clock output that is of importance to the regulation of the rhythm of temperature (Wideman et al., 2000).

In summary, the circadian rhythm in the core body temperature typically shows an advanced phase and a flatter amplitude.

CONCLUSION

The vulnerability of the elderly to respond suboptimal to thermal challenges may result from deficiencies at several levels: thermoreception, thermogenesis and conservation, heat loss, and central regulation. More research is needed to evaluate the relative contributions of these changes. Some changes appear to be secondary to—or aggravated by—a high body mass index and a sedentary lifestyle. This is where efforts to improve the thermoregulatory capacity of old people might best start.

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Chapter | 23 |

Sex Differences in Longevity and Aging

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CHAPTER CONTENTS

Introduction **479**
The Robustness of the Sex Difference in Survival **480**
Do Women Age more Slowly than Men? **482**
The Mortality–Morbidity Paradox **484**
Hypotheses for the Sex Difference in Longevity **485**
  Evolutionary Hypotheses 485
    The Extrinsic Hazards (Williams) Hypothesis 485
    The Heterogametic Sex Hypothesis 487
    The Parental Care Hypothesis 488
  Mechanistic Hypotheses 488
    Sex Differences in Longevity in Laboratory Rodents 488
    Oxidative Stress 489
    Size Difference 491
Conclusions **492**
Acknowledgments **492**
References **492**

INTRODUCTION

Possibly no feature of human biology is more robust than women's survival advantage over men. Women live longer than men in cultures with short life expectancies and those with long life expectancies. Women live longer through periods of war, famine, and pestilence. They are the superior survivors when they are old, when they are young, even in utero. This pattern cannot be attributed to one or a few elevated causes of male death. Women in modern societies die at lower rates from virtually all of the major causes of death. In the United States, if all men's heart disease, their number one cause of death, disappeared tomorrow, they still would not live as long as women.

Intertwined with this robust pattern lies a central paradox of women's survival advantage. They suffer from more illness and chronic health problems than do men. They suffer more disability, make more visits to doctors, and endure more hospital stays.

Despite these major patterns in human biology, the mechanisms underlying them are not even vaguely understood. In fact, research on basic biological mechanisms of the sex difference in longevity is surprisingly sparse. One reason for this is probably the biomedical research establishment's overreliance on the laboratory mouse as the mammalian research model of choice. Laboratory mice have no such consistent sex-specific pattern of mortality or disease. In many studies male mice outlive females; in many other studies there is minimal difference, and in still others females substantially outlive males. To investigate the mechanistic basis of the human sex difference in illness and survival fruitfully, we need either to understand the mechanistic basis of this study-to-study variation or possibly to employ animal species that have a more consistent survival bias. In this chapter, I review the copious evidence for the robustness of women's survival advantage, discuss some of the extant evolutionary and mechanistic hypotheses to explain this pattern, and also examine some pragmatic approaches to investigating this issue. Although some excellent research has been performed on sex differences in survival in insects (see Kawasaki et al., 2008; Carey & Liedo, 1995; Carey et al., 1995), because of limitations of space this chapter focuses primarily on vertebrates.

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THE ROBUSTNESS OF THE SEX DIFFERENCE IN SURVIVAL

Each year the United Nations publishes a *Demographic Yearbook*, which among other things lists life expectancies for every country, principality, island group, and territory for which data are available. Some countries' data are of uneven quality. For instance, the 2007 *Yearbook* lists life expectancy at age 100 for Costa Rican men as 11.7 years (compared with 5.7 years for women). Given that many studies of centenarians in a variety of countries conclude that even with exquisite health care the annual mortality rate of centenarians is 30–50%, which translates to a life expectancy of roughly 1–2 years, these data are clearly not credible. As birth registration was a haphazard to nonexistent affair in many countries for much of the 20th century, anomalies in survival records for the very elderly are not surprising and certainly not confined to Costa Rica (Kannisto, 1988).

Nevertheless, among the 143 such demographic units reported in the most recent (2007) edition of the *Yearbook*, women's life expectancy at birth exceeds men's in 140 of them (Figure 23.1). One country (Afghanistan), with only rudimentary demographic information, lists both men's and women's life expectancy as 43.0 years. The two places in which men are reported to live longer are the Turks and Caicos Islands, a British overseas territory with fewer than 40,000 residents, and Qatar, an Arab emirate of about 1.5 million inhabitants (Table 23.1). Past editions of the *Yearbook* have reported a handful of other countries (e.g., Nepal, Bangladesh, Maldive Islands) with slightly greater male life expectancies, but in the latest report women's life expectancy in these countries has moved slightly ahead of men's. Notably, male life expectancy is suspiciously high in both countries reporting longer-lived men. The Turks & Caicos, for instance, reports that its men live almost as long as Japanese men and Qatar reports that its men live almost 2 years longer (Table 23.1). The best international data sources acknowledge that Japan is currently the country with the longest-living people in the world, for both sexes (Oeppen & Vaupel, 2002). For that reason, I attribute these anomalous reports to poor data quality rather than a real difference. Thus in modern societies the female longevity superiority is virtually universal.

Table 23.1 Sex differences in life expectancy at birth in selected countries

	LIFE EXPECTANCY (YEARS)	
COUNTRY	**MEN**	**WOMEN**
United States (2005)	74.9	79.9
Japan (2007)	79.2	86.0
Russia (2007)	61.4	73.9
Qatar (2007)	81.0	79.2
Turks & Caicos (2007)	79.0	77.4

Source: United Nations *Demographic Yearbook* (2007).

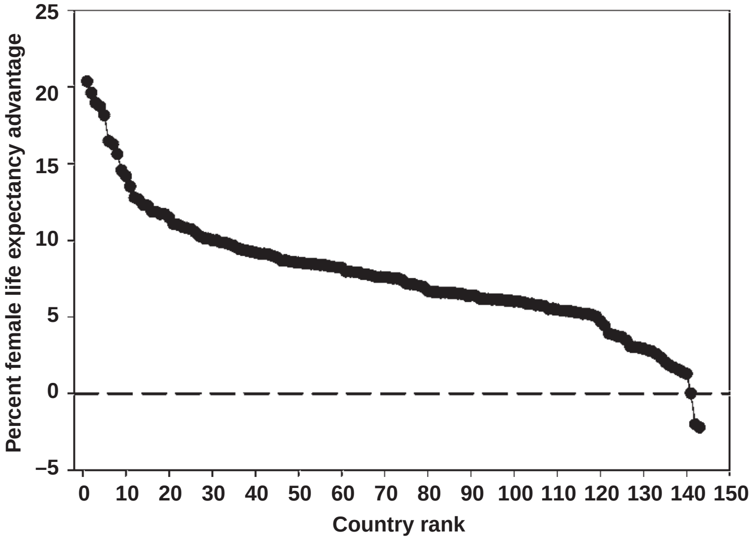

Figure 23.1 Percentage by which female life expectancy at birth exceeds that of males across 143 demographic entities. Source: United Nations *Demographic Yearbook* (2007).

Not surprisingly, there is a considerable range in the sex difference in life expectancy due to complex sociocultural factors. Across the previously mentioned 143 demographic entities, female life expectancy exceeds male life expectancy by an average of around 8%. However, in Russia and several other countries from the former Soviet Union women expect to live about 20% longer than men (Figure 23.1).

A particularly rich source of current and past national mortality and population data is assembled in the Human Mortality Database (http://www.mortality.org/), which catalogs only highly reliable data and therefore includes fewer countries than the United Nations *Demographic Yearbook*. In this data base, women's life expectancy at birth exceeds men's in every one of the 47 countries currently catalogued and in every year of data listed (e.g., from as early as 1751 in Sweden).

During most of the 20th century, the life expectancy gap between the sexes widened internationally as men's mortality declined more slowly than women's. However, this trend has recently reversed, with the gap narrowing between the sexes in most countries because an equivalent decrease in mortality affects life expectancy more in men than in women because of differences in the shape of their survival curves (Glei & Horiuchi, 2007).

Life expectancy at birth is a rather crude indicator of survival at adult ages as it can be heavily affected by infant mortality. Moreover, males in their teens and 20s often display elevated mortality due to accidents, homicides, and suicides, and before modern medical practices, women suffered significant mortality associated with childbirth. What if we consider life expectancy at age 50, when the dangers of infant, testosterone-induced, and maternal mortality are past? In this case too, women's life expectancy exceeds men's in every country and every historical period with reliable records (Human Mortality Database, 2009).

An illustrative example is Iceland, which arguably has the best historical information about its population of any country in the world (Tomasson, 1977). Icelanders are the only European people whose origins occurred in historical time. Even the names of many of the original settlers who arrived between 870 and 930 are known. Iceland's history has been described as a "millennium of misery" as it has been ravaged repeatedly by famine, disease, flooding, volcanic eruptions, and sometimes combinations of these calamities. Just during the 19th century, life expectancy at birth dipped to as low as 18 years during a particularly horrific measles outbreak and reached as high as 57 years during halcyon times (Andreeva, 2008). By contrast, for a time in the 1970s and 1980s Iceland boasted the world's highest life expectancy (Oeppen & Vaupel, 2002). But from the most calamitous to the most salubrious times, women's life expectancy both at birth and at age 50 exceeded that of men, sometimes dramatically. In fact more often than not, the percentage by which women's life expectancy exceeded men's was greater at age 50 than at birth (Figure 23.2).

As a consequence of a lifetime of survival advantage, there are many more extremely old women than men. Among centenarians, there are typically about three women for every man (Kannisto, 1988). At older ages, the sex ratio is even higher. For instance, as of November 2009, there were 77 well-documented supercentenarians (people at least 110 years of age) known to be alive worldwide, according to Robert Young of The Los Angeles Gerontology Research Group; 73 (95%) of these were women (www.grg.org/Adams/Tables.htm).

Did women live longer in the distant human past before the development of agriculture and the consequent aggregation of humans in towns and cities? As preagricultural societies were also preliterate societies, no written records exist. Therefore, we must guess about this from modern studies of cultures that remain preagricultural or did so until recently. Here data quality is particularly problematic, beset by the uncertainties of indirect birth date estimation, small sample sizes, and the complexities of social disruption and disease exposure as regular contact with the outside world became common. It may be overly optimistic to expect that subtle life expectancy differences of 5 to 10%, such as most commonly observed in the modern world, will emerge from inspection of these data.

Given these caveats, extant analyses of modern unacculturated groups reveal little in the way of a consistent pattern of sex difference in survival. For instance, a small female survival advantage from birth is reported among the Yanomama and Xavante tribes of South America, for which life expectancies are estimated to be less than 20 years (Neel & Chagnon, 1968); however, a subsequent analysis of the Yanomama reached an opposite conclusion (Early & Peters, 2000). Perhaps, the most thorough analysis is that of the Ache of eastern Paraguay. Before continuous contact with Westerners, the Ache lived in reasonably rich ecological circumstances. Life expectancy at birth was estimated to be 37–38 years. Males were estimated to have a small survival advantage in childhood (up to age 10), but females survived better in adolescence and adulthood. Female life expectancy exceeded that of males throughout life after the age of 2 years (Hill & Hurtado, 1996).

As much as it would be informative to know whether women survive better than men in a state of nature, such information is simply not recoverable from available data sources. Even with better demographic information there is no reason to expect that these extant hunter–gatherer cultures living in marginal environments would represent the demography of Paleolithic humans (Hill & Hurtado, 1996).

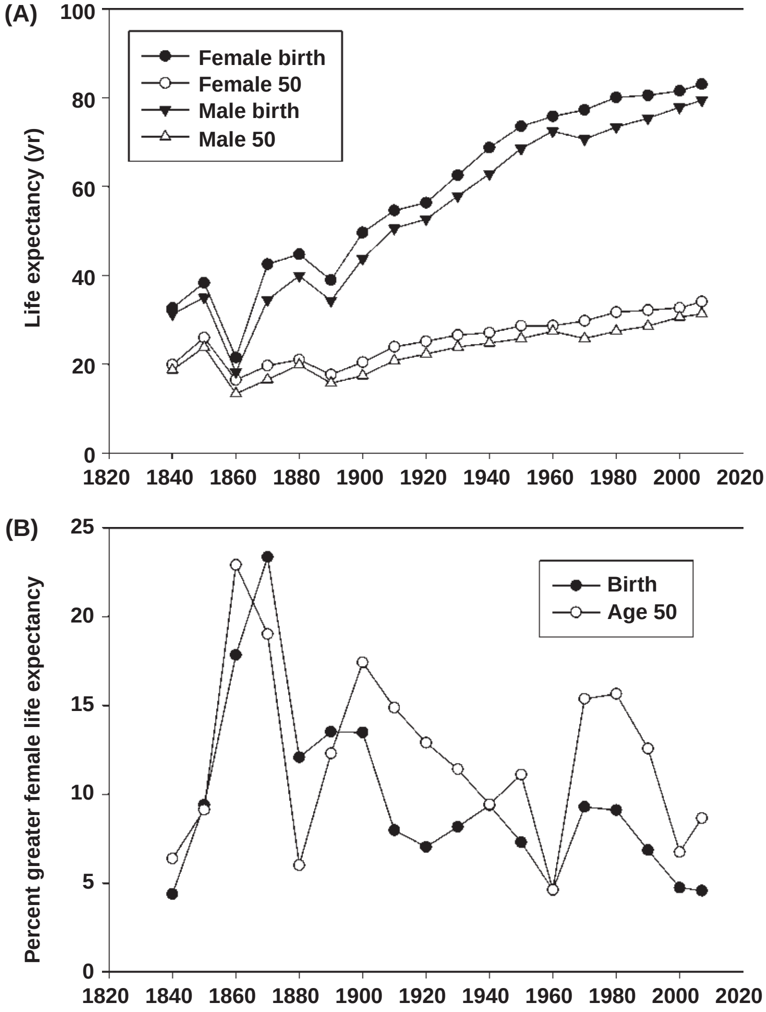

Figure 23.2 Sex difference in life expectancy at birth and at age 50 in Iceland from 1840 to the present. (A) Absolute life expectancy. (B) Percentage by which female life expectancy exceeds that of males. Source: Human Mortality Database (2009).

A related question is whether females survive better in our closest anthropoid relatives. For chimpanzees, the answer is clearly yes. Hill and colleagues constructed a chimpanzee life table combining survival data from five separate long-term field studies and found that female life expectancy was greater than that of males from birth to senescence (Hill et al., 2001). A similar pattern is seen in captive chimpanzees and also among our next closest relatives—gorillas and orangutans (Dyke et al., 1995; Allman et al., 1998). Females live longer in a number of other Old World primate species, too (Bronikowski et al., 2002; Fedigan & Zohar, 1997). However this pattern is not universal. Equal female–male survival or a slight male advantage has been reported for a captive population of a small ape, the siamang (Allman et al., 1998; Smucny et al., 2004). Thus a female survival advantage seems to be common, though not universal, among our anthropoid relatives.

DO WOMEN AGE MORE SLOWLY THAN MEN?

Does the consistent survival superiority of women over men across countries and historical epochs suggest that they age more slowly? This raises the complex issue of exactly how we measure the rate of aging. In an actuarial sense, one might define aging as the age-related rate of increase in the annual probability of dying among adults (Finch, 1990). While in

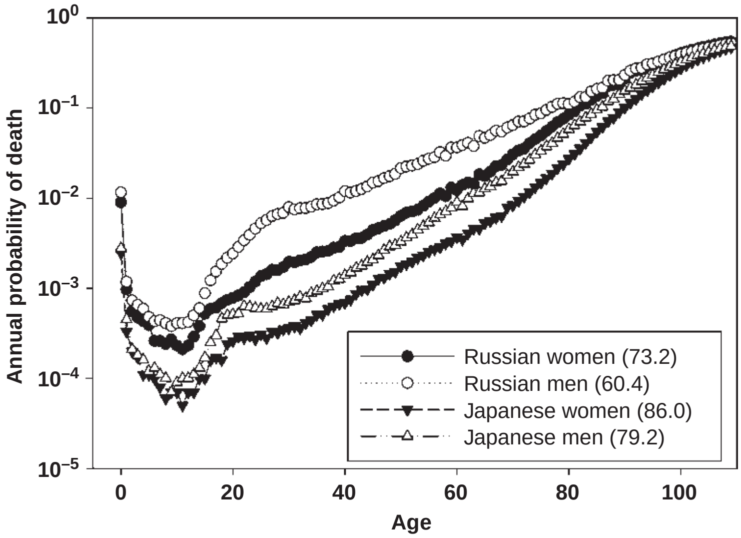

Figure 23.3 Age-specific mortality of the sexes in a country with short-lived (Russia) and long-lived men (Japan). Note that the age-related rate of increase is virtually identical among Russian women and both Japanese sexes, whereas the increase is slower among Russian men, mainly because of elevated mortality at young adult ages. Data are from 2006 for Russia, 2007 for Japan. Numbers in parentheses are life expectancies at birth. Source: Human Mortality Database (2009).

many senses intuitively satisfying, this metric has the disadvantage that any factor that elevates death rate in early adulthood without affecting it at later ages could be said to slow aging. For instance, age-specific mortality increases by more than 15-fold among Russian men between ages 13 and 30. This elevation of early mortality, which is substantially greater than in Russian women or Japanese of either sex, makes the overall rate of mortality increase throughout the rest of life *slower* than that of either Russian women or the Japanese generally (Figure 23.3), all of whom are longer lived. It hardly seems reasonable then to claim that Russian women and Japanese men and women, all of whom have lower mortality rates throughout life and live 1 to 2 decades longer than Russian men, are aging more quickly. Carnes & Olshansky (1997) attempted to overcome the problem of these male mortality humps in adolescence and early adulthood by partitioning mortality rate into components of intrinsic and extrinsic causes. Extrinsic causes would include, for instance, homicide, suicide, and other accidents. If they considered only intrinsic mortality, the male mortality hump disappeared (Carnes & Olshansky, 1997). When this is done, the male and female rates of mortality increase become very similar. Similarly, it is worth noting that Russian women compared to Japanese women display roughly equivalent rates of increasing mortality with age throughout most of the adult life span even though Japanese women have lower mortality throughout life and live more than a decade longer. So from a purely actuarial perspective, it appears that men and women do not age at different rates, but women simply are more robust with respect to survival at all ages even back to birth (Figure 23.3).

In fact women appear to be more robust even before birth. The human sex ratio at birth is roughly 105 males per 100 females—close to the 1:1 ratio that would be expected from simple Mendelian segregation of sex chromosomes (Jacobsen et al., 1999). However, for unknown reasons, the sex ratio of conceptuses or very short term fetuses has often been reported to be considerably male-biased. For instance, a Finnish sample of over 500 abortuses found a M/F sex ratio of 1.64 in embryos of less than 8 weeks gestational age, which had dropped to 1.19 by week 8 and to 1.05 by 14 weeks (Kellokumpu-Lehtinen & Pelliniemi, 1984). A much larger Norwegian study (>1,000,000 preterm births) found almost 2.5 males per female spontaneously aborted at 16–18 weeks gestation (Vatten & Skjaerven, 2004), with that ratio gradually dropping closer to parity as gestation advanced. Furthermore, a French study found that 57% of very early spontaneous births were of males (Zeitlin et al., 2004). This drop in sex ratio from heavily male biased early in gestation to near parity at normal term indicates considerably higher male compared with female mortality in utero. In addition, males born under adverse circumstances, such as at very early term or at very low birth weight, are more likely to die than females born in similar circumstances (Draper et al., 2009; Itabashi et al., 2009). Males seem to be the weaker vessel in utero, in childhood, in adulthood, and in old age.

The robust pattern described above, with females surviving at higher rates than males at virtually all ages, including before birth, in virtually all cultures, and during virtually all historical epochs, suggests that the sexual mortality difference is unlikely to be attributable to one or just a few causes of death. This suggestion is supported by a detailed investigation of causes of death in the United States. In the latest compilation, men had a higher age-adjusted death rate for 13 of the 15 top causes of death, including 6 of the top 7 causes. One cause (No. 3, cerebrovascular diseases) was approximately equivalent between the sexes and for only one (No. 7, Alzheimer disease) did women die at a higher rate. Men were 50% more likely than women to die of heart disease, 40% more likely to die of cancer or infectious diseases, and, less surprisingly, more than twice as likely to die in accidents (Heron et al., 2009).

Mortality patterns are not the final word in defining aging, however. Aging is also about the rate of decline in physiological function as well as an increasing propensity to suffer from chronic health problems. By these measures, the difference between the sexes takes on a somewhat different look.

THE MORTALITY–MORBIDITY PARADOX

As demonstrated above, men die on average earlier than women. However, women, it turns out, display higher overall rates of physical illness at all adult ages. They experience more disabilities and activity limitations, make more doctor visits, spend more days in hospital, and take more medications than do men (Verbrugge & Wingard, 1987; Macintyre et al., 1996; Christensen et al., 2009). Some easy and obvious explanations of this pattern are available—that women are more sensitive to physical discomfort and are more willing to seek medical attention when they perceive it. However, empirical evidence to support such an explanation is uneven and contradictory (Macintyre et al., 1999). Moreover, the mortality–morbidity paradox is not confined to wealthy western European populations, where cultural norms might make such explanations most plausible. It has been reported from many countries, including some such as Bangladesh, where crushing poverty and lack of easy access to medical intervention make such explanations less tenable (Murtagh & Hubert, 2004; Rahman et al., 1994; Gu et al., 2009; Xie et al., 2008). Over the past quarter-century, there has developed a large and complex literature on the impact of more subtle experiential, cultural, and psychosocial factors in this paradox (Macintyre et al., 1996; Verbrugge, 1989). However, it seems to me that such a consistent sex difference found in such diverse cultures and socioeconomic circumstances as the United States, Jamaica, and Bangladesh (Rahman et al., 1994) begs for a biological explanation as well.

One interesting possibility is that the morbidity difference could be a direct result of the mortality difference—the so-called "mortality selection" hypothesis (Manton et al., 1995). The logic of this idea is that as men die at higher rates throughout life, ill men will be more likely to die than ill women, leaving behind a preponderance of ill women. Traces of this effect can be observed. For instance, men die of heart disease at higher age-adjusted rates than women, but heart disease morbidity is more common among women, in at least some reports (Wingard et al., 1989). However, the few studies that have attempted to account for mortality selection statistically have found that despite its impact, the sex difference in morbidity remains (Rahman et al., 1994; Doblhammer & Hoffmann, 2009).

Perhaps, a simpler explanation is warranted. The most consistent finding among dozens of studies investigating the sex-based morbidity difference is that women are subject to more chronic nonfatal health problems and diseases, in particular autoimmune diseases such as rheumatoid arthritis and lupus, as well as non-immune-mediated joint and bone problems such as osteoarthritis, osteoporosis, and idiopathic back pain (Pinn, 2006). Arthritis, because of its attendant pain, is the leading cause of disability among elderly Americans (Crimmins, 2004). Arthritis is also more common and more severe among women than among men (Wan et al., 2005; Verbrugge, 1995). Chronic pain can not only limit activities and cause sufferers to seek medical attention, it can also have more far-reaching secondary health effects resulting from sequelae such as sleep deprivation and chronic stress. One wonders, then, whether the sex difference in morbidity might not be largely a consequence of differential susceptibility to chronic bone and joint diseases. Why women might be more susceptible than men to these chronic diseases specifically is an intriguing question, but one obvious candidate factor is differential sex hormone exposure. Sex hormones are known to affect bone and joint health (Roman-Blas et al., 2009; Karasik & Ferrari, 2008). However, that topic lies beyond the scope of this chapter and the professional competence of this author.

Given the conflicting inferences one might draw from the observations that women live longer than men, but that age-specific mortality increases at a similar rate in both sexes, in addition to which women are more prone than men to chronic age-related physical ailments, there appears to be no simple intuitive answer to the question of whether one sex ages more quickly than the other. However, there is no question that women are better designed for survival.

HYPOTHESES FOR THE SEX DIFFERENCE IN LONGEVITY

Sex differences in longevity are not confined to humans, but have been reported in many other species. In some invertebrates such as honeybees, ants, and tarantulas, females can live many times as long as males. Even within mammals, females can live dramatically longer than males. For instance, female short-finned pilot whales have a life expectancy nearly double that of males (23 vs 12 years) and the oldest female in one study lived 17 years longer than the oldest male (Kasuya & Marsh, 1984). Female African lions, red deer, and vervet monkeys also live longer than males (Clutton-Brock, 2009). As we saw earlier, females of our closest primate relatives live longer. Is it a general pattern, then, throughout animals that females live longer and perhaps age more slowly than males? To interpret the evolutionary pattern of sex differences in longevity and perhaps give insight into the difference in humans, a number of hypotheses have been developed (Williams, 1957; Promislow, 1992; Bonduriansky et al., 2008).

Biologists generally seek to understand features of organisms via immediate mechanistic or proximal causes (answering "how" questions), or via evolutionary or ultimate causes (answering "why" questions). The former seek to identify factors that determine a trait within a single population within a single generation, the latter to identify factors that cause a trait to emerge within a species over many generations (Stearns, 1999). Proximate causation is addressed by investigating how biochemistry, genetics, development, or physiology shapes traits; ultimate causation is addressed by assessing how natural selection, evolutionary constraints, or chance events influence traits. Below I consider evidence for three evolutionary hypotheses and two mechanistic hypotheses about the occurrence of sex differences in longevity.

Evolutionary Hypotheses

The Extrinsic Hazards (Williams) Hypothesis

Evolutionary senescence theory, as developed in the 1950s and 1960s by Medawar, Williams, and Hamilton, implied that one determinant of aging rate was the level of extrinsically imposed, non-senescence-related mortality, such as that due to random predation, famine, or pestilence (Kirkwood & Austad, 2000). Williams (1957) was the first to state specifically the hypothesis that senescence will evolve to be more rapid in populations that experience high levels of extrinsic mortality relative to populations that experience low levels of mortality, and thus it has been called the Williams hypothesis (Williams et al., 2006). The simple verbal model that gave rise to this hypothesis ignored a great deal of biology, including the potential impact on rates of survival and reproduction of population density and individual variation in physical condition (Abrams, 1993; Williams & Day, 2003). Consideration of these factors led to much more complex predictions and may account for some of the studies that fail to find this relationship (Reznick et al., 2004). Nevertheless, an impressive body of evidence from experimental evolution in fruit flies (Stearns et al., 2000) to natural experiments comparing insular and mainland opossums (Austad, 1993) to several comparative demographic analyses supports the prediction (Blanco & Sherman, 2005; Ricklefs, 1998).

With respect to the sex difference in longevity, the prediction made specifically by George Williams was that the sex less prone to die from extrinsic, non-senescence-related, causes would be the longer lived, more slowly aging, sex (Williams, 1957). Evolutionarily, this is a straightforward prediction but encompasses a complex genetic dynamic, because unlike species differences in which genes evolving in one species have no effect on another species, genes selected in one sex will probably be expressed in the other sex as well, unless they are on the Y chromosome (in mammals). However sex-specific effects of longevity-modulating genes appear to be common (Tu et al., 2002; Selman et al., 2007; Holzenberger et al., 2003), so I will ignore this complexity.

Testing evolutionary hypotheses about longevity differences between the sexes is not as straightforward as may first appear. The most rigorous test would employ experimental laboratory evolution in a paradigm in which each sex was subjected, generation after generation, to divergent extrinsic hazard regimes to see if longevity was altered in a sex-specific manner as predicted. A more immediate though less rigorous approach would be a comparative analysis in which one looked for a correlation between sex-specific differences in extrinsic mortality and in longevity. However, there is an inherent circularity in this approach when applied to animals in natural populations. Namely, other things being equal, the sex with lower extrinsic mortality is guaranteed to live longer. That difference is not necessarily due to differential senescence, however.

A useful conceptual approach to thinking about how to interpret mortality data from natural populations is given by Carey et al. (1995). These authors distinguish three sources affecting mortality: (1) constitutional endowment for survival, which includes susceptibility to disease, environmental or physiology stress, and intrinsic deterioration; (2) reproductive biology, such as the effects of male and female hormones, problems in the birth process, or the demands of lactation, which may predispose one sex or the other to greater intrinsic dangers; and (3) behavioral predispositions, such as differential foraging behavior, dispersal pattern, and tendency to engage in intraspecific combat and other risky behaviors. The first of these is most relevant to understanding mechanisms of human aging, but mortality data from field or even many laboratory populations will combine all three (Carey et al., 1995).

An illustration of the problem can be seen in Brandt's bats (*Myotis brandtii*). The longest-lived male in this 7- 8-g species survived at least 41 years in the wild (Podlutsky et al., 2005). In fact, all 67 individuals of this species documented to have lived 20 years or longer were males. Assuming that the probability of recapturing marked bats is not sex-dependent, we might conclude from this observation that female Brandt's bats senesce and die much more quickly than males. However it is also possible that the extreme foraging demands of lactating female bats, who must eat up to 50% of their body weight in insect prey daily, expose them to much greater risks of predation and nutritional deficiencies. Thus the observed longevity differences could be a result of extrinsic hazards alone.

There are several possible ways to address this limitation on inferences that can be drawn from observed sex-specific mortality patterns in nature. First, one could use measures of senescence that do not include mortality or survival per se, but focus instead on age-related changes in other measures of functional performance (Nussey et al., 2009). This approach is just beginning to be employed by field biologists. Second, one might employ survival data from captive populations that have been largely protected from extrinsic hazards.

Where might we expect to find a systematic sex difference in extrinsic mortality? One clear place is among highly polygynous species—that is, species in which some males have many mates, other males none—in which there is intense, potentially disabling or fatal, competition among males for access to females. It is important to note, as some of the hypotheses to be tested will compare patterns in mammals and birds, that mating systems of mammals and birds have very different distributions. More than 95% of mammal species have socially polygynous mating systems, in which males defend groups of females or in which male territories overlap those of multiple females. Males in these species typically contribute nothing to offspring care. By contrast, more than 90% of bird species exhibit a socially monogamous system, in which both parents contribute substantially to the care of offspring (Davies, 1991).

Probably the best comparative test to date of the Williams hypothesis was a survey of field data from 25 long-lived polygynous mammal and bird species compared with 10 monogamous species (Clutton-Brock & Isvaran, 2007). This study included noncanonical monogamous mammals such as beavers and mongooses as well as polygynous birds such as the grouse. These authors found that males were generally shorter lived than females in the polygynous species but not in the monogamous species, as one might expect if polygynous males experienced greater extrinsic mortality. The magnitude of the life-expectancy difference was substantial, such that in 16 of the 21 polygynous species for which appropriate data were available, males lived at least 20% shorter lives than females. More relevant for the Williams hypothesis, reproduction declined more rapidly with age in males compared to females in polygynous species but not in the monogamous species. Moreover, the difference between the sexes in the length of reproductive life was greater in the polygynous species than in the monogamous species. Finally, the degree of sex difference in life expectancy and duration of breeding was associated with the number of females per breeding group—a measure of the intensity of competition among males (Clutton-Brock & Isvaran, 2007).

Details for one particularly well studied polygynous species, the European red deer (*Cervus elaphus*) are informative. In this species, males fight for, and then defend, large female harems (Clutton-Brock et al., 1982). As expected, males in this highly competitive situation survive less well than females. More importantly, male reproduction declines much more abruptly than female reproduction (Nussey et al., 2009). Specifically, numerous measures of female reproductive performance such as annual fecundity, offspring birth weight, and offspring first-year survival reach a peak at 8–9 years of age and then decline slowly. By age 14, females are still reproducing but have only about half the number of offspring surviving to 1 year of age as they had at age 9. Some females were still reproducing at age 16. In contrast, male breeding success increases sharply through early adulthood, reaching a plateau between ages 8 and 10 and then plummeting. By age 14, virtually no males hold harems or are still reproducing.

One would clearly like more evidence with respect to this hypothesis, particularly more evidence on non-mortality-based measures of senescence from natural populations. That sort of information may begin to emerge now that field biologists have discovered that senescence, defined as either increasing mortality or decreasing reproduction with age, is widespread in natural populations (Brunet-Rossinni & Austad, 2006; Monaghan et al., 2008). We can soon expect that field tests of physiological performance will become more common.

Surprisingly, there is little published information on sex differences in longevity from captive colonies of nondomesticated species outside the primates. As useful as domesticated species such as mice, rats, and dogs may be for investigating mechanisms of sex differences, it is hazardous to draw evolutionary inferences from them because they have been so biologically altered by the domestication process itself (Miller et al., 2002). Among primate species commonly kept in large captive colonies, there are few in which it is clear that one sex is more subject to extrinsic hazards in the wild. One of these species in which it is most likely, though, is the gorilla, the males of which defend groups of multiple females. The captive survival bias in favor of gorilla females is even greater than that of humans (Allman et al., 1998). That is also true, however, in various macaque species and chimpanzees, which live in nature in groups of multiple males and females. In this case it is not clear whether the sexes differ in their exposure to extrinsic hazards, although that would be the Williams prediction.

If we assume that the sexes of monogamous species are not likely to have significantly different susceptibility to extrinsic hazards, then a clear prediction from Williams' hypothesis is that there should be no difference in survival between the sexes. Notably, captive colonies of monogamous primates such as siamangs, owl monkeys, and titi monkeys exhibit either no significant sex difference in survival or, in the case of owl monkeys, have a slight (but significant) male survival advantage (Allman et al., 1998), fitting reasonably well with the Williams hypothesis.

A related, but not identical, hypothesis considers sex differences in longevity to be due to sexual selection—commonly defined as competition among males for access to females and choice among females for highly fit males (Promislow, 1992). Under this scenario, sex differences in mortality will be due to aspects of sex-specific morphology, behavior, and reproductive biology. As the focus is not on age-related changes in constitutional endowment for survival per se, but on any source of mortality, differential survival in nature is the currency with which to evaluate the prediction that greater male competition, irrespective of extrinsic hazards, will lead to lower survival. Using the difference in adult body size as an indicator of the intensity of male–male competition within a species, Promislow (1992) found that in a sample of 35 mammal species, the greater the male–female size difference, the greater the male mortality bias and that in monogamous species either there was no sex difference in survival or males actually exhibited greater survival.

Often the most powerful way to test evolutionary theories is to predict anomalies in general patterns. From this perspective, particularly interesting species in which to examine sex differences in aging and longevity would be those from among the 1% of bird species, such as the wattled jacana (*Jacana jacana*), that are polyandrous. Polyandry is sometimes thought of as sex role reversal, in which females assume typically male roles and have many mates, whereas males mate with only a single female. In the wattled jacana, for instance, females are larger than males and compete intensely among themselves for breeding territories. Up to four males build nests within a female's territory and, once egg-laying is finished, virtually all parental care duties are performed by the males. So intense is the competition among females that when a new female takes over a territory she will kill the chicks of the previous female so that males are available more quickly to care for her eggs. Assuming this level of competition leads to higher extrinsic mortality in females compared to males (Emlen et al., 1989), Williams' hypothesis predicts that females should be the more rapidly aging sex in this species. Unfortunately, no data addressing this issue have yet been published for jacanas or other polyandrous bird species (S. Emlen, personal communication, 2009).

How do humans fit into this scenario? In the Standard Cross-Cultural Sample of 186 of the world's ethnographically described societies, more than 80% are described as either "slightly polygynous" or "generally polygynous" (Marlowe, 2000). Assuming this represents the ancestral human state, humans fit the Williams hypothesis in terms of sex differences in survival quite nicely.

All in all, although the evidence is more scant than one would like, sex differences in longevity and aging do appear largely consistent with the Williams hypothesis.

The Heterogametic Sex Hypothesis

A second evolutionary hypothesis is one I call the heterogametic sex hypothesis, although it has also been called the "unguarded X" hypothesis (Trivers, 1985). The heterogametic sex is the one in which the sex chromosomes differ. For instance, in mammals males are the heterogametic sex as they have one X and one Y chromosome, whereas females are the homogametic sex, because they have two X's. In contrast, female birds are the heterogametic sex, having one Z and one W chromosome, whereas males have two Z chromosomes.

The presence of a female's two X's can prevent phenotypic exposure of a sex-linked deleterious allele, whereas males have no second copy to compensate. Thus male mammals might be disadvantaged if the X chromosome commonly bears slightly deleterious alleles (Smith & Warner, 1990). This expectation is sustained even though females of most mammals randomly inactivate one X chromosome in each somatic cell during development, because tissue mosaicism allows functional compensation by nearby cells in which the better allele is active. Males, being hemizygous for such alleles, lack this compensatory advantage. The fact that phenotypes such as hemophilia and Duchenne muscular dystrophy, which are caused by genetic mutations on the X chromosome, are generally not observed in females heterozygous for the allele indicates that such tissue level compensation occurs.

In addition to the potential male disadvantage due to an unguarded X, females might have a positive advantage associated with two X chromosomes if over time the phenotypic effect of the better X becomes dominant because of preferential survival or replication of cells containing the better active X chromosome. Such a view is supported by observations that as women age, one X chromosome tends to become increasingly prevalent in peripheral blood cells (Christensen et al., 2000; Kristiansen et al., 2005).

A nice feature of the heterogametic sex hypothesis is that it makes clear, unambiguous, and easily evaluated predictions. Specifically, it predicts that males will be the shorter-lived sex in *all* mammals, whereas females will be the shorter-lived sex in all birds. The existing data just as clearly and unambiguously fail to support this hypothesis. There may be more mammal species in which males are shorter lived than the reverse (Promislow, 1992), and more bird species in which females are shorter lived than the reverse (Liker & Szekely, 2005), but there are numerous exceptions. For instance, as previously noted, in mammals males are the shorter-lived sex in humans, gorillas, chimpanzees, red deer, lions, and pilot whales, but males are the longer-lived sex in captive populations of multimammate rats (*Mastomys natalensis*), guinea pigs, owl monkeys, and most hamsters (Committee on Animal Models for Research on Aging, 1981; Allman et al., 1998), and there is no significance sex difference in survival in mice (see below). Among birds, females are indeed shorter lived in natural populations of song sparrows, great tits, indigo buntings, and red-billed gulls, but females live as long as or longer than males in blue tits, pied flycatchers, kingfishers, kittiwakes, fulmars, and sparrow hawks (Clutton-Brock, 2009; Newton, 1989). Similarly, among captive birds, females are shorter lived in pigeons, Japanese quail, Bengalese finches, and cockatiels, but not in chickens or a variety of parrots (Austad, 2001).

The Parental Care Hypothesis

Another evolutionary hypothesis that has been advanced to explain sex differences in longevity has to do with patterns of parental care. Specifically, this hypothesis as put forward by Allman and colleagues (1998) is that “the sex that bears the greater burden in the care of offspring will tend to survive longer.” Putting this hypothesis in more direct evolutionary terms, its logic seems to be that natural selection will more strongly favor survival in the sex that provides the most critical postnatal care of offspring. The specific prediction of this hypothesis is that females will tend to live longer than males in species in which the mother does most or all of the parental care, there will be no difference in survival between the sexes when parents contribute about equally to offspring care, and that males will live longer when they provide the bulk of the parental care. Although Allman and colleagues restrict this prediction to slowly developing species with single births (Allman et al., 1998), the same logic should obtain for rapidly developing species or those with multiple offspring at a time,

As with the sexual selection hypothesis, this idea would be most rigorously tested with field data because the source(s) of mortality do not figure into the predictions, only overall survival probability in nature. Initial evidence adduced to support this hypothesis was based on sex-specific survival patterns in 10 captive primate species (Allman et al., 1998). In most of these, males were shorter lived, but among the 4 monogamous species in the sample in which males provided some parental care, such as infant carrying, grooming, or sharing food, survival of males was significantly better than females in one species (owl monkey), trended toward greater male survival in another (titi monkey), or was not significantly different between the sexes (siamang, Goeldi’s monkey).

The problem with interpreting these results is that determining quantitatively which sex “bears the greater burden” of offspring care is difficult. For instance, in both owl and titi monkeys, the males do the vast majority of infant carrying, but over the same period the mother is continuing to provide lactational support to the infant. So who is bearing the greater burden? Estimates of metabolic expenditure in monogamous siamangs suggested that no amount of paternal infant carrying equaled peak lactational demands (Lappan, 2009). Certainly species are known, such as African buffalos and Verreaux’s sifaka, in which there appears to be no difference in survival despite a complete lack of paternal care. Thus this hypothesis does not seem tenable as a general explanation of differences in longevity or aging.

Mechanistic Hypotheses

Field studies are generally not helpful in understanding cellular, molecular, or physiological mechanisms of aging and longevity. To test mechanistic hypotheses, one needs controlled, experimental approaches with well-characterized, easily manipulated laboratory species, preferably with tractable genetics. In mammals, the two conventional laboratory rodent species are mice (house mouse, *Mus musculus*) and rats (Norway rat, *Rattus norvegicus*). How would these species serve to investigate sex differences in longevity?

Sex Differences in Longevity in Laboratory Rodents

A great deal of confusion exists in the aging literature as to the existence of sex differences in longevity in laboratory rodents. In mice one can find claims that males live longer (Ali et al., 2006), that there is no difference between the sexes (Sanz et al., 2007), and that females live longer (Viña et al., 2007). It turns out that these claims are all correct—and all wrong.

To determine whether there was a consistent pattern in mice, I surveyed 118 survival studies that report either mean or median survival for both sexes. I used only studies under standard laboratory conditions without genetic or dietary manipulations. Analysis of these studies indicates quite clearly that unlike humans mice do not display a robust and consistent survival advantage of one sex over the other (Figure 23.4A). Although there is a slight preponderance of studies in which male longevity exceeds females ($n = 65$), there are numerous studies ($n = 51$) that find the reverse. The magnitude of substantial differences in either direction, when they occur, is about equivalent. In only about one-fifth of the studies does longevity of the sexes differ by 5% or more.

Could it be that this variation is due to strain-specific idiosyncrasies? Although strain idiosyncrasies are difficult to rule out, since most strains are represented by one or a few studies, I found 29 independent studies of the C57BL/6 genotype (Figure 23.4A). Even within this single genotype, males can be as much as 20% longer lived than females ($n = 18$) or nearly 20% shorter lived ($n = 11$), depending most likely on the subtleties of individual laboratory environments and husbandry practices. Nor is such variability confined to this genotype. Six studies of DBA/2 mice reveal that males can live as much as 7% longer than females or as much as 6% shorter. Similarly, for 6 studies of BALB/c mice, males can be 5% longer lived or nearly 7% shorter lived. Substantial variation in survival differences is also seen in F_1 genotypes, in mixed genotypes, and even among wild-derived mouse stocks. So the firmest generalization one can make about sex differences in longevity in mice is that one really cannot generalize—even about specific genotypes.

The situation with rats is also less than perfectly clear. Even though rats have been employed in aging research for at least as long as mice, far fewer data are available on sex differences, because the focus of so many studies has been on males alone (see Chapter 14 of this book). However, from the 19 available studies, rat females most often (15 of 18 studies), but not always, live longer than males (Figure 23.4B). Among genotypes that have been assayed multiple times, females live consistently longer in the Wistar stock (4 studies) and in 3 of 4 studies of the F344 strain.

What does this mean about the utility of laboratory rodents for evaluating mechanistic hypotheses about sex differences in aging and longevity? As with any interesting question, the answer is—"it depends." One particularly informative experimental paradigm involves manipulating a presumptive independent trait and determining whether the presumptive dependent trait changes as expected. So, for instance, grave doubts about oxidative stress as a general modulator of aging have arisen in recent years because direct manipulation of oxidative stress has not been found to alter life span as predicted—at least in mice (Perez et al., 2009). An alternative paradigm, less directly informative but still useful, is to manipulate the dependent trait of interest and determine whether the putative causal underlying trait changes as expected. The complication of this latter paradigm is that it establishes correlation but not causation. However, it is quite useful at ruling out hypothesized causal factors and constructing hypotheses to be more directly evaluated.

From this perspective, the laboratory mouse could potentially be enormously informative about mechanisms of sex differences in aging and longevity. Whether this turns out to be the case depends largely on whether researchers are able to determine experimentally the laboratory conditions that favor superior male versus female survival. For instance, in the Mediterranean fruit fly one sex is also not universally more robust than the other (Carey & Liedo, 1995). However, the environmental variables, involving cage conditions, diet, and density, that influence sex mortality differentials have been elucidated such that researchers can create conditions favoring survival of either sex. If investigators could determine these factors for mice, it would allow them to explore causal hypotheses under conditions under which males live longer compared with those under which females live longer, thus providing insight into mechanisms accounting for these sex differences.

As female rats are more consistently long lived, a fruitful experimental approach might be to perform either genetic or physiological manipulations to determine whether these erased the female longevity advantage (e.g., Borras et al., 2003).

General mechanisms underlying variation in longevity or aging have been increasingly well defined in model organisms (Tatar et al., 2003; Henderson et al., 2006; Ladiges et al., 2009). What do we know, and what might we hypothesize, about how these mechanisms might shape sex differences in longevity?

Oxidative Stress

Although little mechanistic research has been performed on sex differences in longevity, more evidence relevant to the oxidative stress hypothesis exists than for any other putative mechanism.

The oxidative stress hypothesis of aging posits that a major contributor to age-related physiological decline is the progressive accumulation of oxidative damage to macromolecules as a consequence of reactive oxygen species (ROS) generated as a by-product of normal metabolism (Harman, 1956). Almost a default hypothesis in the aging field for years because of its intuitive appeal and a smattering of evidence

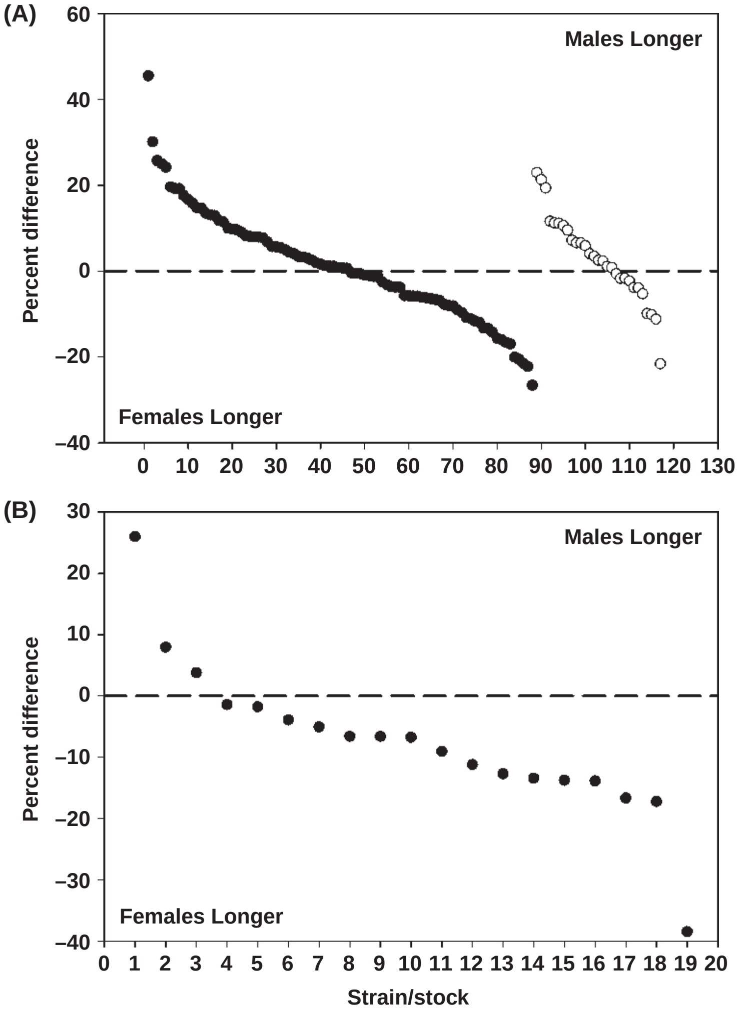

Figure 23.4 Percentage sex difference in longevity in laboratory rodents. (A) 118 mouse studies. The dashed line indicates equal male and female longevity. Open circles, C57BL/6 mice. Closed circles, all other genotypes, including inbred strains, F_1 hybrids, and heterogeneous laboratory and wild-derived stocks. (B) 19 rat studies.

consistent with its predictions, such as findings that oxidative damage to tissues does increase with age in many animal models (Hamilton et al., 2001; Ward et al., 2005) and that some life-extending treatments such as dietary restriction reduced ROS production and tissue damage (Sohal & Weindruch, 1996), nevertheless the hypothesis has more recently fallen on hard times (Perez et al., 2009).

As applied to sex differences in aging and longevity, estrogen is well known to have antioxidant properties (Stice et al., 2009; Mann et al., 2007), so it is reasonable to assume that oxidative stress could underlie a female survival advantage. Some empirical evidence is consistent with this idea. For instance, isolated liver and brain mitochondria from female Wistar rats produced fewer ROS than mitochondria from males and showed higher levels of some cellular antioxidants as well as lower levels of oxidative DNA damage (Borras et al., 2003). Recall that female Wistar rats consistently live longer than males. Moreover, these sex differences in ROS production and antioxidant expression can be obliterated by ovariectomizing the females and reconstituted with estrogen replacement. Several other papers from the same laboratory replicate and extend these observations somewhat (Viña et al., 2005). Unfortunately, no published information is available on the life-span impact of ovariectomy and hormone replacement in this rat genotype. As with sex differences in longevity itself, diverse studies have reached opposite conclusions on the impact of gonadectomy on longevity in rats, dogs, cats, and humans (Hamilton & Mestler, 1969; Hamilton, 1965;

Bronson, 1982; Waters et al., 2009). Additionally, one study of F344 rats found that female heart mitochondria produced fewer ROS than mitochondria from males (Jang et al., 2004).

Problems with the empirical support for the oxidative stress theory of sex differences in longevity arise when mice are considered. In addition to the observations on Wistar rats described above, Borras and colleagues (2003) also found that liver mitochondrial ROS production was lower in female OF1 mice than in males. The problem is that there are no published sex-specific survival data on this outbred mouse stock and, as shown in Figure 23.4A, females do not have a consistent survival advantage in mice. Two further studies associating oxidative stress and sex-specific longevity have been done using C57BL/6 mice. One study assumed from several publications that there is no sex difference in longevity in this mouse genotype and, using a partially inbred strain (94% C57BL/6), found no sex differences in a variety of measures of oxidative stress, mitochondrial bioenergetics, and apoptosis (Sanz et al., 2007). The second study assumed from several different publications that male C57BL/6 mice live longer than females and found that females produce more ROS and express fewer antioxidant enzymes in brain compared with males. This study did not rely solely on literature reports of sex differences but performed survival studies on the mice and observed a statistically nonsignificant (4.4%) greater male life span (Ali et al., 2006).

Evidence supporting an oxidative stress explanation for sex differences in laboratory rodents appears, at this time, at best mixed.

Size Difference

Signaling through the growth hormone/insulin/IGF-1 pathways has been found to modulate longevity in diverse model organisms, including mice, with reduced signaling leading to longer life (Tatar et al., 2003; Ladiges et al., 2009). As these pathways affect body growth, a reasonable hypothesis for sex differences in longevity may be that it reflects differential signaling in one or several of these pathways. The prediction from this scenario is that smaller animals should live longer and age more slowly.

A substantial body of evidence seems consistent with this idea. For instance, an analysis of approximately 400 studies of rats and about the same number of mouse studies (including those manipulating many aspects of diet as well as various genetic parameters) found a highly significant negative correlation between maximum adult body mass and maximum longevity in both species (Rollo, 2002). Whether this is also true for mean or median longevity is unclear as such data were gathered but not presented in the publication. However, given that these studies came from across many decades of the 20th century and were performed under a wide range of husbandry conditions and diets, the fact that any detectable signal emerged from this considerable noise is astonishing. Also, recall that among several dozen field studies of polygynous mammals, the male bias in mortality rate was significantly associated with the sex difference in body size (Promislow, 1992). It has also been asserted that the human sex difference in longevity is due to the size difference between the sexes, such that men and women of equivalent height will have equivalent life expectancies (Samaras et al., 2002).

The accumulated weight of the evidence described above deserves to be taken very seriously. However, as intriguing as the general relationship between size and longevity is across hundreds of mouse studies, there was no statistical sex difference in longevity despite females being on average 11% lighter (Rollo, 2002). This weight difference is somewhat less than that between men and women (roughly 15–20%), but still substantial. Moreover, it is difficult to reconcile this idea with the tremendous variability in sex-specific longevity within single genotypes such as the C57BL/6 mouse. In none of the 29 studies included in Figure 23.4A were females heavier than males, yet in a number they were shorter lived. There are also many exceptions among mammalian species to the general rule of the smaller sex living longer. For instance, male African lions, once they reach sexual maturity, are longer lived than the much smaller females (Packer et al., 1988). Another example taken from a captive rather than wild population is male guinea pigs, which live longer than females despite their larger size (Committee on Animal Models for Research on Aging, 1981).

The human data on height, rather than sex, being the key variable are also problematic. Supporting evidence is of the apples-and-oranges variety, comparing women of one ethnic group to men of another (Samaras et al., 2002). Furthermore, there is a large literature from a diversity of human populations indicating that within a sex taller people—not shorter ones—generally live longer (Song & Sung, 2008; Engeland et al., 2003; Jousilahti et al., 2000), although for specific causes this may not be the case (Miller & Austad, 2006). Thus as attractive as the height hypothesis might be, with the satisfying underlying mechanism to explain it, the evidence at this juncture seems equivocal at best.

This brief discussion does not exhaust the mechanistic hypotheses regarding sex differences in longevity. There is also an immunological hypothesis (Aspinall, 2000), for instance. However, the two hypotheses presented above have the most copious evidence for and against them, and also could be easily seen to predict mortality differences across a wide range of pathologies. The fact that the existing evidence leaves both hypotheses with equivocal support at best emphasizes how far we are from understanding this phenomenon.

CONCLUSIONS

The human sex difference in longevity, and possibly aging, may be as robust an aspect of our biology as any that we do not understand even in broad terms. Information from both the natural world and captive populations indicates clearly that among different species one may find wildly divergent patterns of sex differences. Sometimes females seem more robust, sometimes males. The evolutionary hypothesis that seems best supported by existing evidence is the Williams hypothesis, in which the sex subjected to the greatest extrinsic hazards in the wild will evolve the more rapidly deteriorating phenotype. Gaining insight into the cellular, molecular, and physiological mechanism(s) underlying greater female robustness in humans would potentially be a great boon for enhancing and extending male health. On the other hand, understanding in more detail women's greater susceptibility to chronic nonfatal conditions would be a great boon to their health. Mouse models of the aging process are unlikely to be informative with respect to elucidating mechanisms of these sex differences unless the conditions favoring enhanced male longevity versus female longevity can be discovered. Other model species may prove more suitable for research in this field.

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Author Index

A

Aaron, E. A., 432, 433, 436, 439
Aaron, J. M., 29, 122
Aarsland, A., 122, 123
Abarbanell, A. M., 277
Abbasi, F., 27
Abbi, R., 97
Abbott, D. H., 391, 392, 482
Abbott, N. J., 406, 408
Abdellatif, M., 99
Abdueva, D., 286
Abduljalil, A. M., 68, 95
Abelak, K., 284
Aberg, F., 184
Aboulkassim, T., 412
Abraham, C. R., 336, 337, 338, 340, 391
Abraham, J. L., 190, 191
Abramova, N., 411
Abramovitch-Gottlib, L., 122
Abrams, J. M., 13
Abrams, M. T., 334
Abrams, P. A., 485
Abrassart, D., 249, 253, 262
Abreu, M. T., 281
Accili, D., 27, 224
Acehan, D., 71
Ackermann, M., 216, 485
Acuña-Castillo, C., 281
Adak, S., 349
Adamopoulos, C., 169
Adams, C. E., 306
Adams, G. M., 439
Adams, G. R., 94
Adams, L., 423, 435, 439
Adams, M., 139
Adams, M. R., 391
Adams, R., 386, 387
Adams, S., 183
Adams, V., 95, 96
Adamson, J. S., 428
Adam-Vizi, V., 51
Aderem, A., 277
Ades, P. A., 68, 468
Adey, D. B., 68
Adhihetty, P. J., 64, 67, 68, 85, 95, 96
Adiels, M., 123
Adjaye, J., 145
Adler, A. S., 260
Adler, B., 325
Aerts, A. M., 220
Afeltra, A., 64
Afilalo, J., 64
Agani, F., 358
Agarraberes, F., 306
Agarwal, A. K., 120, 122
Agell, N., 77
Agostini, C., 169, 170
Agostino, A., 219
Aguado, C., 297, 306
Aguilaniu, H., 4, 7, 9, 10, 15, 208, 220, 246
Aguilar, C. C., 34
Aguilar, R. C., 26
Aguilar, V., 208
Aguilar-Gaxiola, S., 338
Aguirre, A., 468
Ahima, R. S., 121, 124, 125
Ahlberg, J., 306
Ahlenius, H., 150
Ahlers, P., 169
Ahlquist, J. E., 448
Ahluwalia, A., 388
Ahmad, N., 253
Ahmed, E., 408
Ahmed, M. U., 27
Ahmed, T., 454, 456, 457
Ahn, B. H., 99, 100, 254
Ahringer, J., 16, 155, 220, 221, 228, 286, 300
Ahuja, N., 254, 255
Aicher, A., 259, 281, 358
Aiello, V. R., 179, 183
Aiken, J. M., 55, 67, 99, 148, 324, 392, 451
Ailhaud, G., 120
Ailion, M., 218
Aimé-Sempé, C., 71
Aiyer, A. N., 169
Aizawa, S., 468
Aizenstein, H., 354, 355
Aizu, T., 28
Ajmo, J. M., 258, 260
Akagi, T., 71
Akahoshi, M., 122, 123
Akaki, T., 121
Akamatsu, H., 145
Akashi, K., 145, 150
Akatsuka, A., 72, 84, 94
Akcakus, M., 286
Akçay, T., 298
Akey, C. W., 71
Akiguchi, I., 351, 352
Akin, D. E., 308
Akishita, M., 385, 387
Akiyama, H., 280, 282
Akkad, A., 168, 169
Akopov, S., 349, 352
Akushevich, I., 282, 284
Alavi, A., 349
Albala, C., 283
Albani, D., 282
Albarado, D. C., 248, 249
Albert, M., 391
Albert, P. S., 222
Alberti, E., 143
Alberti, S., 300
Alberts, S. C., 482
Albinder, B., 217, 222
Albon, S. D., 486
Alcedo, J., 16
Alcendor, R. R., 99, 100, 258, 259
Alder, J. K., 166
Aldridge, J. E., 84
Aleman, A., 32
Alencar, A. I., 482
Alexander, J. J., 406
Alexander, T. M., 449
Alexandre, A., 50
Alford, P. L., 482

Author Index

Alho, H., 180, 184
Ali, A. H., 122, 127
Ali, S. M., 10
Ali, S. S., 181, 186, 388, 489, 491
Alibegovic, A., 382
Alibhai, S. M. H., 324
Alimonti, A., 208
Alison, J. A., 437
Allard, J. S., 453, 456
Allen, D. L., 65, 72, 78, 94
Allen, J. S., 337
Allen, T., 169
Alley, D., 276, 286
Alliouachene, S., 208
Allis, C. D., 244
Allison, D. B., 30, 93, 98, 99, 125, 130, 178, 180, 184, 185, 280, 284, 324, 392, 450, 451
Allison, D. D., 324
Allison, S., 339
Allison, S. J., 258
Allman, J., 482, 487, 488
Allsopp, R. C., 151, 152, 166, 251
Almendro, V., 77
Almond, D., 286
Alnemri, E. S., 71
Al Regaiey, K. A., 14, 26, 29, 229
Al-Regaiey, K. A., 26, 27, 28, 29, 229, 378
Al-Shaer, M. H., 349
Alston, R. L., 333, 335, 336, 337, 339
Alt, F. W., 230, 247, 254, 255, 258, 282
Alt, J. M., 285
Altamura, C., 352
Altmann, J., 482
Altmann, S. M., 262
Altomare, K., 300
Altose, M. D., 425, 426
Altschuler, L. L., 338
Altshuler, L., 336
Altucci, L., 250
Alvarado, C., 32, 33
Alvarez, B., 77, 78
Alvarez, P., 32
Alvarez de Sotomayor, M., 410
Alway, S. E., 64, 65, 67, 68, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 84, 85, 86, 87, 88, 89, 90, 91, 94, 95, 96, 100
Amador-Noguez, D., 29
Amann, M., 421, 433, 438, 439
Amara, C. E., 67, 68, 94
Amaral, D. G., 391
Amasaki, Y., 122, 123
Ambra, R., 300
Amer, M. A., 183
American Diabetes Association, 351, 374, 380
Ames, B. N., 52, 67, 194
Amin, N., 244, 253
Aminova, L., 359
Ammendola, S., 28, 32
Amminger, G. P., 341
Amore, A. M., 262
Amos, N. N., 166
Amouyel, P., 278
An, J. H., 11
Anagnostis, P., 283
Analich, F., 325
Anantharaman, V., 255, 256
Ancel, P. Y., 483
Anckar, J., 300
Andalis, A. A., 12, 220
Andersen, J. K., 52
Andersen, J. S., 244, 254
Andersen-Ranberg, K., 77, 78, 170, 171
Anderson, A. E., 427, 435
Anderson, B., 74, 77, 78, 79, 80, 86, 90, 91, 96
Anderson, C., 360
Anderson, D. C., 448, 451, 452
Anderson, D. O., 423, 435
Anderson, H., 259
Anderson, L. J., 340
Anderson, M. C., 353, 354
Anderson, N. D., 350
Anderson, R. K., 472
Anderson, R. M., 32, 164, 193, 216, 237, 249, 261, 381, 392, 448, 451
Anderson, W. F., 427, 435
Ando, H., 360
Ando, S., 340
Andrade, F. H., 64, 73, 74, 79, 83
Andrade, L., 338
Andrade-Navarro, M. A., 358
Andre, M., 121, 124, 128
Andreasen, N. C., 337
Andreassen, B. U., 77, 78
Andreassen, O. A., 194
Andreeva, M., 481
Andres, R., 283, 321
Andrés, V., 281
Andresen, J. J., 408, 409, 410, 412
Andrew, T., 125, 169
Andrews, D. W., 83
Andrews, N. P., 349
Andreyev, A., 48
Andriambeloson, E., 360
Andriantsitohaina, R., 360
Andziak, B., 196
Angelini, A., 95, 96
Anglard, P., 123, 125
Angleman, S. B., 278
Angus, G. E., 427, 430, 437
Anigstein, D. M., 30, 34
Anigstein, L., 30, 34
Anisimov, V. N., 16, 32
Anne, L. H., 87, 89, 96
Annis, M. G., 83
Anrather, J., 360, 405, 407, 408, 410, 411, 413
Anselme, F., 439
Ansite, J. D., 379
Antebi, A., 26, 28, 489, 491
Antelo, F., 126
Anthonisen, N. R., 433
Anthony, J. C., 338
Anton, S., 96, 281, 284, 453, 456
Anton, S. D., 282, 453, 456
Antoniazzi, J. M., 300
Antonsson, B., 71
Antonucci, A., 99, 358
Antosiewicz-Bourget, J., 142
Antunes, F., 54
Anversa, P., 96, 98
Anwar, S., 124
Aoki, Y., 190, 191
Aon, M. A., 52
Aparicio, O. M., 220, 224, 244
Apfeld, J., 16, 222, 252
Apostolova, L., 341
Apovian, C. M., 121
Appel, L. J., 183
Appell, H. J., 67, 68, 69, 70
Appenzeller, O., 349, 352
Appt, S. E., 389
April, M., 450
Aragaki, A., 323
Arai, F., 147, 152
Arai, K., 408
Arai, T., 193, 437
Arai, Y., 28, 154
Arakawa, S., 165
Araki, K. Y., 152
Araki, S., 127
Araki, T., 261, 262
Arancibia, S., 472
Aranda, C., 227
Aranda, R., 192
Arantes-Oliveira, N., 220, 223, 228
Arase, S., 256
Araujo, D. M., 30
Araujo, J. A., 286
Aravind, L., 255, 256
Arbab-Zadeh, A., 437
Arbeev, K. G., 282, 284
Arbeeva, L. S., 282, 284
Arbuckle, T. E., 286
Arbustini, E., 97
Arch, R., 181, 186
Archer, J. R., 222
Arciero, P. J., 468
Arendash, G., 357
Argentino, D. P., 26, 27, 38
Argiles, J. M., 77, 78
Argmann, C., 120, 230, 248, 261, 263, 412
Arias, E. B., 14, 96, 99, 251
Arias-Loza, P. A., 407
Arif, H., 453, 455, 456

Author Index

Arima, S., 412
Ariyan, C., 68
Ariznavarreta, C., 32, 33
Arjona, A., 278, 281
Arking, R., 221
Arkins, S., 33
Armani, C., 286
Armanios, M., 166
Armellini, F., 120
Armstrong, C. M., 224, 245
Arnaud, E., 121, 124, 128
Arnaud, L., 304
Arndt, S., 337
Arner, E., 126
Arnold, A., 353
Arnold, A. P., 387, 388
Arnold, J. M., 178, 179
Arnold, S., 68
Arnoult, D., 70
Aronis, A., 127
Aronne, L. J., 285
Aronovitz, M., 388
Arora, S., 349
Arp, L. H., 33
Arribas, S. M., 412
Arrick, D. M., 349, 410, 411, 413
Arroyo, J. D., 83
Arsham, A. M., 226, 230
Arslan, P., 85, 95
Artal-Sanz, M., 12
Artavanis-Tsakonas, S., 153
Artese, L., 358
Artham, S. M., 285
Arum, O., 14, 26, 28, 29, 220
Aruoma, O. I., 185
Asai, K., 387, 392, 394, 395, 413
Asakura, A., 84, 85
Ascah, A., 95
Asensi, M., 70
Asghar, M., 285
Ashby, M., 97
Asher, G., 262
Ashford, J. W., 340
Ashida, K., 351
Ashkan, K., 389
Ashraf, J., 5
Ashrafi, K., 12, 220
Asimakis, G. K., 194
Aspinall, R., 491
Asress, S., 261
Asselin-Labat, M. L., 144
Astarita, G., 248, 249, 253, 262
Astier, H., 472
Astle, C. M., 120, 181, 186, 209, 218, 226, 309, 310, 381
Astruc, T., 83
Atalay, M., 248, 249
Athea, Y., 208
Atherton, G. T., 85
Athyros, V. G., 283
Atkinson, C., 32
Atkinson, E. J., 283
Atkinson, H. H., 360
Atkinson, J., 178, 280, 406, 407
Atlan, H., 221
Attaix, D., 66, 148
Attanasio, R., 392
Attar, E. C., 168
Attardo, G. M., 204
Attema, J. L., 152
Atzmon, G., 28, 130, 164, 285
Au, L. T., 205, 206
Au, Q., 258
Au, R., 349
Aubry, N., 396
Auburtin, A., 412
Auffray, C., 148
Aulisa, L., 67
Aunola, S., 374
Aussel, C., 324
Aust, S. D., 193
Austad, S. N., 26, 28, 30, 54, 194, 207, 222, 247, 485, 486, 487, 488, 490, 491
Austriaco, N. R., 225
Ausubel, F. M., 286
Authier, F. J., 77
Auwerx, J., 72, 96, 121, 122, 224, 231, 251
Auyeung, J. S., 324
Avakian, G. N., 360
Avalos, J. L., 245, 249
Avenell, A., 326
Avery, L., 4
Avery, M. A., 262
Avetisyan, A. V., 68
Avila, J., 299
Aviv, A., 167, 168, 169, 171
Aviv, M., 122
Avogaro, A., 169
Avraham, Y., 14
Avruch, J., 10, 204, 208
Aw, T. Y., 302
Awata, T., 412
Axmann, R., 283
Aykin-Burns, N., 258
Aylward, S. R., 406
Ayoub, I. A., 359
Azhar, G., 92, 93, 99

B

Babb, J. S., 337, 355
Babb, T. G., 424, 433
Babcock, M. A., 439
Babendure, J. L., 251, 258, 260
Babich, S., 222
Babikian, V., 349
Babior, B. M., 52
Baccetti, S., 166
Bach, F. W., 466
Bach, J. F., 33
Bachevalier, J., 391
Bachofen, H., 437
Bachschmid, M., 411
Backlund, E. C., 55
Bacou, F., 121, 124, 128
Bacskai, B. J., 407, 408
Bader, B. L., 124
Bader, N., 54, 182
Badia, M. C., 489
Badr, M. S., 421, 439
Badylak, S. F., 468
Bae, S., 72
Baer, D. J., 27, 450
Baesso, I., 169, 170
Baeza, I., 32, 33
Baezner, H., 355
Baghdiguian, S., 81
Bagiella, E., 356
Bahrami, J., 120, 121, 125
Bai, F., 278, 281
Bai, H., 464
Baier, L. J., 125
Bailey, L. J., 55
Bain, B. J., 169
Bain, J. R., 95
Bain, S. C., 286
Baird, A. E., 353
Baird, D. M., 166, 167
Bajenaru, O., 406, 407
Bajic, V. B., 277
Bajpeyi, S., 248, 249
Bakala, H., 34
Bakarev, M. A., 96
Bakaysa, S. L., 170
Baker, B., 84, 85
Baker, B. A., 67, 70, 94
Baker, D. J., 67, 69, 70, 74, 75, 76, 79, 125
Baker, J., 11
Baker, L. D., 285
Baker, M. G., 357
Baker, T. A., 338
Bakker, S. L., 349, 351, 352, 356, 357
Balaban, R. S., 99, 100
Balage, M., 83
Balan, K., 249
Balan, V., 249
Balbi, V., 28
Balch, W. E., 299
Baldacci, S., 437
Baldessari, D., 256, 258
Bale, L. K., 26, 30
Baledent, O., 407
Balistreri, C. R., 387
Ball, S. S., 392, 450
Ballabh, P., 96, 125, 260, 261, 287
Ballam, J. M., 447
Ballard, C. G., 356
Ballester, J. M., 466, 470
Ballinger, S. W., 67, 168, 169
Baloh, R. H., 262, 353

Author Index

Baloh, R. W., 353, 354
Balouch, J., 437
Baltadjieva, R., 353
Baltes, P. B., 339
Bamford, J. M., 352
Bamisedun, O., 472
Bamman, M. M., 94, 256
Ban, E., 33
Banayo, E., 16
Bandeen-Roche, K., 323
Bández, M. J., 179, 182, 195
Bandinelli, S., 278
Bandy, B., 55
Bandyopadhyay, U., 307, 309
Banerjee, M., 224
Banerjee, S., 252
Banerji, M. A., 122
Banfi, G., 283
Banjac, A., 192
Banko, M. R., 4, 5, 7, 9, 10, 206, 208, 229
Banks, A. S., 225, 248, 251, 256
Banon-Maneus, E., 230
Bansal, K., 250
Baracca, A., 410
Barajas, B., 286
Barbatelli, G., 126
Barber, D. F., 217
Barber, J. R., 258
Barber, R., 352
Barbera, J. A., 437, 438
Barbet, J. P., 148
Barbet, N., 226
Barbieri, M., 167
Barbosa, J. A., 337, 338, 339
Bard, J. M., 278
Bardeguez, A., 168, 169
Bardsley, J., 374
Bare, O., 224
Barenz, T. A., 349
Barger, J. L., 14, 96, 99, 125, 251
Barger, S., 280, 282
Bargmann, C. I., 155
Barja, G., 42, 48, 55
Barker, D. J. P., 286
Barker, M. G., 216
Barker, N., 153
Barlow, C. E., 421, 424
Barlow, D. H., 392
Barna, J., 208, 230, 308
Barnard, D. E., 450
Barnes, D. E., 190, 193
Barnes, G. T., 120, 124, 125
Barnes, J. M., 360
Barnes, L. L., 3, 14
Barnes, N. M., 360
Barnes, P. J., 435
Barnes, W. S., 185
Barnett, A. H., 120
Barnett, K. R., 411
Bar-Noy, S., 190, 192, 193
Barnum, S., 280, 282
Baron-Menguy, C., 407
Bar-Or, O., 468, 469
Bar-Peled, L., 204
Barrenetxe, J., 124, 128
Barres, B. A., 333
Barrett, J., 468
Barrett, J. C., 35
Barrett-Connor, E., 324
Barrientos, A., 52
Barry, M. A., 97
Bartali, B., 278
Bartek, J., 146, 472
Bartels, J., 277
Bartholomew, J. C., 68
Bartke, A., 14, 26, 27, 28, 29, 30, 31, 33, 34, 38, 209, 222, 224, 229, 301, 447, 489, 491
Bartoli, M., 81
Bartolome, A., 217
Barton, M., 358
Bartus, K., 408
Bartus, R. T., 30, 391
Bartzokis, G., 336, 337, 339, 340, 341
Barzilai, N., 120, 124, 224, 252, 285
Basagaña, X., 286
Basak, O., 145
Basak, S., 68
Basanez, G., 71
Baseler, W. A., 64
Bass, T. M., 16, 221, 224, 230, 251, 253
Bassik, M. C., 83
Bassiouni, M., 67
Basso, E., 69
Basso, N., 413
Basu, A., 379
Basu, R., 85, 379
Basu, S., 195
Batchvarova, N., 301
Batema, R. P., 121
Bates, D. J., 280
Bates, D. V., 433, 434, 435, 436, 437
Bathum, L., 170, 488
Batkai, S., 100
Batt, J., 95
Battaglia, V., 54
Batterham, R. L., 30, 217, 224, 485
Battista, D., 277
Battistella, L., 220, 225, 247
Battistini, S., 300, 302
Bauer, A. K., 281
Bauer, A. L., 208
Bauer, J., 280, 282, 337
Bauer, J. A., 230, 257, 259
Bauer, J. H., 13, 230, 246, 250
Bauer, M. K., 69
Bauer, R. L., 283
Baukol, P., 468
Baum, S. T., 392, 451
Baumbach, G. L., 349, 406, 407, 408, 410, 412, 413
Baumeister, R., 217
Baumgartner, R. N., 119, 123, 360, 433
Baur, J. A., 14, 16, 166, 248, 249, 250, 251, 254, 259, 283
Bauter, M., 260, 261, 262
Bautmans, I., 327
Bayir, H. A., 70
Baylin, S. B., 255, 256
Baynes, J. W., 27
Bays, H. E., 122
Bazinet, S., 261
Beal, M. F., 282
Beamer, B. A., 327, 328
Beard, C., 142
Beard, C. M., 356
Beard, J. C., 375, 379
Beard, J. L., 471, 472
Beare, R., 353, 355, 356
Beasley, T. M., 30, 32, 164, 216, 381, 392, 451
Beausejour, C. M., 146, 282
Beccafico, S., 83
Bechet, D., 66, 148
Bechinger, B., 71
Bechtel, P. J., 33
Beck, G. J., 435
Beck, P. L., 282
Becker, A. J., 143
Becker, C. F., 254, 255
Becker, L., 181, 185
Becker, L. C., 98
Becker, M. W., 147
Beckermann, B., 286
Beckers, P., 95, 96
Beckers, S., 244, 253, 258
Beckett, L. A., 360
Becklake, M. R., 437, 438
Beckman, D. J., 277
Beckman, K. B., 52, 194
Beckson, M., 333, 334, 335, 336, 337, 338, 339
Bedalov, A., 260, 261
Bedard, A. C., 337, 338, 339
Beecher, C. W., 257
Beecher, G. R., 185
Beekman, M., 28
Beer, B., 30
Beere, P. A., 457
Befroy, D., 68
Begley, D. J., 406, 408
Begun, J., 16, 221, 286
Behar, K., 130
Behrens, M. M., 388, 489, 491
Behringer, R., 29, 34
Beirowski, B., 261
Bejarano, B., 355
Bekaert, S., 168
BelAiba, R. S., 124

Author Index

Belawat, P., 10
Belenky, P., 249, 250
Belham, C., 204
Belikova, N. A., 70
Bell, D. G., 469
Bell, M. Y., 359
Bell, R., 207, 352
Bell, R. D., 357
Bell, S. C., 168, 169
Bellei, M., 469
Bell-Farrow, A. D., 449
Bellino, F. L., 390
Bellizzi, D., 254, 300
Bellush, L. L., 26, 27, 229
Belman, M. J., 433
Belter, J. G., 191
Benabid, A. L., 389
Benayoun, B., 81
Bender, C., 66
Benedict, R. H., 353
Benediktsson, R., 378
Benelli, B., 50
Benes, F. M., 333, 334, 337
Benetos, A., 168, 169
Benezra, R., 85
Benguria, A., 217
Benhaim, P., 129
Ben-Hur, T., 14
Benigni, A., 413
Benini, L., 325
Benit, P., 71
Benjamin, E. J., 169
Benna, C., 219
Bennett, B. J., 286
Bennett, D. A., 360
Bennett, S. A., 31, 457
Bensch, K. G., 221
Benson, R., 353, 354
Bentz, G. L., 281
Ben-Yosef, Y., 358
Benzer, S., 13, 205, 206, 217, 218, 226
Ben-Zvi, A., 299
Berardelli, M., 300
Berber, E., 244, 245, 254, 258, 260
Berdichevsky, A., 16, 224, 225, 230, 246, 250, 261
Berend, N., 428, 430
Beretz, A., 360
Berg, D. J., 413
Bergamini, C., 49, 50
Bergamini, E., 14, 307, 308
Bergamo-Andreis, I. A., 120
Berger, F., 249
Berger, J., 412, 413
Berger, S. L., 144
Berger, Z., 307
Bergeron, M., 358
Berggren, M. M., 192
Bergholm, R., 123
Bergman, A., 285
Bergman, D. A., 122
Bergman, H., 323
Bergman, N. A., 437
Bergman, R. N., 375, 376, 379
Bergmann, M., 123
Bergmann, O., 126
Berkowitz, R., 129
Berlet, H. H., 337
Berlett, B. S., 190, 192, 193
Bernabei, R., 64, 70, 78, 282
Bernal-Mizrachi, E., 252
Bernard, C., 422
Bernard, J. P., 256
Bernard, S., 126
Bernardi, L., 349, 352
Bernardi, P., 68, 69
Bernardi, R., 208
Berndt, J., 129
Berney, T., 377
Bernick, C., 353
Berridge, M. J., 82
Berrocal, J. G., 249, 256, 261
Berry, E. A., 50
Berry, E. M., 14
Berry, M. J., 192
Berryman, D. E., 30, 130
Bersinger, N. A., 358
Berstein, L. M., 16
Bertand, H. A., 231
Bertani, L. E., 5
Bertasso, A. M., 392
Berthoud, H. R., 465
Berthou-Soulie, L., 358
Bertram, C., 145
Bertrand, H. A., 14, 120, 126, 252, 322
Bertsch, K., 407
Bérubé, A., 286
Berzin, T., 357
Besnard, S., 413
Best, A. M., 277
Bestmann, L., 285
Betik, A. C., 70
Betto, D. D., 95, 96
Betto, R., 85, 95
Betz, U. A., 97
Bevan, R., 183
Bevilacqua, L., 248
Beyer, A. M., 412
Bezdek, A. C., 153
Bezlepkin, V. G., 179, 183, 195
Bhalla, K., 69
Bhaskar, P. T., 226, 230
Bhat, G. K., 72
Bhatia, B., 216
Bhattacharya, A., 67
Bhaumik, D., 280
Bhuiyan, M. S., 72
Bhushan, A., 375, 377
Bianchi, A., 279, 281, 411
Bianchi, G., 99, 358
Biarnes, M., 375
Bieganowski, P., 249
Biegelsen, E. S., 358
Bielschowsky, F., 30, 34
Bien-Ly, N., 411
Biery, D. N., 447
Bigbee, A. J., 72, 78
Bigelow, M. L., 55, 68
Biggs, P., 151
Biggs, W., 11
Bigras, E., 218
Bijl, R., 338
Bild, D. E., 169
Bildsoe, H., 65, 84
Billington, B. L., 220, 224, 244
Binart, N., 33
Binder, S. W., 192
Bindokas, V. P., 48, 52
Bindoli, A., 53, 189
Bing, O. H., 97
Binkley, N., 392, 448
Binkley, N. C., 9, 391, 392, 449, 450, 451
Birch, K. E., 277
Birk, R. Z., 122
Birlouez-Aragon, I., 298, 302
Birnbaum, M. J., 205
Birringer, M., 16
Bischoff, C., 170, 171
Bischoff, R., 147
Bish, L. T., 97
Bishop, J. M., 437
Bishop, K. S., 178, 179
Bishop, N., 412
Bishop, N. A., 4, 5, 7, 9, 10, 11, 16, 247
Bisht, K. S., 258
Bisoli, L., 325
Bisschops, R. H., 351, 352
Bissell, M. J., 145
Bistrian, B. R., 285
Biteau, B., 154
Bitetto, D., 285
Bito, J. W., 392
Bito, L. Z., 392
Bitterman, H., 358
Bitterman, K. J., 16, 99, 216, 230, 237, 247, 248, 249, 250, 255, 256, 258, 259, 261, 262
Bjartmar, C., 338
Bjedov, I., 13
Bjelland, S., 194
Bjerknes, M., 153
Bjorge, T., 491
Björkegren, J., 277
Bjorkoy, G., 306
Bjornstrom, L., 388
Bjorntorp, P., 123
Blachly-Dyson, E., 69
Black, A., 392, 449, 450
Black, A. D., 411, 413

Author Index

Black, B. J., Jr., 324, 326, 327, 448
Black, D., 32
Black, L. F., 424
Blackburn, E. H., 163, 166
Blackburn, G. L., 285
Blackett, A. D., 179, 182, 195
Blackie, S., 433
Blackman, M. R., 450
Blackmore, D. K., 182
Blackwell, T. K., 11
Blagosklonny, M. V., 145
Blahak, C., 355
Blair, S. N., 421, 424
Blake, J. T., 33
Blanchard, D., 4, 5, 7, 9, 10, 206, 208
Blanche, H., 28, 156, 285
Blanco, M. A., 485
Blander, G., 225, 256, 257, 258, 259, 260
Blanpain, C., 145
Blanton, C. A., 325
Blasco, M. A., 146, 163, 166
Blatteis, C. M., 473
Blau, H. M., 148
Blaum, C. S., 323, 324, 374
Blauw, G. J., 278
Blazek, D., 261
Bleasdale, J. E., 250, 251
Blenis, J., 205
Blevins, J. E., 325
Blezer, E. L., 337
Blink, E., 71
Blizzard, L., 353, 355, 356
Block, M. L., 286
Bloem, B. R., 353
Bloem, C. J., 379, 380
Blough, E. R., 73, 74, 76
Bloxham, D. P., 49
Bluck, L. J., 27
Bluher, M., 27, 29, 120, 129, 217, 224
Blum, S., 328
Blumenthal, J., 334
Bobyleva, V., 469
Bode, A. M., 28, 29
Bode, R., 430
Bode-Boger, S. M., 412
Bodkin, N. L., 34, 127, 392, 449
Bodner, E. V., 321
Bodyak, N., 72
Boecker, W., 97
Boehm, M., 255
Boeing, H., 123
Boeke, J. D., 224, 244, 245, 249, 254
Boekelheide, K., 248, 251
Boesch, C., 482
Bogan, K. L., 249, 250
Bogardus, C., 125
Bohle, R. M., 281
Böhm, C., 283
Bohr, V. A., 170, 171, 194
Boily, G., 248, 251, 258, 261
Boise, L. H., 71
Boiteux, S., 194
Boivin, L., 127
Bokov, A. F., 28, 29, 56, 182, 184, 186, 187, 189, 190, 191, 192, 193, 196, 489, 490
Boland, B., 307
Boldogh, I., 248, 249
Boldrey, E., 464
Bollen, E. L., 352
Bonada, A., 285
Bonafe, M., 4, 7, 19, 208, 218, 285
Bonawitz, N. D., 230
Bond, J., 484
Bondolfi, L., 154
Bonduriansky, R., 479, 485
Bondy, C. A., 30
Bonenfant, D., 204
Boney, C., 130
Boney, C. M., 128
Boni, A., 99
Bonifazi, M., 168
Bonkowski, M. S., 14, 26, 27, 28, 29, 229
Bonnafous, S., 123, 125
Bonnard, C., 244
Bonner, R. T., 391
Bonner-Weir, S., 373, 375, 377, 378, 379
Bonnet, S., 358
Bonnycastle, L. L., 378
Bonoczk, P., 352
Bonomo, S. M., 325
Bontrop, R. E., 448
Boockvar, K. S., 326
Boomsma, D. I., 170
Booth, C., 143, 153
Booth, F. W., 64, 84, 148, 421, 424
Booth, G. L., 374
Booth, J. A., 491
Bootman, M. D., 82
Bopp, M. M., 324
Borchelt, D., 194
Borchers, A. T., 34
Bordone, L., 13, 225, 247, 252
Boreham, D. R., 179, 183, 195
Borek, A., 51
Boren, H. G., 423, 435, 436
Borg, K. E., 26, 27, 224
Borges, G., 338
Borges, O., 67
Borlaug, B. A., 427
Borra, M. T., 245, 246, 250, 258
Borradaile, N. M., 258, 259
Borras, C., 32, 68, 388, 489, 490, 491
Borrelli, L., 407, 408
Borsch-Galetke, E., 431
Borsos, E., 208, 230, 308
Bortolami, M., 282
Bortoli, S., 148
Bortolus, M., 49, 50
Borton, M., 394
Bosch, J., 178, 179
Bosco, D., 377
Boss, O., 244, 250, 263
Bossemeyer, R. W., 426
Bossy-Wetzel, E., 71
Botelho, N. K., 121, 122
Bouhuys, A., 435
Bouillanne, O., 324
Boulant, J. A., 465, 466, 472
Boullier, A., 277
Boulos, Z., 468
Bouloumie, A., 120, 125, 128, 129
Boultby, R., 169
Bourbon, P. C., 435
Bourgeois, J. M., 55
Bourlier, V., 120
Bourque, B. M., 194
Boutier, V., 321
Bouvy, N. D., 469
Bouwens, L., 375
Boveris, A., 67
Boveris, A. D., 179, 182, 195
Bovina, C., 49, 67
Bowden, D. M., 392
Bowen, J. D., 357
Bowler, J. V., 356
Bowzard, J. B., 277
Boyault, C., 300
Boyce, M., 71
Boyer, B. B., 123
Boyko, E. J., 376
Boyko, S. I., 222
Boyles, J. K., 340
Boylston, W. H., 29
Boylston, W. H. III, 301
Boysen, H. M., 49, 54
Bozzella, M., 167
Braak, E., 340
Braak, H., 337, 340
Braam, B., 167
Brabletz, T., 154
Bracale, R., 247, 259
Brace, C. S., 249, 253, 262
Brach, J., 355
Brachmann, C. B., 224, 244
Brack, A. S., 65, 84, 148, 149, 151, 154, 156
Bradbury, C. A., 256
Bradford, G. E., 26
Bradley, J., 129
Bradley, M., 433
Bradt, B., 280, 282
Braeckman, B. P., 4, 5, 7, 9, 10, 16, 17, 208, 220, 228
Braga, M., 73, 74, 75, 76
Båkenhielm, E., 287
Brancati, F., 374

Author Index

Branch-Mays, G. L., 392
Brand, J., 93, 98, 99, 125
Brand, M. D., 48, 49, 50, 51, 52, 54
Brandes, R. P., 258, 349, 358, 405, 408, 410
Brandon, M., 164, 170, 171
Brandt, B. W., 28
Brandt, J., 353
Brandt, U., 48, 51
Branellec, D., 357, 358
Branshaw, S., 31
Bras, G., 221
Brasky, K. M., 392
Brassil, C. E., 479
Braszko, J. J., 360
Bratusch-Marrain, P. R., 27
Brault, Y., 230
Braun, K. M., 145
Braunstein, M., 244
Bray, G., 120, 121, 122, 127, 128
Brayne, C., 352, 484
Brech, A., 306
Breda, E., 379
Breese, C. R., 26, 30
Breeze, E., 183
Bregegere, F., 302, 304
Bregonzio, C., 360
Breitenbach, M., 250
Breitner, J. C., 340
Breitschopf, K., 168
Brem, G., 34
Brengelmann, G. L., 471
Brenner, C., 68, 71, 249, 250
Brennum, J., 466
Brent, M. M., 261
Breteler, M. M., 349, 351, 352, 357
Brett, J. O., 155
Breuer, P., 298, 300
Breuille, D., 328
Brewer, G. J., 194
Brewer, J. W., 301
Brezden, O. S., 169
Brian, J. E., 407
Brichard, S. M., 285
Brickson, M., 391
Bridges, R. S., 389
Brief, S., 33
Brigelius-Flohé, R., 189
Brigell, M. G., 337
Brignull, H. R., 299
Briles, D. E., 277
Briley, D. P., 352, 353
Brimacombe, M., 166, 167, 170
Brini, M., 82
Brinkman, F. S., 277
Brinster, R. L., 27, 34, 145
Brinton, R. D., 389
Brischetto, M. J., 425, 437, 439
Brisken, C., 144, 145
Brismar, B., 438, 439
Britt-Compton, B., 166, 167
Britten, M., 169
Britto, R. R., 433
Brix, T. H., 488
Broach, J. R., 244
Brock, D. B., 373, 374
Brock-Utne, J. G., 470
Brodaty, H., 355
Brodt, P., 34
Brody, G. M., 464
Brody, S., 391
Brok, H. P., 337
Bronikowski, A. M., 482
Bronson, R. T., 34, 179, 180, 181, 182, 185, 257, 491
Brooks, E. M., 471, 472
Brooks, G. A., 70
Brooks, R., 485
Brooks, R. C., 479
Brooks, R. M., 412
Broom, L., 357
Broske, A., 151
Bross, P., 300
Brot, N., 219
Broughton, S. J., 224
Brouilette, S., 169
Broussard, S. R., 32, 33
Brown, C. K., 337, 347
Brown, C. M., 285
Brown, D., 71, 73, 74, 75, 76
Brown, E. N., 473
Brown, J., 375
Brown, J. P., 283
Brown, K. A., 410, 411, 412
Brown, L. G., 388
Brown, M. D., 67
Brown, P. A., 221
Brown-Borg, H. M., 26, 27, 28, 29, 30, 31, 33, 34, 67, 209, 224
Browne, S. E., 52
Browning, J. D., 122
Brownlee, M., 283
Bruce-Keller, A. J., 32
Bruder, C. E., 55
Brugarolas, J., 205
Brugger, B., 340
Bruhlmann, M., 337
Brümmendorf, T. H., 281
Brun, B., 95
Brunati, A. M., 54
Brunet, A., 3, 4, 5, 7, 9, 10, 11, 12, 15, 16, 17, 221, 224, 229, 247, 248, 255, 256, 261, 262
Brunet-Rossinni, A. K., 486
Brunk, U., 308
Brunk, U. T., 54, 67, 68, 182, 307
Brunner, R. L., 389
Bruno, J., 230, 247
Bruns, C. M., 392
Bruns, N. E., 302
Brunzell, J. D., 125
Bruse, P., 69, 70
Bruss, J., 337
Bruunsgaard, H., 77, 78, 123, 124, 125, 285, 286
Bryan, N., 353
Bryan, R. M., 408, 409, 410
Bryant, M. J., 485
Brydak, L. B., 279
Bryder, D., 147, 150, 151, 152
Bryk, M., 224, 229
Brylka, D. A., 352
Bryner, R. W., 72, 84, 95
Brys, K., 4, 5, 7, 17
Brys, M., 349, 352
Bryson, J. S., 33
Bua, E., 67
Bua, E. A., 55
Bucci, L., 280, 281
Buch, S., 13
Buchan, K. W., 412, 413
Bucher, B., 360
Buchholz, B. A., 126
Buchholz, J. N., 410
Buchler, N. G., 341
Buchmuller, T., 34
Buck, S., 221
Buckingham, J. A., 48, 49, 51, 52, 54
Buckley, B. M., 278
Buckley, M. C., 120, 122
Buckmaster, E. A., 262
Budinger, T. F., 357
Buee, L., 356
Buehner, G., 14, 205, 222
Buettner, G. R., 53
Buffenstein, R., 168
Buist, A. S., 424
Bukau, B., 299
Bukowska, A., 412
Bulcao, C. F., 464, 466, 469, 470
Bullinger, L. V., 408
Bullitt, E., 406
Bulló, M., 285
Bulloch, K., 280
Bullock, R., 282
Bulteau, A. L., 298, 302, 304
Bumgarner, R., 390, 448
Bunce, C. M., 256
Bunik, V. I., 52
Bunkenborg, J., 244, 254, 255
Bunkin, D. A., 123, 124
Bunner, R. L., 323
Buonomo, F. C., 29
Burcelin, R., 231
Burdick, M. D., 127, 128
Bureau, M. F., 413
Burek, M., 407
Burelle, Y., 95
Buret, A. G., 282
Burger, E. J., 433
Burgering, B. M., 224
Burgess, J., 283
Burgoon, P. W., 465

Burgos, F., 437, 438
Burgoyne, P. S., 387
Burkart, F., 427, 437
Burke, A. P., 283
Burke, G., 169
Burke, G. L., 353
Burke, J. T., 230
Burke, P. A., 29, 30
Burman, P., 32
Burnett, R. T., 286
Burr, M. L., 424
Burrows, B., 424, 429, 435
Burstein, E., 78
Burstein, R., 465
Burt, V. L., 347
Burtner, C. R., 220, 222
Burton, F. R., 325
Burton, G. W., 178
Burton, M., 55
Burtt, N. P., 378
Bus, J. S., 193
Buschke, H., 353
Bushnell, M. C., 464
Buskirk, E. R., 468, 472
Busquets, S., 77, 78
Busse, R., 349, 405, 408, 410
Busuttil, R. A., 34, 35, 195
Butcher, S. K., 281
Butel, J. S., 257
Butler, A., 27
Butler, A. E., 373, 375, 379
Butler, C., 427, 438
Butler, D. C., 72, 78, 84, 85, 100
Butler, K. S., 357
Butler, P. C., 373, 376, 377, 379
Butler, R., 169
Butler, R. N., 277
Butler, T. M., 392
Butler-Browne, G., 84, 148
Butov, A., 222
Butt, A. M., 406
Buttemer, W. A., 168
Butterfield, G. E., 32
Buttner, S., 310
Butts, B. D., 68
Buxton, R. B., 437
Buysse, D. J., 468
Byatt, J. C., 27
Byers, T., 324
Bygott, J. D., 491
Byrd, P. J., 151
Byrd, W., 165, 166
Byun, Y., 97

C

Caballero, A., 246
Cabanac, M., 468
Cabello, R., 125
Cabeza, R., 341, 350
Cabrera, C., 195
Cacchio, M., 358
Cacciapaglia, F., 64
Cadenas, E., 54
Cagnacci, A., 473
Cahill, C. M., 11
Cai, J., 56, 69
Cai, L., 257, 308
Cai, W., 277, 285
Cai, Z. L., 65, 66
Caillaud, C., 439
Cain, K. C., 379
Cairns, S. J., 491
Cakatay, U., 298
Cakman, I., 149
Calado, R. T., 170
Calandra, R. S., 26
Caldato, M., 285
Calder, R. B., 34, 35
Calderón-Garcidueñas, L., 286
Calderwood, S. B., 16, 221, 286
Caldwell, S. D., 207, 217, 218, 220, 226, 230, 250, 251
Caliebe, A., 28, 156, 285
Caligiuri, G., 413
Calle, E. E., 286
Calvanese, V., 250
Camacho, R., 33
Cameron, A. J., 321
Cameron, E. E., 244
Cameron, J. D., 387
Cameroni, E., 12, 306
Camilli, A., 435, 436
Camp, J. J., 283
Campbell, E. J., 440
Campbell, J., 225, 256, 257
Campbell, L., 151
Campbell, R., 82
Campisi, J., 64, 125, 146, 163, 167, 195, 277
Campochiaro, P. A., 262
Campodonico, S., 427, 437
Canaday, D. H., 306
Canaider, S., 300
Canali, R., 300
Cande, C., 71
Candido, E. P., 222
Candore, G., 285, 387
Canning, A. M., 394
Cannon, B., 123
Cannon, R. O. III, 168
Cannon, W. B., 422
Canto, C., 96, 230, 251, 252, 258
Cantwell, A. M., 392
Canty, J. M., Jr., 97
Cao, B. Y., 65, 66
Cao, G., 179, 180, 181, 182, 185
Cao, L., 248, 251, 256, 257, 302
Cao, P., 55, 56, 187, 188
Cao, Q., 209
Cao, R., 287
Cao, S. X., 301
Cao, X., 166, 167, 169
Cao, Y., 262, 287
Cao, Z., 67, 148
Capasso, J. M., 96, 98
Capoluongo, E., 32
Capone, C., 405, 413
Cappola, A. R., 323, 328
Capri, M., 280, 281
Capurso, C., 285
Capurso, S. A., 285
Caputo, J. L., 433
Capuzzi, D. M., 279, 281, 411
Carafoli, E., 82
Caraveo-Anduaga, J., 338
Carbo, N., 77, 78
Carcel, J., 297
Cardell, A. F., 471, 472
Cardellini, M., 258
Cardile, A., 247, 259
Cardin, R., 282
Cardus, J., 437, 438
Carey, B. W., 142
Carey, J. R., 216, 479, 485, 486, 489
Carey, K. D., 390, 482
Carling, S., 96, 247, 259, 453, 456
Carlisle, K., 466, 472
Carlson, B. A., 193
Carlson, B. M., 148
Carlson, C. J., 421, 424
Carlson, C. S., 27, 31, 392
Carlson, E. C., 34
Carlson, E. J., 186, 187, 190, 191
Carlstrom, K., 391
Carman, C. V., 190, 191
Carmelli, D., 119
Carmona, J. J., 248, 249, 254, 260
Carmona-Gutierrez, D., 220, 310
Carnes, B. A., 483
Carnethon, M., 386, 387
Carney, D. P., 230, 248, 250, 251, 262, 263
Caro, A. A., 52
Carobbio, S., 254, 255
Caron, C., 300
Carotenuto, L., 285
Carp, R. I., 406
Carr, A., 182
Carr, D., 4, 7, 11, 216, 231
Carr, D. B., 125, 376, 379
Carr, J. J., 169
Carr, P., 146
Carra, S., 186, 187
Carrano, A. C., 4, 5, 7, 10
Carrard, G., 298, 302, 304
Carraro, U., 85, 95
Carrel, L., 388
Carrick, J., 286
Carrozzi, L., 437
Carson, J. A., 65, 84, 94
Carter, C. S., 27, 31, 64, 70, 74, 77, 78, 79, 80, 86, 90, 91, 96, 282, 360

Author Index

Carter, M., 338
Carter, S., 127
Carter-Su, C., 25
Cartron, P. F., 71
Cartwright, A., 119, 122, 123, 124, 126, 129
Cartwright, J., 55, 191
Cartwright, M. J., 119, 122, 123, 124, 126, 129
Carty, J. L., 183
Casagrande, V., 258
Cascajo, M. V., 14, 35
Casciola-Rosen, L., 82
Cases, S., 128
Cash, A., 16
Cashion, A. B., 34
Caskie, G., 339
Caslake, M. J., 123
Cassada, R. C., 223
Cassaro, M., 282
Casscells, S. W., 281
Cassis, P., 413
Castaneda, S., 484
Castano, A., 301
Castedo, M., 51
Casteilla, L., 125
Castellanos, F. X., 334
Castelli, W. P., 282
Castillo, C., 32, 33
Castrillon, D. H., 152
Castro, O., 169
Castronovo, V., 254, 255
Cataldi, A., 99, 358
Cattaneo, A., 16, 216, 230
Cauley, J. A., 123, 323
Caulo, M., 352
Cavalcante, P., 254
Cavalieri, T. A., 92, 93, 98
Cavallini, G., 14
Cawthon, P. M., 323
Cawthon, R. M., 169, 170, 171
Cayuela, M. L., 166
Ceccatelli, S., 66
Cecchini, G., 51
Cecconi, F., 71, 208
Cecim, M., 26, 27, 29, 30, 34
Ceconi, C., 95
Cederbaum, A. I., 52
Cederna, P. S., 84
Cefalu, W. T., 391, 449, 457
Celada, A., 124
Celic, I., 224, 245, 254
Cella, S. G., 325
Cellerino, A., 16, 216, 230
Centers for Disease Control and Prevention, 353, 455
Cerami, A., 283, 284
Cerella, J., 339
Ceresi, E., 126
Certa, U., 286
Cesari, M., 32, 64, 96, 281, 284, 360
Chabi, B., 64, 68, 85, 95
Chabot, J. G., 30
Chabriat, H., 355
Chada, K., 155
Chadeneau, C., 166
Chaffotte, A. F., 304
Chagnon, N. A., 481
Chahal, H., 281
Chai, C. M., 287
Chai, W., 166, 169
Chaiken, R. L., 122
Chakravarthy, M. V., 421, 424
Chamany, S., 283, 284
Chambers, S. M., 150, 151, 152
Chambon, P., 388
Champagne, C., 453, 456
Champy, M. F., 120
Chan, E., 208
Chan, G., 129
Chan, J. C., 283
Chan, L. Y., 283
Chan, P. H., 194, 388
Chan, S. H., 194
Chan, S. R., 166
Chan, Y., 120, 121, 125
Chandel, N. S., 52
Chandra, A., 4, 7, 12, 206, 208, 226, 230, 308
Chandra, M., 97
Chandra, S. B., 146
Chandrashekar, V., 26, 34
Chang, A. M., 373, 374, 375, 376, 379, 380, 381
Chang, C., 13, 246
Chang, C. F., 32
Chang, G. R., 120
Chang, H. Y., 71
Chang, I., 302, 304
Chang, J. W., 14, 35, 217, 221, 222
Chang, K., 256
Chang, S., 166
Chang, T. S., 191
Chang, Y., 14, 26, 28, 205, 222
Chanson, P., 33
Chanturiya, A., 71
Chao, D. T., 70, 71
Chao, T. H., 120
Chao, Y., 286
Chapman, I. M., 324, 325, 326
Chapman, K. R., 425
Chapman, N., 360
Chapman, T., 27, 216, 221
Chardigny, J. M., 68
Charette, S. L., 94
Charmantier, A., 486
Charriere, G., 121, 124, 128
Chartrand, P., 216
Chase, N., 349
Chatenay-Lapointe, M., 230
Chatterjee, S., 97
Chaudhrai, A., 72, 73, 74, 76, 79, 80, 85, 86, 87, 89, 90, 94
Chaudhuri, A. R., 190, 191, 192, 195, 196
Chaunchaiyakul, R., 429, 430, 431, 432, 433, 436, 439
Chauvet, S., 16
Chaves, P., 323, 328
Chavey, C., 123, 125
Chavez, E. A., 165
Chavez, J. C., 358, 359
Chavez, J. D., 68
Chawluk, J. B., 349
Cheatham, R. A., 453, 456, 457
Cheema, P., 26
Cheignon, M., 34
Chello, M., 64
Chen, A., 261, 263
Chen, C., 209
Chen, C. J., 258
Chen, C. N., 67
Chen, D., 4, 5, 7, 9, 10, 11, 13, 16, 204, 205, 206, 207, 208, 209, 220, 224, 226, 227, 230, 247, 248, 254, 255, 261, 262, 358
Chen, H., 378
Chen, H.-I., 433
Chen, H. L., 32, 282
Chen, H. W., 34
Chen, H. Z., 259
Chen, J., 33, 97, 146, 250, 255, 256, 288, 304, 353, 355, 356, 390, 408, 411
Chen, J. H., 455
Chen, J. W., 97
Chen, K. D., 84
Chen, K. Y., 248, 254, 260
Chen, L., 248, 262
Chen, L. D., 390
Chen, L. F., 261, 288
Chen, M., 379
Chen, M. J., 169
Chen, M. L., 219
Chen, Q. M., 55
Chen, R., 28, 156
Chen, S., 72
Chen, S. F., 194
Chen, W., 99, 100, 166, 167, 168, 169, 252, 261, 263
Chen, W. Y., 120, 255, 256
Chen, X., 251, 277, 283, 285
Chen, X. D., 119
Chen, X. P., 413
Chen, X. Z., 34
Chen, Y., 56, 277
Chen, Y. H., 358
Cheng, A., 67, 69
Cheng, C., 12, 220, 225, 247, 470
Cheng, C. M., 31
Cheng, D. S., 388
Cheng, E. H., 69

Cheng, H., 153
Cheng, H. L., 230, 247, 254, 255
Cheng, S. L., 70
Cheng, W., 96, 97, 98
Cheng, Y., 302
Cherkas, L. F., 125, 166, 169, 170
Cherkasova, V. A., 227
Cherniack, E. P., 326
Cherniack, N. S., 425, 426
Chernov, N. N., 53
Chernyak, B. V., 68
Cheshier, S., 151
Chettier, R., 207
Chevalier, J., 34
Cheynier, V., 360
Chi, Y. I., 51
Chiado-Piat, L., 95
Chiang, E., 406
Chiang, H., 306
Chiarelli, D. A., 10
Chiba, T., 306, 309
Childress, A. M., 218
Chin, C. S., 206
Chinkes, D., 122, 123
Chinopoulos, C., 52
Chinta, S. J., 52
Chinte, U., 51
Chippindale, A. K., 13, 205
Chipuk, J., 66
Chipuk, J. E., 78
Chisholm, D. J., 121, 122
Chittenden, T., 71
Chiu, C. H., 127
Chiu, H. C., 120
Chiu, R. K., 304
Chiu, Y. S., 120
Cho, D. J., 283
Cho, J. S., 16
Cho, J. Y., 359
Cho, M. O., 28, 164, 285
Cho, R. H., 150
Cho, S. K., 71
Cho, Y. I., 283
Chobot, S. E., 50
Choe, E., 71
Choi, B. H., 301
Choi, D., 359
Choi, S. Y., 70
Choi, Y. H., 285
Choksi, K. B., 29, 301
Chondrogianni, N., 302, 303, 304
Chong, Z. Z., 249
Chopra, K., 360
Chou, C. J., 120, 124, 125
Chou, J., 32
Chou, M. Y., 277
Chou, T. C., 473
Choudhary, C., 254
Choudhury, A. I., 14, 30, 120, 208, 209, 217, 224, 381, 485
Choueiri, N. E., 349
Chow, C. C., 283
Chow, N., 357
Chowdhury, I., 72
Chowdhury, S. K., 51
Chowienczyk, P. J., 170
Choy, L., 122
Choy, W., 254
Chretien, D., 70
Chrissobolis, S., 405, 408, 410, 411, 413
Christensen, J. D., 9, 449, 450, 451
Christensen, K., 164, 286, 484, 488
Christensen, K. C., 250
Christian, C., 307, 309
Christian, P. J., 389
Christiansen, J. S., 30
Christiansen, M., 126
Christiansen, P., 351
Christodoulou, D. C., 244, 245, 254, 255
Christy, B. A., 85
Chrunyk, D., 250, 251
Chrysant, G. S., 360
Chrysant, S. G., 360
Chu, C. P., 356
Chu, C. T., 55, 56, 187, 188
Chu, F. F., 192
Chu, G., 259
Chu, K. U., 301
Chu, P. G., 192
Chu, S. W., 286
Chu, X., 15, 16, 286
Chu, Y., 410, 411, 412
Chua, K. F., 224, 225, 248, 255, 256, 258, 261, 262
Chucher, I., 300
Chumlea, W. C., 119
Chung, H., 169
Chung, H. Y., 64, 71, 74, 75, 76, 96, 280, 281, 284, 412
Chung, L., 73, 74, 75, 76, 83
Chung, M. Y., 130
Chung, N., 252
Chung, S. S., 392
Chung, Y. J., 124
Chypchar, T., 439
Chyu, D. W., 194
Cianflone, K., 231
Cider, N. L., 13
Ciechanover, A., 297, 302
Ciemerych, M. A., 377
Ciesielski, W. A., 70
Cifone, D., 38
Cigan, A. M., 227
Cignolle, C. T., 323, 374
Cilenti, L., 71
Cini, R., 413
Cinti, H. C., 126
Cipolla, M. J., 405, 406, 407, 408, 410, 411, 412
Cipolletta, D., 120, 121, 125
Cirillo, P. M., 484
Cirocco, W., 221
Cishek, M. B., 360
Cisotto, P., 219
Civelek, V. N., 121
Civitarese, A. E., 96, 247, 259, 453, 456
Cjte, S., 338
Clancy, D. J., 8, 205, 217, 224, 229
Clanton, T. L., 433
Clare, M., 221
Claret, M., 30, 217, 224, 485
Clark, D. F., 429, 430, 433, 436
Clark, J. A., 387
Clark, J. D., 224
Clark, R., 33
Clark, S., 53
Clarke, J. R., 429, 430, 431, 432, 433, 436, 439
Clarke, K., 69, 70
Clarke, M. F., 377
Clarkson, T. B., 389, 390, 391
Clarkston, W. K., 325
Clasen, L., 334, 336
Claudio, L., 356
Clay-Farrace, L., 146
Clement, K., 120, 121, 125
Clements, M., 30, 217, 224, 485
Clements, M. N., 486
Clemmons, D., 26, 27, 229
Clemmons, D. R., 30
Clermont, G., 277
Cleutjens, J. P., 388
Cleveland-Donovan, K., 130
Clever, B., 491
Clevers, H., 143, 145, 153
Cliffer, K. D., 465
Cline, J. M., 34, 389, 390
Clodfelter, K. H., 388
Cluett, T. J., 55
Clutton-Brock, T. H., 485, 486, 488
Coast, J. R., 433
Cobbe, S., 278
Cocchi, A., 360
Coccia, R., 64
Cochrane, A. L., 359
Cochrane, B. B., 323
Cock, T.-A., 412
Cockell, M. M., 244
Codran, A., 66
Coe, C. L., 392
Coenen-Schimke, J., 55, 68
Coffey, A. C., 277
Coffey, C. S., 324
Coffey, R. G., 360
Coggan, A. R., 68, 95
Cohen, D., 13, 225, 247, 285
Cohen, G. M., 66
Cohen, H., 237, 249
Cohen, H. Y., 16, 99, 230, 247, 248, 249, 250, 255, 256, 259, 261, 262

Author Index

Cohen, I., 71
Cohen, J. A., 337
Cohen, J. C., 122
Cohen, R. D., 484
Cohn, B. A., 484
Colacicco, A. M., 285
Colditz, G. A., 123
Cole, G. M., 280, 282, 358
Cole, S. L., 282
Colebatch, H. J. H., 429, 430
Coleman, A. J., 437
Coleman, M. P., 261
Coles, L. S., 165
Colige, A., 125
Collen, D., 125
Collier, B., 30
Collier, R. J., 27
Collins, F., 490
Collins, J., 449
Collins, K. J., 464, 466, 468, 469, 470, 471, 472
Collins, P., 385, 387, 388
Colman, R., 391, 451
Colman, R. J., 9, 27, 30, 32, 164, 216, 381, 389, 391, 392, 448, 449, 450, 451
Colom, B., 96
Colonna, R., 68
Colonna-Romano, G., 285, 387
Colonnier, M., 336, 337
Colosimo, E., 428
Colton, C. A., 285
Combaret, L., 66, 148
Combs, C. A., 99, 100
Comet, S., 34
Comijs, H., 282
Committee on Animal Models for Research on Aging, 488, 491
Communal, C., 97
Comroe, J. H., 437
Comstock, J. A., 448
Conboy, I. M., 147, 148, 149, 151, 154, 156
Conboy, M. J., 65, 147, 148, 149, 151, 154, 156
Conconi, M., 302, 304
Conde, S. V., 425, 426
Condorelli, G., 97
Conforti, L., 261, 262
Conley, K. E., 67, 68, 70, 94
Connell, J. M., 360
Connell, L. J., 468
Connell, M. L., 412
Connor, J. R., 338
Connor, K. E., 32, 282
Connor, S., 472
Conover, C. A., 26, 30, 301
Conraads, V. M., 95, 96
Conrad, C. C., 191, 194
Conrad, M., 192
Considine, T., 250
Constancia, M., 455
Constantine, G., 277
Conti, F. G., 29, 34
Conti, S., 413
Contursi, C., 69, 99, 100
Conwit, R., 64
Cook, J. E., 277
Cook, N. R., 178, 179, 324
Cooke, J. P., 358
Cooke, M., 183
Cooney, P. T., 31
Cooper, B. Y., 466
Cooper, C., 283
Cooper, J. A., 284
Cooper, K. E., 473
Cooper, T. M., 247
Copeland, N. G., 85
Copello, J. A., 388
Coppe, J. P., 125, 146, 281, 282
Coppola, J. D., 325
Coracina, A., 169, 170
Corberand, J. X., 125
Corcondilas, A., 436
Corcoran, C. D., 340
Corder, E. H., 340
Cordum, H. S., 388
Corkey, B. E., 121
Corman, B., 34
Corna, D., 413
Cornoni-Huntley, J., 324
Corradi, D., 96
Corral-Debrinski, M., 67
Correa, J. E., 95
Correia, M. L., 349
Correia, S., 357
Corsellis, J. A., 333, 335, 336, 337, 339
Corsi, A., 278
Corsi, M. M., 283
Cortassa, S., 52
Cortez, L. A., 34, 56, 190
Corti, M. C., 124
Cortopassi, G., 302
Coschigano, K. T., 26, 27, 30, 31, 130, 224, 229
Cosentino, F., 358
Cosopodiotis, G., 13
Costa, D. L., 286
Costall, B., 360
Costford, S. R., 248, 249
Cote, S., 338
Cotes, J. E., 424
Cotter, R. J., 245
Cotton, S. M., 341
Couillault, C., 16
Coull, A. J., 405
Coulombe, C., 321
Coupal, J., 357
Court, D. L., 9, 12
Cousin, B., 121, 124, 128
Cousin, J. L., 121
Coussens, M. J., 251
Couture, J. G., 437, 438
Covello, G., 254
Covinsky, K. F., 324
Cowell, S., 437
Cowie, A., 83
Cowie, C. C., 373, 374
Cox, K., 321
Coyle, T., 337
Cozijnsen, M., 153
Cozzi, V., 247, 259
Craddock, J., 302
Craggs, G., 85
Craig, C. G., 154
Craig, D., 282
Craig, D. B., 437, 438
Craigen, W. J., 69
Crane, F. L., 184
Cranney, A., 283
Crapo, R. O., 427, 437, 438
Crapper McLachlan, D. R., 359
Cras-Meneur, C., 252
Cravatt, B. F., 100
Crawford, D., 4, 7, 9, 12, 205, 206, 207, 208, 217, 227, 229, 230
Crawford, F., 357
Crawford, K., 448, 450, 451, 489
Crawford, K. S., 392
Cree, M. G., 122, 123
Cregg, J. M., 305
Crenshaw, E. B. III, 26
Crespo, J. L., 204
Cress, M. E., 94
Crimi, G., 279, 281, 411
Crimi, M., 50
Crimmins, E. M., 276, 278, 286, 484
Cripps, R. L., 455
Cristofalo, V. J., 303
Crivello, A., 285
Crockett, A., 435
Croft, K. D., 183
Croft, M. A., 392
Crofts, A. R., 50, 51, 55, 56
Croley, A. N., 149
Crompton, M., 81
Cross, J. M., 94
Cross, R. J., 33
Crossno, J. T., Jr., 127, 128
Croston, T. L., 64
Crow, M. T., 72
Crowell, M., 3, 14
Crowell, M. F., 120, 228
Csapo, I., 352
Csermely, P., 301
Csiszar, A., 29, 96, 125, 259, 260, 261, 287, 358, 410, 411, 413
Csukly, K., 95
Cubbon, R. M., 170
Cuculi, F., 169
Cuervo, A. M., 12, 297, 305, 306, 307, 308, 309, 310

Cui, K., 34
Cui, X., 29, 123, 124, 125
Cullen, D. E., 152
Culmsee, C., 191
Culotta, V. C., 12, 220
Cumming, R. C., 308, 309
Cummings, J. L., 337, 339, 340, 341, 358
Cummins, P., 129
Cunningham, D. A., 425, 439
Cupples, L. A., 168, 169, 349, 357
Curcio, M. J., 224
Curran, S. P., 207, 208, 218, 219, 220, 228
Currie, A. R., 64
Curry, B. B., 301
Curry, C., 358
Curtis, R., 4, 7, 9, 16, 222, 250, 256
Curtiss, S. W., 428
Curtsinger, J. W., 217, 218, 224, 300
Cushman, M., 353
Cusumano, A. M., 34
Cutler, J. A., 347
Cutler, N. R., 357
Cutler, R. G., 181, 186, 392, 450
Cutlip, R. G., 94
Cviko, A., 65
Cybulski, N., 231
Cynober, L., 324
Cypess, A. M., 122, 123, 469
Cypser, J., 222
Cypser, J. R., 222
Cypser, J. W., 222
Czeisler, C. A., 473
Czopik, A., 13, 225, 247

D

Daanen, H. A. M., 464, 466, 470, 471
Dabholkar, A. S., 334, 339
Dabkowski, E. R., 64
d'Adda di Fagagna, F., 146
Daeberlein, M., 220
D'Agati, V., 283
Dagenais, N. J., 360
Dagon, Y., 14
Dahlmann, M., 249
Dahmen, N., 341
Dai, D. F., 56
Dairaku, N., 68
Dakak, N., 349
Dale, A. M., 337
Dali-Youcef, N., 120, 224
Dalla, L. L., 85, 95
Dalla Man, C., 379
Dallner, G., 184
Dalton, A. J., 359
Daly, G., 193
Damasio, H., 333, 334, 337
Damian, M., 244, 245, 254, 258, 260
D'Amore, A., 28
Danesh, J., 278
Dang, I., 191
Dang, N., 4, 5, 7, 9, 10, 12, 206, 207, 217, 218, 219, 220, 226, 227, 228, 229, 230, 247
Dang, W., 220
D'Angelo, M. A., 146
Dani, C., 121
Danial, N. N., 70, 71
Daniele, N., 81
Danielson, E., 282
Dann, S. G., 14
Danson, J., 433
Dantzer, R., 32
Dapp, C., 64
Daram, P., 10
Dardenne, M., 33
Dardevet, D., 66, 83, 148
Darley-Usmar, V. M., 168, 169
Darlington, G., 29
D'Armiento, F. P., 169, 279, 281, 411
D'Armiento, M. R., 279, 281, 411
Darr, K. C., 94
Dart, A. M., 387
Dart, G., 427, 429, 436, 438
Das, S., 190, 191
Das, S. K., 453, 454, 456, 457
Dasen, J. S., 26
Dasgupta, B., 16, 251
da Silva, C. J., 72
Dasuri, K., 302
Datar, R., 153
Dato, S., 254, 285, 286, 300, 388
Datta, S., 73, 74, 75, 76
Daugas, E., 71
Daun, S., 277
Daussin, F., 230, 248, 261, 263
D'Autreaux, B., 53
Davicco, M. J., 283
Davidge, J. T., 224
Davidson-Moncada, J., 277
Davies, C. T. M., 439
Davies, J. E., 307
Davies, K., 298, 304
Davies, K. J., 70, 298, 304
Davies, N. B., 486
Davies, S., 216
Davis, A. A., 301
Davis, B. T., 327
Davis, C., 4, 7, 11, 216, 231
Davis, J., 302, 308
Davis, J. A., 250
Davis, J. M., 359
Davis, L. S., 251
Davis, R. L., 85
Davis, S. W., 341
Davison, A. J., 55
Davison, L. E., 119, 120, 122, 123
Dawson, D. R., 392
Dawson, H., 32
Dawson, S., 152
Dawson, V. L., 307
Dawson, W. W., 392
Dax, E. M., 127
Day, T., 485
DCCT Research Group, 374
de, T. P., 97
Dean, R. L. III, 30
Dean, W., 144
Deane, R., 357
De Angelis, A., 99
Deary, I. J., 170, 171, 353
De Bacquer, D., 168
de Bakker, P. I., 378
DeBarros, K. A., 484
Debeljuk, L., 26
De Benedictus, G., 285
de Boer, R. A., 169, 170
de Brito, J. M., 33
Debska-Vielhaber, G., 69
De Buyzere, M. I., 168
de Cabo, R., 4, 5, 7, 9, 10, 125, 221, 228, 229
DeCarli, C., 349
Decary, S., 148
de Castro, I. N., 29
De Caterina, R., 413
Decaudin, D., 51
Deckert, G., 259
de Craen, A. J., 352
Dedon, P. C., 193
Deed, R. W., 85
Deeg, D. J., 32, 77, 78
Deeks, S. G., 279
DeFelice, A., 13, 14, 205, 222
DeFelice, C., 168
DeFord, J. H., 29, 34, 301
Defossez, P. A., 12, 206, 219, 220, 229, 247, 258
DeFronzo, R. A., 27
Degenhardt, K., 70, 71
Degens, H., 65, 72, 73, 74, 75, 76, 79, 80, 84, 85, 86, 87, 89, 90, 94
De Giorgi, F., 69
Degli Esposti, M., 50
Degraff, L. M., 281
de Grey, A. D. N. J., 277
de Groot, J. C., 349, 351, 352
De Groot, L. C. P. G. M., 326
Degterev, A., 71, 308
de Haan, E. H., 32
Deisseroth, A., 54
Dejean, L. M., 68, 69
Dejgaard, A., 466
de Jong, G. I., 357
de Jonge, L., 30, 258, 456
Dekhuijzen, P. N., 85
Dekio, S., 121
DeKosky, S. T., 357
de Kreutzenberg, S. V., 169, 170
Dela, F., 55

Delacourte, A., 356
De la Fuente, M., 32, 33
Delagrange, P., 406, 407
DeLance, N., 411
de Lange, T., 163
Delanney, L., 388
Delany, J. P., 30, 258, 456
de la Torre, J. C., 356, 357
Del Buono, A., 32
Del Castillo, D., 285
Delclaux, B., 437, 438
de Leeuw, F. E., 349, 351, 352
De Leo, V., 168
de Leon, M. J., 349
Deleuze, S., 412
Deleuze, V., 413
Della Bella, S., 149
Dellacha, J. M., 26
Dell'Agnello, C., 219
Deloche, O., 12, 204, 205, 207, 217, 227, 306
DeLorey, D. S., 424
de Lorgeril, M., 359
de los Santos-Arteaga, M., 32
del Rio, J. C., 301
Demanet, C., 327
Demaree, S., 185
De Martinis, M., 328
de Mello Coelho, V., 33
Demer, L. L., 283
De Meyer, T., 168
Demissie, S., 166, 168, 169, 170
Demong, N. J., 487
Dempsey, J. A., 421, 424, 432, 433, 436, 438, 439, 440
Demuth, H. U., 375
Denavit-Saubie, M., 359
Denckla, M. B., 334
Deng, C. X., 224
Deng, F. Y., 119
Deng, H., 245
Deng, W., 145, 154
Deng, X., 32
den Heijer, T., 349, 356, 357
de Nigris, F., 279, 281, 411
Denislic, M., 464, 466, 467
Denke, M. A., 123
Dennis, N. A., 341
Dennison, E. M., 283
Denu, J. M., 224, 245, 246, 249, 250, 254, 258
de Oliveira, T. A., 433
DePinho, R., 151, 152
DePinho, R. A., 166
Depinho, R. A., 163
Depino, A., 277
Deplancke, B., 225, 246
DePonte, M., 129
Deppe, M., 349
Depre, C., 387, 389, 390, 395, 396, 397, 398
Dequiedt, F., 256, 258
De Rango, F., 254
DeRango, F., 300
Derbyshire, M. K., 244
Derenne, J., 437, 438
Derenne, J. P., 425
Deretic, V., 306
Derhovanessian, E., 279
de Rooij, D. G., 145
DeRousseau, C. J., 392
Derr, J. A., 471, 472
D'Errico, A., 428
Deruisseau, K. C., 67
Dervisefendic, A., 13, 15
Derynck,R., 122
Desagher, S., 71
Desai, M., 120, 124
Deschenes, M. R., 94
Deshmukh, M., 64
De Simone, G., 386, 387
Desmond, D. W., 356
DeSouza, C. A., 168, 169, 170
de Souza-Pinto, N. C., 194
Desprez, P. Y., 146
Dessain, S. K., 4, 13, 16, 224, 255, 256, 261
Dessen, P., 71
DeTeresa, R., 336, 337
Detrano, R., 169
Detrano, R. C., 169
de Troyer, A., 430
Deuschl, G., 360
Deutsch, W. A., 96, 247, 259, 453, 456
Devens, B. H., 33
Deveraux, Q. L., 71, 72
De Vescovi, V., 300
de Villiers, W., 279
Devine, S. E., 244
De Virgilio, C., 12, 226, 230, 306
Devoto, E., 283
De Vreese, A., 4, 5, 7, 16, 17
de Vries, H. A., 439
de Vries, W. R., 32
de Vries-Smits, L. M., 224
DeWaal, E. M., 196
de Weerd, H., 357
De Wolfe, L. K., 181, 185
Dhahbi, J. M., 16, 29, 33, 301
Dhawan, S., 375, 377
Dhonukshe-Rutten, R. A. M., 326
Di, L. F., 68, 69
Di, W. J., 121, 122
Diabetes Prevention Program Research Group, 374
Diais, S., 277
Dialynas, E., 33
Diamond, T. H., 64
Diano, S., 469
Diapro, A., 218, 220
Diaz, G., 396
Diaz, O., 437, 438
Diaz, V., 34, 35, 282, 284
Diaz-Troya, S., 208
Di Baldassarre, A., 358
Di Bari, M., 360
Dibner, C., 262
Dice, J., 306, 307, 309
Dice, J. F., 306, 309
Di Cianni, F., 254
Dickinson-Anson, H., 154
Didion, S. P., 408, 410, 411, 412, 413, 415
Diebolt, M., 360
Diem, J. E., 423, 429, 434, 435, 436
Diemer, T., 16
Dietrich, M., 251
Dietschy, J. M., 340
Dietz, W. H., 120
Dieu, M., 302, 304
Di Francescomarino, S., 358
Di Francisco, V., 324, 325
Di Giulio, C., 99, 358
Dijk, D. J., 473
Dijk, E., 437
Dik, M. G., 32
Dikic, I., 304
Dikshit, P., 300
Di Lisa, F., 69
Dill, D. B., 439, 472
Dillin, A., 3, 4, 5, 7, 8, 9, 10, 15, 208, 219, 220, 221, 223, 228, 229, 299
Dilova, I., 3
Dimayuga, E., 302
Dimmeler, S., 168, 256, 259
Di Monte, D. A., 388
Dimri, G. P., 167
Ding, C., 283
Ding, D., 190, 195, 352
Ding, Z., 259
D'Introno, A., 285
Dioli, A., 325
Dionne, L., 55, 191
Di Pede, F., 437
Dipp, M. A., 258
Dirks, A., 64, 67, 70, 72, 74, 75, 76, 83, 84, 87, 88, 89, 91, 94, 95, 96
Dirsch, V. M., 360
DiSalvo, T. G., 97
Di Stasi, V., 472
Disteche, C. M., 387
DiStefano, P. S., 4, 7, 9, 16, 222, 250, 256
Di Valerio, V., 358
Divecha, N., 224, 261
Divoux, A., 120, 121, 125
Dixit, V. D., 32
Dixit, V. M., 71
Dixon, G. F., 433
Djian, P., 129
Djordjevic, T., 124
Djouadi, F., 208

D'Mello, N. P., 218
Do, K. L., 68
Do, S. I., 168
Dobashi, H., 33
Dobbin, M. M., 251, 262
Dobbins, R., 122
Doblhammer, G., 164, 484
Dobrin, R., 412
Dobrogowska, D. H., 406
Dobson, D. E., 120, 122, 127, 129
Docherty, J. R., 394
Docherty, M., 67, 70, 94
Dockery, D. W., 423, 424, 435
Dodane, V., 34
Doebeli, M., 485
Doecke, W. D., 302, 304
Doerr, H. W., 281
Doevendans, P. A., 388
Doherty, T. J., 179, 183, 322, 433
Doi, C., 340
Doi, T., 29, 34
Doi, Y., 279, 281
Dolle, M. E., 34, 35
Dollé, M. E., 187, 188, 189
Dollery, C. T., 437
Dolman, D. E. M., 406, 408
Dolovich, M. B., 433, 437
Dombrowksi, L., 127
Domenici, L., 16, 216, 230
Dominici, F. P., 14, 26, 27, 29, 38
Domma, F., 388
Don, H. F., 437, 438
Donadio, V., 472
Donata, A., 412
Donato, A. J., 411, 413
Donehower, L. A., 150, 151, 257
Donevan, R. E., 437
Dong, H., 261, 263
Dong, L. M., 285
Dong, Q., 352
Dong, Y., 217, 219, 220, 228
Dong, Z., 70, 71
Donlon, T. A., 28, 156
Dontas, A. S., 436
Dooldeniya, C., 408
Dor, Y., 375
Dore, C., 466, 470
Dore, J., 11
Doriguzzi, C., 95
Doring, A., 386, 387
Doring, C., 96
Dorman, J. B., 10, 25, 217, 222
Doroshow, J. H., 192
Dorr, A., 261
Dorsey, J. A., 220
Dorshkind, K., 33, 150, 156
Dosman, J., 430
Douck, D. C., 33
Douglas, P. S., 99
Doulabi, B. Z., 122
Dounce, A. L., 54
Dovgan, D., 472
Dowd, G. J., 245
Dowlatshahi, D., 4, 5, 7, 9, 10, 206, 208, 229
Downes, M., 16
Downs, J. L., 390, 450
Doyonnas, R., 148
Drabe, N., 285
Drage, C. W., 425, 437
Dragoni, F., 387
Dragowska, W., 151, 165
Drahota, Z., 51
Drake, J., 433
Drake, V. J., 300
Draper, E. S., 483
Drayson, M. T., 256
Drent, M. L., 32
Drew, B., 67, 491
Drexler, H., 349, 405, 410
Dreyfuss, Y., 35
Driege, Y., 13, 224
Drincic, A., 97
Dringen, R., 53
Drinkwater, D., 321
Driscoll, M., 4, 7, 12, 16, 206, 208, 221, 226, 230, 308
Driver, C., 283, 284
Driver, C. J., 13
Droge, W., 307
Drose, S., 51
Drossaerts, J. M., 469
Droyvold, W. B., 324
Drozhinin, O., 71
Drucker, D. J., 375
Drummond, G. R., 410, 413
Drummond-Barbosa, D., 144
Dryden, S. C., 258
D'Souza, R., 352
Du, S., 51
Du, Y., 52
Duan, H. J., 34
Duan, W., 32
Duara, R., 357
Duarte, J. A., 67, 68, 69, 70
Dube, M. G., 99
Dube, P. H., 392
Dubey, D. P., 190, 191
Dubner, R., 464
Dubocovich, M. L., 473
Dubouchaud, H., 68
Dubouloz, F., 12, 306
Ducharme, M. B., 468
Duckett, C. S., 78
Ducos, B., 29, 217, 224, 485
Ducrest, A. L., 244
Ducret, T., 68
Ducrey, O., 72
Dudaronek, J. M., 221
Dudash, H. J., 67, 70, 94
Duden, R., 299
Dudgeon, C., 78
Duerink, H., 279
Duffy, A., 83
Duffy, J. F., 473
Duffy, S. J., 358
Dufouil, C., 352
Dufour, E., 55
Dufour, S., 68
Dugan, L. L., 181, 186, 388, 489, 491
Dugichi-Djordjevich, M. M., 280
Dujka, M. E., 146
Duke, R. C., 64
Dumble, M., 152
Dumond, M. E., 249, 256, 261
Dumont, M., 473
Dumont, P., 55
Duncan, G. H., 464
Dunn, S. E., 35
Dunn, W. A., Jr., 305
Dunne, P. J., 277
Dunnill, M. S., 427
Dunning, L. M., 301
Dupont, J., 29, 217, 224, 485
Dupont, S., 388
Dupont-Belmont, C., 324
Dupont-Versteegden, E. E., 64, 70, 71, 73, 74, 78, 79, 83, 95
Dupre, M. E., 484
Dupree, J. L., 340
Dupuis, F., 406, 407
Durand, D., 67
Duren, W. L., 378
Durieux, A. C., 64
Durieux, J., 4, 7, 9, 10, 15, 208
Duriez, M., 413
Durkee, S. J., 26, 28, 487
Durrani, J. F., 166, 169
Durrant, J. R., 412
Duss, S., 144, 145
Dussault, S., 170
Dustman, R. E., 338, 339
Dutta, J., 78
Dutton, P. L., 50
Duvel, K., 209
Duxbury, A., 321
Dwolatzky, T., 325
Dwork, A. J., 338, 341
Dyke, B., 482
Dymecka, A., 425, 426
Dysko, R. C., 26, 28, 487
Dzierzak, E., 169
Dziura, J., 68

E

Eagger, S., 357
Eagon, J. C., 285
Earl, S., 283
Earle, M. S., 68
Early, J. D., 481
Earnshaw, W. C., 71
Easlon, E., 3, 230, 247

Author Index

Eastman, A., 97
Eaton, E. N., 255, 256, 261
Eaves, C. J., 144
Ebenezer, P., 302
Eberhardt, M. S., 373, 374
Eberling, J. L., 357
Ebersole, J. L., 392
Ebert, R. V., 427, 428, 429, 430, 433, 435
Ebihara, S., 185
Ecclestone, N. A., 439
Eckel, J., 124
Eckel, R. H., 120, 122, 123, 124, 125, 126, 129
Eckfeldt, J. H., 168, 169
Eckhardt, E., 279
Eckman, D. M., 407
Economou, E. V., 360
Edamatsu, R., 181, 186
Edelman, N. H., 430, 431, 434, 435, 436, 437
Edelman, S. W., 72
Eden, S., 26
Edenhofer, F., 204
Edgar, B. A., 10
Edge, J. R., 424, 430
Edgerton, V. R., 65, 94
Edgley, M. L., 222
Edland, S. D., 151
Edmundowicz, D., 169
Edo, M. D., 281
Edouard, C., 122
Edqvist, A., 151
Edwards, D. P., 388
Edwards, J., 180, 184, 185
Edwards, J. G., 358, 410, 411, 413
Edwards, L. M., 69, 70
Edwards, M. G., 193
Edwards, N., 333, 334, 335, 336, 337, 338, 339, 340, 341
Effros, R. B., 33, 279, 283
Egan, D. F., 204
Egan, K. M., 388
Egashira, A., 193
Egbarya, R., 349
Eggo, M. C., 120
Egormin, P. A., 16
Egushi, K., 360
Ehrenfried, J. A., 301
Ehrsam, R. E., 427, 437
Ehsani, A. A., 439, 453, 454, 456, 457
Eikelenboom, P., 282
Eilertsen, D. E., 337
Eilken, H. M., 169
Eini, T., 81
Einstein, F. H., 130
Einstein, G., 357
Eipper-Mains, J., 128
Eisenberg, T., 310
Eisenhauer, D. A., 304
Eisenstein, R. S., 191, 195
Ekbom, A., 3
Ekenstedt, K., 27, 31
Eklund, J., 26
Elahi, D., 380
Elas, M., 48, 52
El-Assar, M., 405, 410, 411, 413, 414
Elchuri, S., 191, 195
Eldrup, E., 77, 78
el-Gamal, N., 466, 470
Elgavish, A., 256
El Hajjaji, F. Z., 359
Elia, M., 468
Eliasson, P., 145, 152
Eller, G., 33
Elliott, A. J., 279
Elliott, J. L., 190
Elliott, K. S., 378
Elliott, P., 262
Elliott, P. J., 244, 250, 263
Ellis, R. E., 64
Elmfeldt, D., 360
Elmquist, J. K., 473
Elouardighi, H., 99
Elrick, H., 26, 491
El Sayed, R. M., 277
El Shikh, M. E., 277
Eltoum, I. A., 256
Ema, H., 150
Embler, E., 16
Emerson, S. S., 125
Emirgil, C., 427, 437
Emlen, D. J., 487
Emlen, S. T., 487
Emmanuel, F., 413
Emmerson, R. Y., 338, 339
Emond, M., 56, 189, 218
Emond, M. J., 56, 188
Emr, S. D., 305
Enders, F. T., 427
Enerback, S., 123
Engel, H. M., 392
Engel, J., 450
Engeland, A., 491
Engelman, R. W., 32, 282
English, R. J., 357
Enrietto, J. A., 353
Enright, P. L., 321, 433, 435
Ensrud, K. E., 323
Enstrom, E., 179, 183
Entenmann, G., 129
Enzinger, C., 351
Enzler, T., 282
Epe, B., 190, 193, 194
Epstein, A., 325
Epstein, C. J., 56, 182, 186, 187, 189, 191, 193, 194
Epstein, D., 217, 224, 485
Epstein, L., 325
Epstein, P. N., 52
Epstein, S., 218
Er, E., 71
Erbs, S., 96
Erdelyi, P., 208, 230, 308
Erdi, B., 230
Erecinska, M., 193
Erhardt, P., 78
Erickson, R. P., 56, 192
Erickson, R. R., 301
Erikkson, J. G., 374
Eriksson, J., 491
Eriksson, O., 69
Eriksson, P., 120, 121
Eriksson, P. O., 84
Erion, D. M., 251
Erker, L., 195
Erkinjuntti, T., 356
Erlanger, B. F., 186
Ermini, F., 154
Ernerudh, J., 77
Ernster, L., 184
Ershler, W. B., 323, 328, 392, 448, 450, 451, 489
Ervin, J. F., 357
Escalante-Semerena, J. C., 245
Eskelinen, E. L., 226, 308
Eskes, R., 71
Eskurza, I., 411, 413
Espeland, M. A., 389
Espinoza, S., 327
Esposito, L., 411
Esposito, R. E., 244
Esquifino, A. I., 33
Esquivel, P., 33
Esselman, P. C., 67, 94
Essen-Gustavsson, B., 67
Esser, C., 300
Essig, D. A., 83
Esteban, I., 297
Estenne, M., 430
Esteve, J. M., 297
Estey, C., 248
Estornell, E., 50
Esworthy, R. S., 192
Eto, K., 120, 121, 125
Eto, M., 256
Ettinger, W. H., Jr., 124
Eva, W. S., 87, 89, 96
Evans, A., 278
Evans, E., 472
Evans, H. M., 178, 179
Evans, J. M., 284
Evans, M. S., 31
Evans, P. C., 300
Evans, W. J., 64, 122
Even, P. C., 29, 217, 224, 485
Eveno, E., 148
Evers, B. M., 301
Ewbank, D. C., 285
Ewbank, J. J., 16
Ewing, S. K., 323

Exton-Smith, A. N., 464, 466, 468, 469, 470, 471, 472

F

Fabre, J. E., 358
Fabris, C., 285
Fabris, N., 286
Fabrizio, P., 12, 207, 216, 217, 218, 220, 225, 247
Facchini, F. S., 27
Factor, S. M., 97
Fadini, G. P., 169, 170
Faeder, J., 277
Fahie, K., 244, 245, 254, 255
Fahy, G. M., 16
Fain, J. N., 26
Fairbarn, M. S., 433
Fairhead, J. F., 405
Faith, M. S., 324
Falcon-Neri, A., 360
Falk, B., 468, 469
Falkenberg, M., 55
Fallavollita, J. A., 97
Falleti, M. G., 32
Fallin, D. M., 285
Faloia, E., 126
Fan, G., 78
Fan, H., 251
Fan, W., 70
Fan, X., 151
Fan, Y., 78, 412
Fang, C., 357
Fang, G., 261
Fang, H., 67, 69
Fang, J., 26
Fano, G., 83
Faraci, F. M., 349, 405, 406, 407, 408, 410, 411, 412, 413, 415
Faragher, E. B, 285
Farese, R. V., Jr., 128, 285
Fargnoli, J., 300
Farias-de-Oliveira, D. A., 33
Farina, A., 300
Farina, F., 97
Farinati, F., 282
Farkas, E., 357, 405, 406, 407, 408
Farlow, M. R., 357
Farol, P., 333, 334, 337
Farquhar, W. B., 472
Farrall, A. J., 406
Farrell, P. A., 471, 472
Farris, J. W., 68
Fato, R., 49, 50, 410
Faulkner, J., 191, 193
Faulkner, J. A., 148
Faure-Delanef, L., 285
Fazekas, F., 337, 349, 351
Fazio, S., 178
Febbraio, M. A., 284, 285
Fedarko, N. S., 327, 328
Fedigan, L. M., 482
Fehrenbacher, J. W., 277
Feier, C., 431
Feige, J. N., 120, 230, 231, 251, 252, 258
Feigin, J., 336
Feinberg, A. P., 254
Feletou, M., 408, 409
Felicio, L. S., 389, 390
Fellingham, G. W., 379
Felts, P. A., 338
Feng, W., 70
Fenn, W., 432, 435
Fennema-Notestine, C., 337
Fenske, N. A., 470
Fenton, A. C., 483
Ferber, M., 301
Ferbeyre, G., 216
Ferder, L., 413
Ferguson, A. V., 473
Ferguson, D., 282
Ferguson, M., 96, 180, 184, 185
Ferguson, S. J., 48
Ferguson, T. B., 386, 387
Fergusson, M. M., 99, 100, 247, 248
Ferketich, A. K., 94
Fernandes, G., 34
Fernandes, P., 412
Fernandez, A. M., 28
Fernandez-Arias, C., 217
Fernandez-Kim, S. O., 302
Fernández-Marcos, P. J., 166
Fernando, M. S., 352
Fernéndez, H. N., 26
Fernstrom, M., 68
Ferrando, A. A., 94
Ferrante, A. W., Jr., 120, 124
Ferrante, R. J., 190, 194
Ferrari, S. L., 484
Ferrario, M., 386, 387
Ferreira, A. J., 413
Ferreira, J. R., Jr., 181, 185
Ferreira, R. M., 67, 68, 69, 70
Ferrero, J. A., 70
Ferretti, A., 352
Ferrieres, J., 278
Ferriero, D. M., 358
Ferrigno, P., 220
Ferrington, D. A., 67, 302, 304
Ferrini, M. G., 73, 74, 75, 76
Ferris, B. G., 423, 424, 429, 431, 432, 433, 435, 436, 439
Ferrucci, L., 83, 124, 278, 285, 323, 328, 353
Ferry, A. L., 64, 73, 74, 79, 83
Feskens, E. J., 359
Festing, M. F., 182
Feuerer, M., 120, 121, 125
ffrench, M., 307
Fiatarone Singh, M. A., 122
Fiaux, G. W., 428
Fichtlscherer, S., 169
Fidzianska, A., 65
Fiegler, H., 146
Fiehn, E., 95, 96
Field, A. E., 324
Field, C. E., 30
Fields, S., 4, 5, 7, 9, 10, 12, 207, 216, 217, 218, 220, 221, 226, 228, 229, 230, 247
Figueiredo, P. A., 67, 68, 69, 70
Figueiredo-Pereira, M. E., 304
Figueras, M., 77
Figueredo, R., 285
Fikrig, E., 278, 281
Filey, G. F., 427, 429, 436, 438
Filippi, M., 356
Fillmore, M. C., 300
Finch, C. E., 222, 275, 276, 277, 278, 279, 280, 281, 282, 284, 285, 286, 389, 390, 482
Finel, M., 49
Fink, H. A., 323
Fink, K. B., 408
Finkel, T., 145, 224, 247, 248, 410
Finley, D., 304
Finley, K. D., 308, 309
Finn, J. T., 261
Finnegan, T. P., 64
Finot, P. A., 283
Firbas, C., 169
Fire, A., 9, 12
Firestein, R., 225, 256, 257
Fischer, A., 251, 262
Fischer, M., 33, 450
Fischer, M. R., 255, 258
Fisher, C. I., 27
Fisher, C. M., 351
Fisher, D. E., 145
Fisher, N. D., 169, 350, 360
Fisher, R. S., 337
Fishman, A. P., 425, 426
Fisk, C. J., 150, 151
Fiskum, G., 52
Fitzpatrick, A. L., 168, 353
Fitzpatrick, D. F., 360
Fitzpatrick, E., 389
Fitzpatrick, P. A., 4, 7, 9
Fjell, A. M., 337
Flachsbart, F., 28, 156, 285
Flanders, D., 324
Flatt, T., 13, 246
Flaum, M., 337
Flechsig, P., 335
Fleckenstein, J., 94
Fleg, J. L., 424
Flegal, K. M., 373, 374, 386, 387
Fleming, D. M., 279, 391
Fleming, H. E., 151
Fleming, I., 349, 405, 408, 410
Fleming, J., 282
Fletcher, A. E., 183

Author Index

Fletcher, E., 349
Fletcher, J. M., 277
Fletcher, Q., 485
Fleuriel, C., 248
Fliers, E., 473
Fligiel, S., 13, 33, 35
Flood, D. G., 190, 195
Flores, I., 166
Flores, J. M., 166, 217
Flores, L. G., 357
Florez-Duquet, M., 468, 470
Floryk, D., 51
Floto, R. A., 307
Flower, L., 124
Floyd, R. A., 181, 186, 209
Fluckey, J. D., 95
Flurkey, K., 26, 27, 30, 33, 34, 35, 120, 181, 186, 209, 218, 224, 226, 309, 310, 381
Flynn, F. W., 387
Flynn, S. E., 26
Flyvbjerg, A., 224
Fodde, R., 154
Fogel, R. W., 286
Fogelstrand, L., 277
Fogo, A. B., 412
Foiani, M., 227
Folch, H., 33
Folger, S., 34
Folian, B. J., 412
Folkow, B., 405
Folsom, A. R., 169
Foltz, N., 4, 7, 9
Fomenko, D. E., 193
Fonseca, F. A., 282
Fontaine, E., 68, 95
Fontaine, K. R., 324
Fontana, L., 120, 125, 164, 285, 453, 454, 456, 457
Fontanilles, A. M., 121
Fontan-Lozano, A., 32
Foraker, A. G., 427, 435
Forbes, I. J., 97
Forbes, M. E., 31
Forbes, W. F., 428
Force, T. L., 97
Ford, D., 286
Ford, D. A., 120
Ford, E., 245, 252
Ford, E. S., 120, 247
Ford, G., 484
Ford, I., 169
Ford, J., 255, 256
Ford, L. L., 121
Ford, R. J., 259
Forloni, G., 282
Forman, D. E., 352
Forman, H. J., 53
Formiggini, G., 49, 67
Fornace, A. J., Jr., 300
Fornallaz-Mulhauser, M., 218
Forrest, N. C., 144
Forse, R. A., 127, 128, 129
Forsey, R. J., 77
Forsmark, P., 184
Forster, C. Y., 407
Förster, H., 191
Forster, M. J., 96, 179, 182, 284
Forsythe, J. A., 358
Forte, M., 68, 69
Forte, M. A., 69
Forti, E., 122
Fortin, T., 357
Foschini, E., 472
Fostel, J., 281
Foster, C., 322
Foster, G. L., 374
Foster, J. A., 428
Foster-Schubert, K., 285
Fotuhi, M., 341
Foulstone, E. J., 78
Fourquet, S., 53
Fowler, D. R., 283
Fowler, K., 13
Fowler, R. W., 424, 436
Fowler, W. S., 437
Fox, C. S., 283, 284
Fox, L. A., 12, 206, 207, 217, 218, 219, 220, 226, 227, 228
Fox, R. H., 466, 470
Frampton, G., 128, 129
Franceschi, C., 85, 95, 281, 286, 302, 328
Franco, S., 225
Frane, J. L., 142
Frank, L. L., 285
Frank, N. R., 424, 429, 431, 432, 433, 435, 436, 439
Frank, S. M., 464, 466, 469, 470
Frankel, S., 13, 205, 217, 229, 246, 247
Franklin, D. S., 218
Franklin, S. S., 258
Fraser, A., 155
Fraser, A. G., 16, 220, 221, 223, 228, 286, 300
Frattali, A. L., 30
Frayling, T. M., 378
Fre, S., 153
Freeman, J. R., 13, 32
Freeman, M. R., 262
Freer, M., 85
Frei, B., 182
French, H. A., 306
French, J., 35, 92
French, R. A., 33
French, S., 99, 100
French, W. J., 169
Frenkel-Denkberg, G., 358
Frick, K. M., 389
Fridovich, I., 186
Fried, J., 308
Fried, L. P., 321, 323, 324, 328, 374
Fried, S. K., 120, 123, 124
Friedland, R. P., 357
Friedlander, A. L., 32
Friedman, D. B., 4, 7, 9
Friedman, L., 324
Fries, S., 388
Friesen, C., 82
Frigerio, F., 120, 205, 209, 231
Friguet, B., 298, 302, 303, 304
Frippiat, C., 55
Frisard, M. I., 30, 258, 453, 456
Frisoni, G. B., 356
Fritah, S., 300
Frizzell, K. M., 249, 256, 261
Froelich, S., 72, 224
Froguel, P., 121, 285
Frolich, K. U., 220
Frölich, M., 282
Frontera, W. R., 122
Frost, G., 468, 469
Frushour, B. P., 250, 251
Fry, D. L., 435
Frydman, J., 299, 300, 301
Frye, D. M., 392
Frye, R. A., 13, 224, 244, 254, 255, 256, 260, 261
Frys, K., 360, 413
Frystyk, J., 28
Fu, J. H., 352
Fu, M. H., 67, 69, 70
Fu, Q., 437
Fu, Y. C., 258
Fu, Z., 281
Fuchs, E., 143, 145
Fueger, B., 208
Fugere, N. A., 67, 302, 304
Fujii, K., 407
Fujioka, S., 122
Fujisaka, S., 251
Fujisaki, J., 151
Fujisawa, M., 165
Fujita, H., 49
Fujita, T., 277
Fujiyoshi, A., 455
Fukuchi, Y., 430, 439
Fukuda, M., 193
Fukuhara, A., 249
Fukumoto, K., 218, 302
Fukunaga, K., 72
Fukuto, J. M., 53
Fukuuchi, Y., 337
Fukuyama, H., 351
Fulda, S., 82
Fulle, S., 83
Fuller, C. D., 194, 490
Fuller, D. K., 433
Fulop, T., 149
Funahashi, T., 123, 125
Funakoshi, M., 191
Funatsu, M., 470

Fung, K., 374
Fung, Y. C., 427, 428, 429, 436, 438, 439
Funk, J. A., 250, 251
Furlanetto, R. W., 26
Furong, Z., 413
Furukawa, T., 70
Furuya, T., 308
Fuss, P. J., 453, 456, 457
Fuster, J. M., 338
Fuster, V., 358
Fuyama, Y., 221

G

Gabbert, H. E., 72
Gabel, S. A., 388
Gabriel, I., 169
Gabriely, I., 252
Gacayan, R., 186, 187
Gadbury, G. L., 324
Gaddy, D., 95
Gadek, J. E., 433
Gadella, B. M., 340
Gades, M. D., 34
Gaede, P., 374
Gaesser, G. A., 433
Gage, F. H., 145, 154, 155
Gage, T. B., 482
Gagne, D. J., 230, 248, 250, 251, 262, 263
Gagnerault, M. C., 33
Gagnon, M., 349, 360
Gajic, O., 434, 439
Galecki, A. T., 26, 28, 379, 380, 382
Galetke, W., 431
Galic, S., 285
Galkina, E., 281
Gallagher, D., 119, 123, 433
Gallardo, N., 124, 128
Galli, M., 260, 261
Gallina, S., 358
Gallo, C. M., 247, 249
Galloway, M. T., 360
Galluzzi, L., 68, 71
Galluzzi, S., 356
Galonek, H., 250, 254
Galonek, H. L., 250, 251
Gambardella, A., 28, 32
Gambini, J., 490
Gamble, M. J., 249, 256, 261
Gamboa, A., 349, 352
Gamboa, J., 349, 352
Gamboa, J. L., 64, 73, 74, 79, 83
Gamie, Z., 283
Gamst, A. C., 337
Gan, Q., 256
Ganassini, A., 439, 440
Gander, P. H., 468
Gandy, S., 262
Ganesh, V., 168, 169
Gano, L. B., 413
Ganz, E., 357
Gao, B., 261, 263
Gao, E., 95
Gao, L., 277
Gao, S., 99, 100, 258, 259
Gao, W., 92, 93, 99
Gao, X., 10
Gao, X. M., 221
Garantziotis, S., 97
Garber, G. B., 230, 250
Garcia, A. M., 34, 35, 195
Garcia, C. I., 338
Garcia, C. K., 163
Garcia, J. G. N., 406
Garcia, J. H., 351
Garcia, M., 355, 356
Garcia, R., 120
Garcia-Alloza, M., 407, 408
Garcia-Esteban, R., 286
García-Lorda, P., 285
Garcia-Martinez, C., 77
García-Río, F., 425
Garcia-Sala, D., 388, 489, 490, 491
Garcis-Palmer, F. J., 96
Garcis-Sala, D., 68
Gard, P. R., 360
Garde, E., 351
Gardin, J. F., 392
Gardin, J. M., 353
Gardiner, P. F., 95
Gardner, A. W., 468
Gardner, E. M., 33
Gardner, J. P., 125, 166, 167, 168, 169, 170
Gardner, P. R., 187
Gardner, R. M., 427, 437, 438
Gardy, J. L., 277
Garfinkel, D. J., 224
Garg, A., 120, 122
Garigan, D., 16, 221, 286, 300
Garinis, G. A., 195
Garofalo, R. S., 217, 224, 250, 251
Garry, D. J., 94
Garry, P. J., 119
Garsin, D. A., 16, 221, 286
Garthwaite, G., 408
Gasch, A. P., 219
Gasco, E., 70
Gasdaska, P. Y., 192
Gash, D. M., 3, 32
Gaskell, P. C., Jr., 340
Gast, D., 154
Gatbonton, T., 258
Gatfield, D., 262
Gattazzo, C., 220, 225, 247
Gatza, C., 150, 151
Gatzka, C. D., 387
Gaughan, J. P., 95
Gaurente, L., 246
Gaus, S. E., 473
Gautel, M., 306
Gauthier, M. S., 251
Gauthier-Fisher, A., 145
Gautini, D., 349
Gavilan, E., 301
Gavilan, M. P., 301
Gavrilova, O., 255
Gaziano, J. M., 178, 179
Gaziev, A. I., 179, 183, 195
Gazzaniga, F., 250
Ge, H., 12, 99, 100, 207, 397
Ge, X., 251
Ge, Y., 337
Geary, G. G., 410
Gebhardt, S. E., 185
Geddawy, A., 408
Gee, N. A., 390
Geiger, H., 152
Geleijnse, J. M., 359
Gelinas, C., 78
Gellon, L., 255, 258
Geloen, A., 29, 217, 224
Gems, D., 8, 205, 217, 222, 224, 229, 230, 251, 253
Genade, T., 16, 216, 230
Gendron, C. M., 12, 207, 216, 217
Genest, J., 282
Genius, J., 181, 185
Genova, M. L., 49, 410
Gens, D., 16
Gensch, E., 217, 221, 222
Gentina, S., 218
Genton, L., 122
George, A. E., 349
George, L., 285
George-Hyslop, P. H., 357
Georges, R., 427, 437, 438
Georgiou, D., 169
Gerber, S. A., 249, 250
Geresh, S., 122
Gerhardt, G. A., 3, 32
Gerhart-Hines, Z., 224, 230, 248, 251, 261, 263
Gerhold, T. D., 412
Gerich, J. E., 27
German, J. B., 360
Germinario, E., 95, 96
Geromel, V., 85, 95
Geronilla, K., 84, 85
Geronilla, K. B., 67, 70, 94
Gerrits, J., 483
Gershman, B., 217, 224
Gershon, D., 358
Gerstbrein, B., 221
Gerstenblith, G., 98
Gertler, A., 14
Gertsberg, Z., 99
Gertz, K., 408
Gertz, K. H., 438
Gertz, M., 254, 255
Gerzoff, R. B., 324

Geschwind, D., 337, 339, 340, 341
Geschwind, D. H., 339, 340, 341
Gesta, S., 129
Getachew, R., 261
Getchell, M. L., 411
Ghaeni, L., 256, 258
Ghalambor, C. K., 485
Ghalambor, D. E., 485
Ghelli, A., 50
Gherardi, R. K., 77
Ghezzo, H., 430
Ghislat, G., 297
Ghisletta, P., 339, 341
Ghosh, A., 406
Ghosh, A. P., 78
Ghosh, G., 68
Ghosh, K., 387, 389, 390, 395, 396, 397, 398
Ghosh, P. K., 26
Ghosh, S. P., 78
Ghoshal, S., 279
Ghribi, O., 31
Giaccia, A. J., 11
Giacconi, R., 300
Gianaldi, L., 328
Giannakis, C., 97
Giannakou, M. E., 13, 120, 217, 224, 229, 261
Giannogonas, P., 285
Giannoukos, G., 144
Gibbons, L. W., 421, 424
Gibbs, M., 27
Gibbs, R. A., 390, 448
Giblin, F. J., 191
Gibson, G. E., 282
Gibson, G. J., 429, 431, 436
Gibson, J. E., 193
Gibson, J. M., 128
Gicquel, E., 81
Gidalevitz, T., 299
Gidday, J. M., 358
Giedd, J. N., 334
Gielen, S., 96
Giesler, G. J., Jr., 465
Gietzer, O. W., 325
Gigante, A., 64
Gigot, D., 249
Giladi, N., 353
Gilardi, K. V., 390
Gilbert, J. R., 357
Gilbert, T. M., 473
Gilbey, M. P., 473
Giles, M. F., 405
Giles, W. H., 120
Gilhooly, C. H., 453, 454, 456, 457
Gill, R. G., 127, 128
Gill, S., 389
Gillespie, A. M., 186, 187, 190
Gillessen, S., 282
Gillette, J. A., 349
Gillick, K., 81
Gilligan, D. M., 349
Gilliland, F., 286
Gillon, W. S., 120, 126, 127, 128, 129
Gillooly, M., 428
Gilquin, B., 300
Gilson, J. C., 424
Gimbrone, M. A., Jr., 413
Gingeras, A. C., 204
Gingerich, R., 375
Gioia, D., 168
Giordano, G., 96
Giordano, S., 388
Giorgadze, N., 121, 125, 127, 128, 129
Giorgio, M., 69, 99
Giovannini, S., 64, 71, 74, 75, 76, 77, 78, 79, 80, 86, 90, 91, 96, 281, 284
Girgis-Gabardo, A., 147
Girma, E. R., 148, 149, 151, 154, 156
Girouard, H., 360, 405, 408, 410, 411, 413
Gitlin, M., 340
Giuliano, G., 49
Giunta, S., 281
Gladyshev, V. N., 192, 219
Glaser, R., 32
Glasser, A., 392
Glatt-Deeley, H., 13
Glaumann, H., 306
Glei, D. A., 278, 481
Gleiser, C. A., 3, 14, 203, 207, 222, 322, 325
Glickman, J. N., 120, 121, 125
Glickman, L. T., 26
Glindmeyer, H. W., 423, 429, 434, 435, 436
Globerson, A., 145, 150
Glotzer, L. D., 333, 334
Gnad, F., 254
Goas, J. A., 30
Gobbo, V., 95, 96
Gobel, C., 217
Goddell, M. A., 150, 151
Godefroy, O., 407
Goderie, S. K., 411
Godfrey, J. A., 408, 410, 412
Goehle, S., 258
Goetz, C. G., 360
Goetzman, E., 248, 256
Goff, B. L., 33
Gogg, S., 125
Goglia, F., 469
Gogtay, N., 334, 336
Goh, K. C., 250, 256, 258, 260
Gokce, N., 358
Gold, H. K., 283
Gold, P. E., 30
Goldberg, P. B., 392
Goldberg,A. L., 302, 304
Golden, J., 453, 454, 456, 457
Golden, J. K., 453, 454, 456, 457
Golden, J. W., 223
Goldfarb, D. S., 250
Goldfine, A. B., 122, 123, 469
Goldman, A. S., 472
Goldman, N., 278
Goldsmith, S., 280
Goldstein, D. E., 373, 374
Goldstein, D. S., 464, 466, 469, 470
Goldstein, J., 125, 167, 282
Goldwasser, E., 52
Goloen, A., 485
Golstein, D. B., 286
Gomez, C., 179, 182, 195
Gomez, C. R., 281
Gomez, D. M., 426, 427
Gomez, L. A., 68
Gomez, R., 121
Gomez-Cerezo, J., 325
Gómez-Mendieta, A., 425
Gomez-Reino, J., 121
Gompf, A., 147, 151, 170
Goncharova, N. D., 392
Gondry-Jouet, C., 407
Gong, K. W., 286
Gong, X., 302, 304
Goni, J., 355
Gonos, E. S., 55, 302, 303, 304
Gonyea, W. J., 65, 94
Gonzalez, A., 227
Gonzalez, C., 425, 426
González, H., 179, 182, 195
Gonzalez-Campoy, J. M., 122
Gonzalez-Reyes, A., 143
Gonzalvez, F., 70
Good, R. A., 32, 282
Good, T. P., 221
Goodall, A. H., 169
Goodall, J., 482
Goodell, M. A., 152
Goodgold, A., 349
Gooding, G. A., 32
Goodman, S., 285
Goodman, S. I., 191
Goodpaster, B. H., 77, 78, 119, 123
Goodrick, C. L., 13, 14, 182
Goodwin, D., 408
Goovaerts, I., 170
Gopinath, S. D., 145
Gora, F., 351
Goraksha-Hicks, P., 204
Goran, M. I., 468
Gordon, D., 122, 178, 179
Gordon, J. I., 12, 220
Gordon, M. N., 32, 282
Gordon, S. E., 65, 421, 424
Gordon, T. P., 448, 451, 452
Gore, A. C., 389
Gori, Z., 14

Gorlach, A., 124
Gorman, A. M., 66, 82, 83
Gorman, W., 349
Gorski, D. H., 357
Gosele, C., 407
Goss, M., 13, 120, 217, 224, 229
Gostout, B. S., 387
Gostynski, M., 386, 387
Goswami, A., 300
Goto, S., 127, 248, 249, 302, 412
Goto, T., 123
Gotoh, S., 407
Gottdiener, J., 323
Gottfried-Blackmore, A. C., 280
Gottlieb, E., 70
Gottlieb, G. J., 166, 223
Gottlieb, R. A., 72
Gottlieb, S., 10, 25, 222, 244
Gotto, A. M., Jr., 282
Gottschling, D. E., 220, 224, 244
Gottsdanker, R., 339
Gough, M., 277
Gould, K. G., 448, 451, 452
Goupil, S., 230, 250
Goustin, A. S., 258
Govaert, G., 220
Goyal, M., 34
Goymer, P., 205
Graakjaer, J., 170, 171
Graciarena, M., 277
Gradisar, M., 473
Grady, C. L., 349, 357
Grady, D., 32, 391
Graeber, R. C., 468
Grafnetter, D., 386, 387
Graham, L. S., 283
Graham, S., 283
Grahn, D., 470
Gralla, E. B., 218, 220
Grammas, P., 357
Grandison, R. C., 13, 15, 221
Granhall, C., 375
Granström, M., 195
Grant, B. F., 338
Grantham, J., 246
Grassi, F., 304
Grassi, V., 427, 437, 438, 439, 440
Gray, D. A., 304
Gray, R., 124
Gray, S. L., 323
Gray-Donald, K., 321
Grazette, L. P., 97
Grazia, T., 127, 128
Greaves, I. A., 429, 430
Greco, V., 300
Gredilla, R., 67
Greeley, E. H., 447
Green, A., 13
Green, B. G., 464
Green, D. R., 66, 78
Green, J. E., 34
Green, P. S., 285
Green, R. C., 357
Greenberg, A. S., 123, 124
Greenberg, S. M., 407, 408
Greenman, Y., 27
Greenstein, D., 334, 336
Greenwood, C., 324, 392
Greer, E. L., 3, 4, 5, 7, 9, 10, 11, 12, 15, 16, 17, 206, 208, 221, 229, 247
Greer, P. L., 224, 248, 255, 256, 261, 262
Gregerman, R. I., 127
Gregersen, N., 300
Gregg, D., 169
Gregg, E. W., 324
Gregory, L., 166
Greider, C. W., 165, 166
Greifenstein, F. E., 437
Greiner, L. H., 356
Greiner, P. A., 356
Gresl, T. A., 9, 449, 450, 451
Gress, R. E., 279
Grieco, M., 360
Griendling, K. K., 169, 413
Griffey, S. M., 325
Griffiths, H. R., 182, 183
Griffiths, R. D., 70
Grigioni, W. F., 428
Grillo, J., 32
Grimaldi, B., 262
Grimaldi, M. P., 387
Grimaldi, P., 120
Grimes, A., 146
Grimm, A. A., 248, 249, 252
Grimm, S., 69
Grimminger, F., 358
Grinberg, M., 81
Grindeland, R. E., 72, 78
Gringhuis, S. I., 407
Grisendi, S., 208
Grisson, S. F., 281
Grizzle, W. E., 256
Groban, L., 73, 74, 79, 80, 86, 87, 89, 90, 91, 95
Groebe, K., 298
Groeller, H., 421, 424, 429, 430, 431, 432, 433, 436, 439
Groeneweg, M., 279
Groh, S., 412
Grohe, C., 388
Groleau, J., 170
Groom, L., 67, 69
Gross, L., 35
Gross, M. D., 178
Gross, S. J., 359
Grossardt, B. R., 387
Grossin, N., 283
Grossman, R. I., 337, 355
Grove, J. S., 28, 156
Grubeck-Loebenstein, B., 149
Grubisha, O., 245
Gruenler, S., 96
Gruenwold, D. A., 325
Grundy, S. M., 120, 122, 123, 124
Grune, T., 54, 182, 298, 302, 304
Grünenfelder, J., 285
Grunfeld, C., 32
Grunnet, L. G., 382
Grunstein, M. M., 425
Grunwald, Z., 95
Gruss, P., 147
Grutter, M. G., 71
Gruver, A. L., 33, 279
Gryaznov, S. M., 165
Grynpas, M. D., 392
Grzywa-Celinska, A., 436
Gu, D., 484
Gu, M., 189
Gu, W., 224, 250, 255, 256, 261
Gu, Y., 72, 97
Gualillo, O., 121
Guan, K. L., 14, 204
Guan, Y., 412
Guarente, L., 4, 5, 7, 9, 10, 11, 12, 16, 28, 206, 217, 219, 220, 224, 225, 229, 230, 243, 244, 245, 246, 247, 248, 250, 252, 255, 258, 259, 260, 261, 447
Guarente, L. P., 252
Guariglia, R., 349
Gudiene, D., 406
Gudkova, O. A., 360
Guenard, H., 437
Guénot, F., 285
Guerci, A. D., 169
Guernah, I., 208
Guertin, D. A., 207
Gueydan, C., 260, 261
Guido, D., 94
Guinness, F. E., 486
Gulyas, B., 391
Gundersen, H. J., 336, 338
Gunes, T., 286
Gunnarsson, L., 438, 439
Gunnett, C. A., 413
Gunsalus, R. P., 51
Gunstad, J., 352
Guo, F., 278
Guo, H., 357
Guo, L., 68
Guo, S. S., 119, 122, 128
Guo, W., 122, 123, 124, 125, 128, 300
Guo, X., 258, 261
Guo, Y., 229
Guo, Z., 194, 282, 490
Gupta, D., 95
Gupta, G., 120
Gupta, M., 251, 259, 260, 282
Gupta, M. P., 251, 254, 259, 260, 282

Gupta, N., 78
Gupta, S., 82, 83
Gur, T., 357
Guralnik, J. M., 83, 323, 328, 353
Gurbuxani, S., 71
Gurevich, T., 353
Gurland, P., 391
Gurley, C. M., 64, 71, 95
Gurlo, T., 375, 377
Gurnani, P., 224
Gurol, M. E., 352
Gussekloo, J., 170, 171
Gustafson, B., 125
Gustafson, T. A., 127
Gustafsson, A. B., 72
Gustafsson, B., 67
Gustafsson, L., 220, 246
Guthrie, D., 13, 33, 35
Gutierrez, J., 168, 169
Gutierrez-Juarez, R., 225, 248, 251, 256
Gutsmann-Conrad, A., 300
Gutteridge, J. M., 54, 194
Guttmann, C. R., 353, 354
Gutviller, F., 386, 387
Guyton, A. C., 193, 465, 470, 471, 472, 473
Gwinn, D. M., 204
Gygi, S. P., 224, 247, 249, 250

H

Haan, J., 353
Haas, W., 224, 247
Habauzit, V., 283
Habener, J. F., 375
Haberl, R. L., 349, 352
Hachinski, V. C., 356
Hack, B., 406
Hacker, T. A., 14, 96, 99, 251
Hackett, S. F., 262
Haddad, P., 170
Haddy, T. B., 169
Hadfield, R. M., 392
Hadley, E. C., 323
Hadrup, S. R., 33
Haegebarth, A., 145, 153
Haendeler, J., 358
Haendl, T., 349, 352
Hafen, E., 8, 120, 204, 205, 217, 224, 229
Haff, G. G., 67, 70, 94
Haffa, A. L., 391
Haffner, S. M., 283
Haffor, A. S., 29
Hagberg, J. M., 434, 439
Hage, F. G., 277
Hagemann, D., 407
Hagen, J. L., 67, 69, 70
Hagen, T. M., 67, 68, 185
Hagen-Larsen, H., 488
Haggerty, C., 123
Hahn, W. C., 83
Hahne, M., 72
Haider, N., 97
Haigis, K. M., 244, 245, 254, 255
Haigis, M., 247
Haigis, M. C., 244, 245, 252, 254, 255, 263
Hailey, D. W., 306
Haine, S. E., 170
Haines, J. L., 389
Hajdu, I., 26
Hajdu, M. A., 349, 406, 412
Hajduk, C. L., 453, 456, 457
Hajjar, I., 349
Hajjar, R. J., 97
Hajos, M., 469
Hakim, A., 348
Hakim, Z. S., 53
Hakkinen, A. M., 123
Hal, A. S., 169
Halabi, C. M., 412
Halachmi, S., 257
Halavaara, J., 123
Halban, P. A., 375
Halcox, J. P., 169
Hald, J., 349
Halicka, D., 360
Hall, A., 167, 204
Hall, C., 353
Hall, C. B., 353, 354
Hall, D. A., 179, 182, 195
Hall, D. H., 226, 308
Hall, D. M., 300
Hall, J. E., 193, 389
Hall, M. A., 33
Hall, M. N., 120, 204, 205, 207, 226, 231
Hallfrisch, J., 321
Halliwell, B., 54, 185
Hallows, W. C., 245, 254
Halter, J. B., 127, 327, 373, 374, 375, 376, 379, 380, 381, 382
Hamadani, L., 16
Hamaguchi, I., 147, 152
Hämäläinen, M., 286
Hamann, A., 123
Hamasaki, A., 128
Hamazaki, J., 303
Hamberg, M., 194
Hamel, E., 408
Hamer, N. A., 427, 437
Hamida, C. B., 148
Hamilton, B., 217, 219, 220, 228
Hamilton, J. A., 121, 325
Hamilton, J. B., 490
Hamilton, J. S., 325
Hamilton, K., 92
Hamilton, M., 190
Hamilton, M. L., 191, 193, 194, 490
Hamilton, M. T., 421, 424
Hammarqvist, F., 68
Hammarstedt, A., 125
Hammer, R. E., 27, 34
Hammerstone, J. F., 359
Hammes, A., 407
Hamner, J., 349
Hamon-Vilcot, B., 324
Hamprecht, B., 53
Hamuro, J., 189, 219
Han, D., 54
Han, E. S., 194, 195
Han, H., 453, 456
Han, J., 122, 126, 127, 128, 129
Han, L., 256
Han, S., 259
Han, S. H., 357
Han, X., 128
Han, Y., 282
Han, Z. S., 204, 208
Hanby, J. P., 491
Hancock, R. E., 277
Handa, H., 406
Handwerker, H. O., 464
Handy, A., 449, 450
Handy, A. M., 450
Hankins, L. L., 30
Hanna, J., 142
Hannah, R., 411
Hannan, P., 436
Hano, T., 170
Hans, D., 119, 122, 123
Hans, J., 379
Hansen, B. C., 34, 127, 392, 449
Hansen, I. A., 204
Hansen, L. A., 336, 337
Hansen, L. F., 277
Hansen, M., 4, 7, 9, 12, 205, 206, 207, 208, 217, 219, 220, 226, 227, 228, 229, 230, 308
Hansen, R. D., 64
Hansford, R. G., 48, 49
Hanson, G. T., 69
Hansotia, T., 375
Hansson, A., 55
Hansson, G. K., 413
Hansson, L., 360
Hansson, O., 375
Hanze, J., 358
Hao, L. Y., 165
Hao, X., 246
Haour, F., 33
Haponik, E. F., 435, 440
Hara, K., 204
Hara, T., 306, 309
Harada, S., 218, 302
Haramizu, S., 72, 85, 100
Harashima, S., 227
Harchelroad, F. P., 466, 470
Hard, R., 97
Harder, D. R., 407
Hardie, D. G., 16

Author Index

Hardies, J., 337
Hardt, J. I., 186
Hardy, J., 356
Hardy, J. D., 466
Hardy, S., 127
Haring, H. U., 122
Harkins, J. M., 124
Harley, C. B., 151, 152, 166
Harman, D., 47, 67, 69, 70, 177, 489
Haromy, A., 358
Haroon, S., 353
Harper, J. M., 14, 26, 28, 205, 222, 247, 487
Harper, T., 13, 205, 206, 217, 218, 226
Harrigan, S. M., 341
Harris, A. G., 32
Harris, K., 97
Harris, M. E., 300, 301
Harris, M. I., 373, 374
Harris, N., 220
Harris, N. L., 220
Harris, S. E., 170, 171
Harris, T., 120, 321
Harris, T. B., 77, 78, 123, 124, 324, 360, 373, 374
Harrison, D. E., 14, 16, 26, 27, 30, 33, 34, 35, 120, 181, 186, 209, 218, 222, 224, 226, 309, 310, 381
Hars, E., 308
Harshman, L., 216
Harshman, L. G., 229
Hart, B. A., 337
Hartl, F. U., 298, 300
Hartman, P. S., 72
Hartvigsen, K., 277
Harvey, B. K., 32
Harvey, D., 349
Harvey, D. J., 349
Harvey, M., 257
Harvey, N. C., 283
Hasegawa, K., 250
Haselton, J., 31
Hasenstaub, A., 482, 487, 488
Hashikawa, T., 71
Hashimoto, N., 407
Hashizume, K., 195
Haskell, D., 449
Hasko, G., 52, 100
Hass, B. S., 322
Hass, R., 145
Hass, R. W., 322
Hassall, D. G., 412, 413
Hassan, A., 352
Hastings, R., 168, 169
Hasty, P., 195
Hata, M., 407
Hatake, K., 405, 410
Hatakeyama, S., 262
Hatano, M., 306
Hatano, Y., 410
Hatazawa, J., 351, 407
Hato, F., 33
Hattori, K., 55
Hatzer, M. W., 146
Hatzianthis, K., 220
Hatzis, P., 153
Hatzopoulos, A. K., 192
Hauck, S., 26, 29, 38
Hauck, S. J., 28, 29
Haug, H., 336, 337
Hauner, H., 124, 129
Hausdorff, J. M., 353
Havenith, G., 471, 472
Havighurst, T. C., 451
Hawke, T. J, 94
Hawker, G., 283
Hawkins, B. J., 52
Hawkins, M., 252, 285
Hawse, J. R., 304
Hawse, W. F., 245
Haxby, J. V., 349, 357
Hay, N., 226, 230
Hay, P., 324
Hayakawa, M., 55
Hayashi, K., 406
Hayashi, T., 248
Hayashida, K., 360
Hayden, R., 256
Hayer-Hartl, M. K., 298, 300
Hayflick, L., 165
Haynes, W. G., 349
Hays, N. P., 325
Haytowitz, D. B., 185
Hayward, C. S., 385, 387, 388
He, C., 305, 306
He, D., 282
He, J. C., 277, 285
He, L., 68, 229
He, M., 259
He, Q., 28, 156, 455
He, W., 251
He, X., 261
He, X. C., 151
He, X. H., 248, 251, 258
Heath, C., 324
Heath, D., 427
Heath, G. W., 439
Heber, D., 127, 128
Hedden, T., 339
Hedenstierna, G., 438, 439
Hedjazifar, S., 125
Hedrick, M., 129
Heeren, G., 250
Heeschen, C., 259, 358
Heft, M. W., 466
Hegazi, R. A., 123
Hegde, R., 71
Hegedus, V., 51
Heglind, M., 469
Heijdra, Y. F., 85
Heijmans, B. T., 28
Heikaus, S., 72
Heikkinen, S., 120, 412
Heilbronn, L. K., 30, 96, 247, 258, 259, 453, 456
Heilman, M. J., 285
Heiman, M. L., 26
Heindel, W., 351
Heiniger, H. J., 34
Heinrich, K. R., 180, 184, 185
Heisinger, P. R., 262
Heiss, C., 169
Heistad, D. D., 349, 406, 407, 408, 410, 411, 412, 413
Heitman, J., 204, 226
Heitmann, A., 468
Hejtmancik, J. F., 304
Hekimi, S., 4, 7, 9, 10, 12, 28, 207, 208, 218, 219, 228, 229
Hekking, B., 99, 247, 248, 255, 256, 259, 261, 262
Helbock, H. J., 194
Helder, M. N., 122
Helfand, S. L., 13, 16, 205, 217, 221, 224, 225, 229, 230, 246, 247, 250, 251
Heller, H. C., 470
Heller, R. C., 224
Hellerstein, M. K., 126, 287
Helliwell, S. B., 226
Heltweg, B., 217, 218, 230, 250, 251, 258
Heman, M. A., 387
Hemann, M. T., 165, 166
Hemberger, M., 144
Hemio, K., 374
Hemp, E., 466
Hen, R., 299
Hendel, H. W., 77, 78
Henderson, J. A. M., 433, 437
Henderson, S. T., 4, 7, 9, 16, 19, 208, 217, 218, 222, 286, 489
Henderson, V. W., 337, 389
Hengstler, J. G., 190, 193, 194
Henkelman, E. A., 84
Henkenius, A. L., 334, 337
Hennighausen, L., 34
Henrikssen, E., 387
Henriques-Pons, A., 82
Henriquez, N. V., 281
Henríquez-Roldán, C., 286
Henry, J. F., 389
Hensel, H., 464, 465, 466, 471, 472, 473
Hensley, K. C., 181, 186
Hensley, K. L., 209
Henson, D. A., 359
Hentges, K. E., 204
Heo, H. S., 96, 260
Hepple, R. T., 67, 69, 70, 74, 75, 76, 79, 302
Heras, M., 360

Herbert, A., 169
Herbert, J., 355
Herbert, K. E., 182, 183
Herbert, W. H., 427, 437
Herbst, A., 67
Herbst, L., 491
Hercus, M. J., 221, 224
Herholz, K., 351
Herker, E., 220
Herman, A. G., 281
Herman, T., 353
Herman, W. H., 375, 382
Hermanns, W., 34
Hermes, M., 407
Hernandez, C., 217
Hernandez, E. J., 325
Hernandez, F., 299
Hernandez, J. A., 427, 435
Hernandez-Diaz, S., 387
Hernanz, A., 325
Herndon, F. J., 33
Herndon, J. G., 391, 392
Herndon, L. A., 221
Herold, K., 277
Heron, M., 484
Herranz, D., 13, 248, 251, 256, 260, 261
Herrera, E., 166
Herrera, M. D., 410
Herrero, A., 42, 48, 55
Herrero, L., 120, 121, 125
Herrero, P., 120
Herrero-Beaumont, G., 484
Herritt, L., 286
Herrmann, A., 336
Herrmann, J. L., 277
Hershey, J. W. B., 205
Herskowitz, I., 224, 244
Hertog, M. G., 359
Hertweck, M., 11, 217
Herzog, H., 427, 437
Heshka, S., 119, 123
Hesselink, M. K., 127
Heston, L., 357
Hetzel, M. R., 424, 436
Hetzer, R., 388, 389
Heuser, J. E., 71
Hevener, A. L., 284, 285
Heydari, A. R., 300, 301
Heymsfield, S. B., 433
Hickner, R. C., 149
Hicks, J. B., 224, 244
Hickson, M., 327
Hida, Y., 256
Hidalgo, J., 64, 96
Hieber, K., 78
Higami, Y., 125
Higginbotham, M. B., 457
Higgins, E., Jr., 194
Higgins, I. T. T., 424
Higgins, M., 347, 435
Higgins, M. B., 387
Higuchi, M., 73, 83, 84
Hikasa, M., 256
Hikida, R. S., 65
Hildebrand, C., 338
Hilderbrandt, J., 428
Hill, A. M., 285
Hill, B., 33, 450
Hill, J. M., 30, 169
Hill, K., 482
Hill, K. H., 481
Hillier, L., 388
Hillyard, S. D., 472
Hilz, H., 245
Hilz, M. J., 349, 352
Himms-Hagen, J., 123
Hindhede, M., 452
Hine, C., 167
Hines, M. W., 392
Hinman, J. D., 338, 340
Hinnebusch, A. G., 204, 209, 227
Hiona, A., 47, 55, 69, 70, 74, 92, 95, 96, 98, 489, 491
Hirabayashi, Y., 189, 219
Hirai, H., 84, 85
Hirai, T., 428
Hirano, M., 193
Hirao, A., 147, 152
Hiraoka, M., 195
Hiratsuka, M., 245, 258
Hirayama, J., 262
Hirokawa, K., 32
Hirota, H., 97
Hirsch, C., 301, 323
Hirsch, C. H., 323
Hirsch, T., 51
Hirschey, M. D., 248, 256
Hirschfield, S. L., 360
Hirshman, C. A., 425
Hirst, J., 48, 49
Hisahara, S., 248
Hishida, S., 405, 410
Hislop, A. D., 306
Hitchcock, C., 439
Hixon, J., 217, 218, 230, 250, 251
Hixon, M., 248, 251
Hjelmborg, J. B., 77, 78, 166, 170, 287
Hjelmborg, J. V., 170
Ho, B. C., 337
Ho, C. W., 471, 472
Ho, D. S., 191
Ho, K. H., 299
Ho, L., 3, 262, 282
Ho, M., 99
Ho, S. C., 324
Ho, Y. S., 191, 195
Hoang, K., 4, 5, 7, 9, 10, 206, 208, 229
Hoberg, J. E., 256, 260
Hobi, H. J., 437
Hochmuth, C. E., 154
Hochshild, R., 305
Hochstrasser, M., 302
Hock, C. E., 92, 93, 98
Hodgson, J. M., 349
Hoefnagels, W. H. L., 326
Hoeijmakers, J., 147, 151, 152, 195
Hoeijmakers, J. H., 195
Hoeijmakers, W. A., 146, 281
Hoek, J. B., 53
Hoenel, B., 302, 304
Hoerstrup, S. P., 285
Hoetzer, G. L., 168, 169, 170
Hof, P. R., 356
Hofer, T., 68, 453, 456, 489, 491
Hoffman, E. K., 190
Hoffman, H. J., 183
Hoffman, T. A., 259
Hoffmann, A., 68, 78
Hoffmann, J., 281, 358
Hoffmann, K., 123
Hoffmann, R., 484
Hoffstedt, J., 120, 121
Hofker, M. H., 279
Hofman, A., 244, 253, 341, 349, 351, 352, 356, 357, 359, 360
Hofmann, K., 72
Hofmann, W. K., 259
Hogan, D. B., 323
Hogh, P., 351
Hogikyan, R. V., 382
Hogue, B. A., 48, 49
Hohfeld, J., 300
Hokari, S., 412
Holbrook, M., 358
Holbrook, N. J., 300, 302
Holden, J. M., 185
Holder, J. C., 139
Holland, A. N., 224
Holland, C. M., 352
Holland, J., 433, 434, 435, 436, 437
Hollander, J. M., 64
Hollander, W., 338, 340
Holle, E., 99, 100, 258, 259
Hollenbach, S., 190, 193
Hollenberg, C. H., 120, 124, 126, 127, 128, 129
Hollenberg, N. K., 350, 360
Holliday, R., 203, 222
Hollman, P. C., 359
Holloszy, J. G., 321
Holloszy, J. O., 125, 164, 179, 183, 424, 433, 434, 435, 436, 437, 439, 453, 454, 456, 457
Holloway, L., 32
Holly, J. M., 78
Holly, T. A., 97
Holm, C., 26
Holm, P., 180, 184
Holman, B. L., 357
Holmen, J., 324

Author Index

Holmes, S. G., 244
Holston, K. A., 389
Holtz, J., 96
Holtzman, J. L., 301
Holzenberger, M., 29, 217, 224, 485
Hombach-Klonisch, S., 143
Honda, K., 68, 464
Honda, S., 16
Hong, K. M., 127, 128
Hong, Z., 352
Honig, L. S., 170, 171
Honigbaum, S., 340
Honjoh, S., 4, 5, 7, 9, 10, 11, 206, 208
Honke, K., 340
Hood, D. A., 64, 67, 68, 85, 92, 95, 96, 98, 99, 100
Hood, L., 206
Hoogenraad, N. J., 84
Hoozemans, J. J., 299, 301
Hope, G. M., 392
Hopkins, S. R., 437
Hoppel, C. L., 70
Hopper, B. R., 389
Hori, S., 70
Horibe, T., 84
Horio, Y., 248
Horiuchi, S., 481
Horiuchi, T., 483
Horner, J. W., 152
Horner, J. W. II, 166
Hornig, B., 349
Hornsby, P. J., 146
Horovitz, Z. P., 360
Horowitz, M., 325
Horoz, M., 121
Horsefield, R., 51
Horst, M., 306
Horton, E. S., 468
Horton, J. D., 122
Horvath, T. L., 251, 469
Horvitz, H. R., 64, 225
Horwich, A., 299
Horwitz, B. A., 123, 325, 468, 470
Horwitz, J., 304
Hoshi, H., 357
Hoshide, S., 360
Hosier, S., 13
Hosoda, T., 99
Hosokawa, M., 428
Hosono, R., 4, 5, 7, 302
Hosono, T., 473
Hossmann, K. A., 407
Hosya, H., 325
Hotamisligil, G. S., 123, 124, 125
Hoth, K. F., 352
Hotta, K., 127
Hou, X., 16, 248, 251
Houser, S., 95
Housset, B., 437, 438
Houten, S. M., 230, 251, 252, 258
Houthoofd, K., 4, 5, 7, 9, 10, 16, 17, 208, 228, 230, 251, 253
Howald, L., 226
Howard, A., 425
Howard, C. F., Jr., 392
Howell, K. S., 218
Howitz, K. T., 16, 230, 247, 250, 251, 258
Hoyer, P. B., 389
Hoyert, D. L., 484
Hoymans, V. Y., 95, 96
Hron, J. D., 249, 261
Hsieh, C. C., 34
Hsieh, E. A., 287
Hsieh, J., 31
Hsin, H., 220, 222, 223, 228
Hsu, A. L., 4, 7, 11, 16, 219, 220, 221, 222, 223, 228, 286, 300
Hsu, C. P., 258, 259
Hsu, D., 12
Hsu, F. F., 120
Hsu, H. C., 33
Hsu, H. K., 473
Hsu, Y. T., 71
Hu, A., 95
Hu, C., 308
Hu, D., 4, 5, 7, 9, 10, 12, 55, 56, 187, 188, 206, 207, 217, 218, 219, 220, 226, 227, 228, 229, 230, 247
Hu, J., 12, 207
Hu, P., 245
Hu, W., 306
Hua, N., 27
Huang, C., 33, 78, 221
Huang, D., 337, 339, 340
Huang, F. F., 302, 304
Huang, H. Y., 183, 298
Huang, J., 245, 256
Huang, J. H., 68, 70
Huang, K., 428, 433
Huang, L., 51
Huang, M. E., 53
Huang, S. Y., 127
Huang, T. T., 186, 187, 190, 191, 193, 194, 195
Huang, Y., 282, 287, 328, 340
Huang, Y. Y., 64
Huang, Z., 70, 451
Hubbard, A., 207
Hubbard, G. B., 34
Hube, F., 120
Huber, A., 12, 204, 205, 207, 217, 227
Huber, C., 490
Huber, M., 302, 304
Hubert, H. B., 484
Huck, J., 450
Huckell, C. B., 392
Hudis, C., 97
Hudson, D. B., 222
Hudson, D. J., 185
Hudson, J. C., 392, 451
Hudson, L. L., 33, 279
Huebner, R. J., 34
Huffman, D. M., 130, 256
Hughes, B., 218
Hughes, C., 68
Hughes, C. L., 391
Hughes, L. F., 27, 28
Hughes, M., 338
Hughes, S. M., 65, 84
Hughes, V. A., 122, 323
Huh, A., 4, 7, 11
Huhtala, H., 180, 184
Hui, A. S., 208
Hulbert, A. J., 168
Hulette, C., 357
Hulette, C. M., 357
Hull, C. J., 27
Hull, R. L., 125, 376, 379
Hulley, S. B., 169
Hulmes, D. J., 428
Hulo, N., 12
Hultenby, K., 55
Hulver, M. H., 96, 247, 259, 453, 456
Humala, N., 3, 282
Human Mortality Database, 481, 482, 483
Humke, E. W., 71
Hummel, S., 323
Humphrey, J., 357
Humphreys, S. M., 27
Humphries, A., 145
Humphries, K. M., 304
Humphries, S. E., 286
Hung, L. W., 51
Hunkin, J. L., 169
Hunt, B. J., 352
Hunt, K., 484
Hunt, N. D., 453, 456
Hunt, S. C., 168, 169
Hunter, R. B., 83
Hunter, S. A., 411
Hunter, T., 4, 5, 7, 10
Huntly, B. J., 152
Hurley, R. L., 205
Hurst, D. N., 424
Hurst, T. L., 77
Hurtado, A. M., 481
Hurtig, H. I., 349
Husain, S., 349
Huska, M. R., 151
Husom, A. D., 302, 304
Hussain, S. G., 301
Hussein, K., 307
Hutchins, P. M., 31
Hutchinson, J., 423, 434, 435
Huttenlocher, P. R., 334, 339
Hutter, E., 55
Huttermann, M., 68
Huuskonen, J., 260

Huycke, M. M., 192
Huyghe, M., 153
Hwang, D., 28, 164, 285
Hwang, J. C., 64, 70, 78
Hwang, J. S., 302, 304
Hwang, J. T., 16
Hwang, J. Y., 11
Hwangbo, D. S., 217, 224
Hyatt, R. E., 424, 433, 435
Hyde, D. E., 471, 472
Hyman, B., 277
Hyman, B. T., 336, 337
Hyun, D. H., 125

I

Iachine, L., 287
Iadecola, C., 405, 407, 408, 410, 411, 413
Ibaraki, M., 407
Ibayashi, S., 407
Ibrado, A. M., 69
Ichimaru, S., 122, 123
Ichisaka, T., 204
Ido, Y., 251
Iemitsu, M., 358
Igarashi, H., 122, 193
Igel, M., 120
Iggo, A., 466
Ihara, M., 351
Ihling, C., 358
Ihm, S. H., 379
Iida, M., 279, 281
Iijima, K., 68, 256
Ikeda, M., 180, 182, 184
Ikeda, Y., 412
Ikeno, Y., 27, 28, 31, 34, 182, 184, 187, 189, 190, 191, 192, 196, 282, 324, 326, 327, 448, 489, 490, 491
Ikeya, T., 224
Ikeyama, S., 302
Ikram, M. A., 341
Ikushima, M., 414
Illouz, K., 349
Iloputaife, I., 349, 360
Ilynsky, O. B., 464
Imada, K., 32
Imahiyerobo, T. A., 255, 256, 261, 262
Imai, H., 191
Imai, M., 414
Imai, S., 13, 99, 224, 245, 248, 249, 254, 255, 257, 258
Imai, S. I., 224, 255, 256, 261
Imaizumi, M., 351
Imamura, R., 470
Imamura, T., 251, 258, 260, 408
Imamura, Y., 195
Imanishi, T., 170
Imatani, A., 68
Imboden, H., 360
Imlay, J. A., 51
Inagaki, N., 254
Inayoshi, M., 185
Ince, P., 356
Incefy, G. S., 33
Inda, M. C., 32
Ingelsson, M., 277
Inglese, M., 355
Ingold, K. U., 178
Ingraham, J., 407
Ingram, D. K., 4, 5, 7, 9, 10, 13, 27, 28, 32, 34, 125, 154, 221, 228, 229, 389, 390, 392, 449, 450, 453, 456
Ingram, R. L., 26, 30, 31, 34, 457
Ingram, V. M., 164
Ingram-Drake, L., 388
Inohara, N., 72
Inoki, K., 14, 204
Inoue, I., 412
Inoue, K., 305
Inoue, M., 168
Inoue, R., 184
Inoue, S., 180, 182, 184
Inoue, T., 245, 258
Inoue, W., 277
Inoue, Y., 471, 472
Inserra, F., 413
Inzitari, D., 355
Iorio, A. M., 149
Iorio, E. L., 283
Iotti, R., 34
Iqbal, S., 68
Irie, K., 359
Irmiger, H. M., 168, 169, 170
Irmler, M., 72, 181, 185
Irrcher, I., 64
Irvine, E., 14, 120, 208, 209, 217, 381
Irving, W. L., 285
Irwin, I., 388
Isaka, Y., 351
Isbatan, A., 260
Isberg, R. R., 277
Isensee, J., 388, 389
Iser, W. B., 4, 7, 9, 229
Ishibashi, J., 84, 85
Ishida, T., 33
Ishii, N., 16, 72
Ishikawa, F., 259
Ishikawa, J., 360
Ishikawa, K., 414
Ishikawa, Y., 170, 392
Ishiwata, T., 97
Ishizaki-Koizumi, S., 189, 219
Islam, A., 65
Islam, M. N., 464
Islam, M. S., 433
Israelian, K., 250, 254
Issac, B., 144
Issuree, P. D., 287
Isvaran, K., 486
Itabashi, K., 483
Itahana, K., 167
Itakura, E., 305
Itani, Y., 483
Ito, H., 407
Ito, K., 147, 152, 435
Ito, M., 251, 261
Ito, T., 71, 125, 227, 285, 360
Itoh, M., 128, 351
Itoh, T., 170
Itokawa, Y., 70
Itzhaki, R. F., 285
IUPS Thermal Commission, 469
Ivanishina, N. S., 360
Ives, D. G., 353
Ivessa, A., 99, 100
Ivy, J. M., 224, 244
Iwahara, N., 248
Iwahashi, S., 410
Iwaki, K., 180, 182, 184
Iwakoshi, N. N., 209
Iwakuma, T., 193
Iwama, A., 147
Iwasaki, K., 3, 14, 203, 207, 222
Iwata, J., 306, 309
Iwatsubo, T., 299
Iyer, N. V., 358
Izyumov, D. S., 68

J

Jablonski, K. L., 413
Jacinto, E., 204
Jack, C. R., Jr., 341
Jacks, T., 257
Jackson, J. R., 73, 74, 77, 78, 79, 80, 81
Jackson, M., 302
Jackson, M. D., 245, 249
Jackson, M. J., 70
Jackson, S. E., 277
Jackson, S. J., 27
Jackson, S. P., 146
Jackson Roberts, L., 191, 195
Jacob, A., 406
Jacob, G., 349
Jacob, M.-P., 413
Jacobs, D., 391
Jacobs, D. R., 436
Jacobs, H. T., 13
Jacobs, I., 469
Jacobsen, R., 483
Jacobson, J., 224
Jacobson, K. M., 353
Jacobson, L. A., 4, 5, 7, 10
Jaffe, D. B., 144
Jagger, C., 484
Jagtop, P., 411
Jagust, W. J., 357
Jahngen, E. G., Jr., 304

Jahngen, J. H., 304
Jakab, M., 354
Jakobson, H., 195
Jakupoglu, C., 192
Jamali, F., 360
James, A. M., 184
James, M. L., 285
James, M. W., 285
Jamieson, H. A., 13, 230, 248, 250, 251, 259
Jammes, H., 33
Jan, Y. N., 206
Jana, N. R., 300
Jang, M., 257
Jang, Y., 193
Jang, Y. C., 56, 67, 187, 188, 195
Jang, Y. H., 359
Jang, Y. M., 491
Janowsky, J. S., 349
Jansen, A. S., 473
Jansen, G., 219
Jansky, L., 469
Janson, J., 373, 379
Janssen, B. J., 388
Janssens, J. P., 427, 438, 439, 440
Jantz, T. D., 394
Jantzen, P., 360
Japan Ministry of Health, Labor, and Welfare, 455
Jaquet, M., 231
Jaras, M., 151
Jardine, A. G., 360
Jardine, K., 248, 251, 258, 261
Jarolim, S., 250
Jarosch, E., 301
Jaruga, E., 12, 206, 220, 229
Jarvis, W. D., 81
Jarzebowicz, A. A., 151
Jasper, H., 154
Jaworski, M., 147, 151, 170
Jayakumar, P., 261
Jayasankar, V., 97
Jayashankar, L., 392
Jazwinski, S. M., 12, 30, 206, 216, 217, 218, 220, 229, 300
Jeanclos, E., 168
Jeang, K. T., 192
Jeffery, S., 352
Jeffrey, P. D., 72, 81
Jeffries, N. O., 334
Jeganathan, K., 125
Jeisy-Scott, V., 277
Jejurikar, S. S., 84, 95
Jemmerson, R., 69
Jenal, U., 216
Jenkins, D. P., 359
Jenkins, G. M., 70
Jenkins, N. A., 85
Jenkins, N. L., 222
Jenndahl, L., 125
Jenner, P., 302
Jenny, N. S., 168
Jensen, M., 122
Jensen, P. B., 120
Jensen, R. A., 29, 30, 452
Jensen, T. S., 466
Jensen, U. B., 300
Jeon, H., 359
Jeong, W., 191
Jernigan, A., 190, 193, 194
Jernigan, T. L., 334, 337
Jerome, C. P., 392
Jerrett, M., 286
Jerschow, E., 124
Jesina, P., 51
Jessen, C., 466
Jeune, B., 77, 78, 488
Jeyapalan, J. C., 125
Jezova, M., 360
Jhala, U. S., 252
Ji, G. J., 388
Ji, H., 259
Ji, L. L., 68
Jia, K., 4, 7, 12, 204, 206, 207, 208, 226, 230, 302, 308
Jian, W. X., 119
Jiang, B., 16, 170, 251
Jiang, B. H., 358
Jiang, D., 29
Jiang, H., 95, 147, 151, 170, 408, 411
Jiang, J., 70, 218
Jiang, J. C., 12, 206, 220, 229, 300
Jiang, L., 281
Jiang, L.-Q., 413
Jiang, M., 255, 256
Jiang, N., 218
Jiang, S., 71
Jiang, X., 71
Jiang J., 70
Jiao, X., 95
Jilma, B., 169
Jimenez, R., 96, 125, 260, 261, 287
Jimenez, S., 301
Jimenez, W., 360
Jin, D., 208
Jin, F., 125
Jin, H., 72
Jin, L., 254, 411
Jin, S., 282, 308
Jing, E., 248, 256
Jinnouchi, S., 357
Jirousek, M., 263
Jo, D. G., 125
Jo, M., 168
Joaquin, M., 120, 205, 209, 231
Jobe, H., 73, 74, 79, 80, 86, 87, 89, 90, 91, 95
Johannsen, B. N., 470
Johannsson, G., 30
Johansson, B., 33, 77, 170
Johns, R. A., 358
Johnson, A. W., 413
Johnson, B. D., 421, 424, 432, 433, 436, 439, 440
Johnson, C. R., 81
Johnson, E., 449
Johnson, F. B., 220
Johnson, G., 355
Johnson, H. R., 391
Johnson, J. K., 324
Johnson, J. M., 470, 471
Johnson, K. A., 357
Johnson, L. R., 424
Johnson, R. W., 32, 280
Johnson, S. A., 280
Johnson, S. C., 32, 164, 216, 381, 392, 451
Johnson, T., 222
Johnson, T. E., 4, 5, 7, 9, 10, 11, 16, 19, 30, 207, 208, 217, 218, 220, 222, 228, 284, 300, 489
Johnson, T. J., 286
Johnson, W. A., 35
Johnston, J. R., 216
Joiner, M. L., 219
Jokinen, E., 286
Jokubka, R., 375
Jolette, J., 391
Jonason, T., 387
Jones, D. P., 56
Jones, D. R., 256, 260
Jones, G., 283
Jones, H. C., 406
Jones, J. I., 30
Jones, K. M., 95
Jones, N. L., 439
Jones, P. B., 407, 408
Jones, P. H., 145
Jones, R. N., 349, 423, 429, 434, 435, 436
Jones, T. T., 194
Jonker, C., 32
Jonsdottir, I., 378
Jonsson, J. I., 145, 152
Jonsson, P. V., 355, 356
Joo, S. H., 357
Joost, H. G., 120
Jorgensen, T., 77, 78
Joseph, A. M., 64, 68
Joun, N. S., 178, 179, 183
Jousilahti, P., 491
Joutel, A., 407
Joyner, M. J., 85
Joza, N., 71
Ju, Z., 147, 151, 170
Jubrias, S. A., 67, 68, 94
Jucker, M., 154
Juhan-Vague, I., 278
Juhasz, B., 259
Juhasz, G., 230
Juin, P., 71
Julian, D., 308, 491
Jung, K. J., 96, 260, 280, 358

Jung, S. B., 259
Jung, S. Y., 250, 255, 256
Jung, T., 54, 182
Junger, M. A., 120, 217, 224
Jung-Hynes, B., 253
Jungreis, C. A., 353
Jungreis, R., 82
Jungwirth, H., 220
Junn, E., 299
Junzhu, C., 413
JUPITER Trial Study Group, 282
Jurd, K. M., 352
Jurgensmeier, J. M., 71
Just, H., 349, 405, 410

K

Kaarniranta, K., 260
Kabe, K., 483
Kablar, B., 84, 85
Kabra, N., 248
Kachholz, B., 254, 255
Kaczor, J. J., 67, 94
Kadenbach, B., 68, 69
Kadi, F., 148
Kaeberlein, M., 4, 5, 7, 9, 10, 12, 206, 207, 208, 216, 217, 218, 220, 222, 224, 225, 226, 227, 228, 229, 230, 231, 245, 246, 247, 250, 251
Kaeberlein, T. L., 4, 15, 217, 218, 219, 221, 228, 229
Kaehlcke, K., 261
Kaes, T., 333, 336, 337, 339
Kagan, V. E., 70
Kagawa, Y., 452, 455
Kahl, J., 55, 68
Kahn, B. B., 27, 29, 120, 127, 217, 224
Kahn, C. R., 27, 29, 120, 217, 224
Kahn, S. E., 376, 379
Kai, T., 144
Kail, R., 338, 339
Kaiser, M., 485
Kaiser, S. M., 283
Kaitosaari, T., 286
Kaiyala, K. J., 124
Kaji, M., 466, 470
Kaji, Y., 466, 470
Kajstura, J., 96, 97, 98
Kakafika, A. I., 283
Kakarla, S. K., 92
Kakishita, E., 405, 410
Kakiuchi, T., 408
Kakuma, T., 121, 122
Kalaany, N. Y., 14, 30, 35
Kalant, N., 32
Kalaria, R. N., 356
Kale, S. P., 218
Kaley, G., 358, 410, 411, 413
Kalhan, S. C., 123
Kalinina, E. V., 53
Kalka, C., 169
Kallio, K., 286
Kalnin, A., 349
Kalow, W., 359
Kalra, A., 13, 230, 248, 250, 251, 259
Kalra, S. P., 99
Kalu, D. N., 424
Kaluzova, M., 248, 249, 253, 262
Kalyanasundaram, A., 82
Kamath, R. S., 16, 155, 220, 221, 228, 286, 300
Kamath, T. V., 258
Kamei, H., 28
Kamel, C., 224, 261
Kamidono, S., 165
Kamijo, T., 147
Kaminski, M., 483
Kaminski, P. M., 259, 260, 261
Kamitani, H., 258
Kamper, U., 169
Kampert, J. B., 421, 424
Kamzalov, S., 180, 184, 185
Kanaganayagam, G. S., 170
Kanai, S., 325
Kanapuru, B., 323, 328
Kanatani, Y., 251
Kandarian, S. C., 83
Kane, G. C., 427
Kaneko, K., 433, 437
Kang, J. G., 255
Kang, M. J., 358
Kang, M. R., 250
Kang, P. M., 72
Kang, S. H., 255
Kang, S. S., 168
Kang, S. W., 191
Kangro, T., 387
Kanim, L. E., 179, 183
Kannel, W. B., 347
Kannisto, V., 480, 481
Kanno, I., 407
Kanosue, K., 473
Kanters, E., 279
Kantorow, M., 304
Kao, T. C., 78
Kapahi, P., 13, 203, 205, 206, 207, 208, 209, 217, 218, 220, 226, 230, 381
Kapetanios, K. J., 360
Kaplan, G. A., 484
Kaplan, J. R., 389, 391, 392
Kaplan, R. C., 353
Kaplun, L., 249
Kappagoda, C. T., 360
Kappelle, L. J., 352
Kapphahn, R. J., 302
Kapral, M. K., 374
Kapralov, O., 70
Kaptoge, S., 278
Karagiannides, I., 121, 122, 124, 126, 127, 128, 129
Karagiannis, A., 283
Karalis, K. P., 285
Karanth, N., 411
Karanth, S., 471
Karas, R. H., 387, 388
Karasik, D., 484
Kari, F. W., 35
Karim, B., 166
Karim, M., 360
Karin, M., 78
Kario, K., 360
Karsegard, L., 122
Karunananthan, S., 323
Karuppagounder, S. S., 282
Karwowska-Polecka, W., 360
Kasahara, E., 195
Kashon, M. L., 94
Kashyap, A., 250
Kass, D. A., 98
Kassiri, Z., 71
Kaste, M., 356
Kastelein, J. J., 282
Kastman, E. K., 32, 164, 216, 381, 392, 451
Kastrup, J., 466
Kasuya, T., 485
Kaszubowska, L., 146
Katagishi, T., 168
Katan, M. B., 359
Katayama, K., 421, 433, 438, 439
Katewa, S. D., 13, 205, 206
Kato, B. S., 166, 169, 170
Kato, K., 68
Kato, T., 194
Katsanos, C. S., 122, 123
Katsiaras, A., 123
Katsuta, H., 128
Katta, A., 92
Kattel, R., 216
Katz, M. J., 353
Katz, M. S., 324, 326, 327, 353, 448
Katze, M. G., 390, 448
Katzman-Rooks, S. M., 468
Kaufman, P. L., 392, 448, 450, 451, 489
Kaufmann, S. H., 71
Kauhanen, S., 64
Kaul, S., 412
Kauppinen, A., 260
Kauser, K., 146
Kaushik, S., 306, 307, 309
Kavanagh, K., 390, 391
Kavazis, A. N., 67
Kawachi, H., 470
Kawahara, T. L., 260
Kawai, T., 283
Kawakishi, S., 185
Kawamura, J., 351

Kawasaki, N., 479
Kawase, M., 194
Kayali, R., 298
Kaye, J. A., 349
Kayo, T., 14, 96, 99, 251, 301
Kazama, K., 360, 413
Kealy, R. D., 447
Keaney, J. F., Jr., 358
Kearney, M., 358
Kearns, J. D., 68, 78
Keck, F. S., 464
Keefe, D. L., 97, 166
Keen, H. L., 412
Kehlenbrink, S., 285
Kei, T., 128
Keijser, J. N., 357
Keith, R. S., 168, 169, 170
Kekonius, L., 411
Keller, B. K., 326
Keller, C., 65, 149, 151, 154
Keller, J. N., 302, 304
Keller, M. D., 256, 260
Kelley, D. E., 123
Kelley, K. W., 32
Kelliher, M., 304
Kellogg, D. L., Jr., 470, 471
Kellokumpu-Lehtinen, P., 483
Kelly, J. W., 299
Kelly, K. L., 121
Kelly, M., 251
Kelly, R. P., 385, 387, 388
Kelm, M., 169
Kelsen, S. G., 425, 430, 433
Kemerink, G. J., 469
Kemnitz, J. W., 9, 388, 389, 391, 392, 448, 449, 450, 451, 489
Kemp, K., 77, 78
Kemper, C., 280
Kemper, T., 333, 334, 336, 337, 338, 339
Kemper, T. L., 336, 337, 391
Kempermann, G., 154
Kempf, T., 72
Kendaiah, S., 491
Kendziorski, C. M., 193
Kengeri, S. S., 491
Kenna, M. A., 224
Kennaway, D. J., 472
Kennedy, B. K., 4, 12, 15, 207, 216, 217, 218, 219, 220, 221, 222, 225, 226, 227, 229, 230, 231, 247
Kennedy, C. C., 283
Kennedy, K. M., 341
Kennedy, M. A., 29
Kennedy, T. C., 429, 430, 433, 436
Kenney, L. W., 471, 472
Kenney, R. D., 250, 251
Kenney, W. L., 468, 471, 472
Kenny, R. A., 356
Keno, Y., 122
Kenshalo, D. R., 464, 466
Kenyon, C., 4, 7, 9, 10, 11, 12, 16, 25, 28, 205, 206, 207, 208, 217, 219, 220, 221, 222, 226, 227, 228, 229, 230, 243, 286, 300, 308
Kerber, K. A., 353
Kerber, R. A., 169, 170, 171
Kerkhof, G. A., 468
Kerr, C. W., 97
Kerr, E. O., 4, 5, 7, 9, 10, 12, 206, 207, 217, 218, 219, 220, 226, 227, 228, 229, 230, 247
Kerr, J. F., 64
Kerr, K. S., 262
Kerschan-Schindl, K., 387
Kerscher, S., 48
Kessler, B., 99, 247, 248, 255, 259, 262
Kessler, M. J., 392
Kessler, R. C., 338
Ketsawatsomkron , P., 412
Kewitt, K., 27
Keylock, K. T., 285
Keymel, S., 169
Keys, A., 436
Khachaturian, A. S., 340
Khan, A., 31
Khan, R., 407
Khan, Y., 333, 334, 337
Khanim, F. L., 256
Khaodhiar, L., 285
Kharazi, S., 151
Khazaeli, A. A., 218
Khazan, B., 281
Kho, J. H., 250
Khodr, C., 29
Khokhlova, O. N., 285
Khosla, S., 122, 127
Khritankov, A. M., 486
Khvorostov, I., 55
Kiauta, T., 349, 352
Kiecolt-Glaser, J. K., 32
Kiefer, T. L., 450
Kiel, M. J., 145, 147
Kiely, D. K., 349, 360
Kiess, W., 30
Kiffin, R., 307, 309
Kihara, S., 123, 125
Kikinis, R., 354
Kikugawa, K., 194
Killampali, V., 281
Killiany, R. J., 338, 340, 352, 354, 391
Kilo, J., 411
Kim, A. R., 359
Kim, C. N., 69
Kim, C. S., 259
Kim, D., 251, 262, 359
Kim, D. H., 16, 221, 286, 358
Kim, D. K., 285
Kim, E., 204
Kim, E. J., 250
Kim, E. M., 97
Kim, F. S., 151, 156
Kim, G., 190, 193, 260
Kim, H., 359
Kim, H. J., 358
Kim, H. K., 358
Kim, H. M., 16
Kim, H. S., 248, 251, 254, 256, 257, 258
Kim, H. Y., 219
Kim, I., 155
Kim, I. S., 359
Kim, J. E., 250, 255, 256
Kim, J. H., 96, 125, 285
Kim, J. J., 224
Kim, J. K., 130, 276, 286
Kim, J. S., 68, 94, 256, 301
Kim, J. W., 285
Kim, J. Y., 96, 154, 260
Kim, K., 280, 284
Kim, K. D., 12, 191, 206, 207, 247
Kim, K. K., 51
Kim, P. K., 306
Kim, S., 217, 220, 302, 304
Kim, S. B., 13, 205
Kim, S. H., 190, 191, 224
Kim, S. J., 396
Kim, S. K., 4, 5, 7, 10, 16, 224, 230, 250, 261
Kim, S. U., 190, 191
Kim, W. H., 195
Kim, Y., 218
Kim, Y. J., 359
Kim, Y. M., 16, 299
Kimata, K., 34
Kimble, J., 10, 168
Kim-Han, J. S., 186
Kimura, H., 49
Kimura, J., 351
Kimura, K., 302
Kimura, K. D., 222
Kimura, M., 125, 166, 167, 168, 169, 170
Kimura, N., 258
Kimzenbaw, D. A., 413
Kincaid, K., 285
Kindler, S., 120, 126
King, F. A., 448, 451, 452
King, K. V., 222
Kingwell, B. A., 387
Kinnally, K. W., 68, 69
Kinney, B., 29
Kinney, B. A., 30, 31
Kinney, N. E., 31
Kinney-Forshee, B. A., 31
Kinnula, V. L., 261
Kinoshita, Y., 33
Kinzenbaw, D. A., 410, 411, 412, 413
Kioi, M., 244, 245, 254, 258
Kip, K. E., 169
Kipling, D., 166, 167

Kirby, J. E., 277
Kirby, T. E., 94
Kirchman, P. A., 220
Kirchner, H., 149
Kireev, R., 32
Kiriazis, H., 221
Kirkin, V., 304
Kirkland, J. L., 120, 121, 122, 124, 126, 127, 128, 129, 327
Kirkland, K. T., 12, 229, 230, 247
Kirkpatrick, C., 357
Kirkpatrick, D. S., 249, 250
Kirkwood, T. B., 153, 154, 156, 166, 167, 243, 280, 287, 485
Kirshenbaum, L. A., 99, 258
Kirtland, J. L., 130
Kishi, C., 305
Kishimoto, I., 245
Kishimoto, K., 249
Kishner, J. A., 375, 377
Kissebah, A. H., 121
Kitabchi, A. E., 122
Kitadai, Y., 282
Kitani, K., 181, 185, 186
Kitano, H., 257
Kitazono, T., 407
Kitchen, C. M., 283
Kitzman, D. W., 457
Kiuchi, K., 392
Kiyohara, Y., 279, 281
Kjaer, M., 67
Klar, A. J., 224, 244
Klass, M. R., 9, 217, 221
Klebanov, S., 277
Klechi, S., 169, 170
Klein, A. M., 194
Klein, C., 147, 151, 170
Klein, K. P., 391
Klein, M. A., 179, 183
Klein, S., 125, 285, 453, 454, 456, 457
Kleindorp, R., 28, 156, 285
Kleinerman, J., 427, 432, 433, 435, 438
Kleinman, J. C., 324
Kleinman, M., 286
Klein-Nuland, J., 122
Klemm, D. J., 127, 128
Klerman, E. B., 473
Kleschyov, A. L., 360
Kliegel, T., 169
Klier, C. M., 341
Klijn, C. J., 352
Kling, R. M., 437
Klionsky, D. J., 301, 302, 305, 306
Klivenyi, P., 194
Klocke, B. J., 78
Klocke, F. J., 97
Kloetzel, P. M., 302
Klopp, R. G., 10, 180, 184, 185, 302
Klotman, P. E., 34
Klotz, L. O., 302
Klungland, A., 193
Klurfeld, D. M., 26
Knapka, J. J., 450
Knapp, D., 322
Knauer, H., 310
Knecht, E., 297, 306, 307, 309
Knecht, S., 349
Kneer, N., 469
Knepper, L., 353
Knight, C., 225, 248, 251, 256
Knippenberg, M., 122
Knoblaugh, S. E., 56, 188
Knoke, J. D., 324
Knopman, D. S., 341
Knowlton, A. A., 490
Knox, M., 95
Knudsen, G. P., 488
Knudsen, K. E., 224
Knudson, D. E., 429, 430, 433, 436
Knudson, R. J., 423, 424, 427, 429, 430, 433, 434, 435, 436, 437, 438, 439, 440
Ko, G. T., 283
Kobatake, T., 122
Kobayashi, Y., 249, 253, 262
Kobes, S., 125
Koc, A., 193, 219
Kochanek, K. D., 484
Kochunov, P., 337
Kochunov, V., 337
Kockx, M. M., 281
Kodama, K., 125, 376, 379
Kode, A., 260, 261, 262
Kodela, E., 285
Koehl, C., 72, 224
Koehler, K. M., 119, 433
Koehler, R. C., 407
Kogianni, G., 490
Kogishi, K., 428
Koh, A., 378
Kohama, T., 168
Kohan, E., 129
Kohl, H. W., 421, 424
Kohlmeyer, M. M., 408, 410
Kohrt, W. M., 321, 424
Koike, T., 68, 262
Kojima, T., 28
Kokkonen, G., 302
Kokkotou, E., 121, 127
Koklu, E., 286
Kokmen, E., 356, 426
Kolipinski, M. C., 13, 205, 206
Kollb-Sielecka, M., 408
Koller, A., 358, 410, 411, 413
Koller, B., 388
Køllgaard, T., 33
Kolli, M., 92
Kollias, N., 221
Kolling, E. A., 302, 304
Kollipara, R., 152, 259
Kolodgie, F. D., 97, 283
Kolodny, E. H., 349, 352
Kolson, D. L., 337
Koltai, E., 248, 249
Kolvraa, S., 300
Komatsu, M., 306, 309
Kon, K., 340
Kon, N., 225, 248, 251, 256
Kondo, H., 70
Kondo, N., 189, 219
Kondo, T., 194
Koneru, S., 259
Konishi, J., 351
Konje, J. C., 168, 169
Konrad, A., 341
Kontopoulos, E., 262
Koo, A., 129
Koo, G. C., 33
Kooij, G., 407
Kooijman, R., 32
Koos, R. D., 358
Kop, W., 323
Kopchick, J. J., 26, 27, 28, 29, 30, 31, 130, 224, 229, 301
Kopelman, A., 217, 224
Koppeschaar, H. P., 32
Kops, G. J., 224
Korach, K. S., 388
Korc, M., 97
Korenda, L., 283, 284
Koretz, J. F., 392
Korner, J., 285
Kornfeld, K., 221
Korolchuk, V. I., 65, 66
Korosi, J., 375
Korsmeyer, S. J., 70, 71, 83, 98
Kory, R. C., 423, 435, 436
Koseki, T., 72
Koshel, N. M., 221
Kosiba, W. A., 470, 471
Kosik, L. M., 340
Kosiol, S., 169
Koskenvuo, M., 287
Koslover, D., 13, 205, 206, 217, 218, 226
Kosmatka, K. J., 32, 164, 216, 381, 392, 451
Koss, B., 357
Kossovsky, M. P., 119, 123
Kosteas, T., 280, 284
Kostelc, J. G., 27
Kosugi, H., 194
Kotani, K., 122
Kothari, R., 280
Kotliarova, S., 300
Koto, A., 337
Kotz, C. M., 325
Koubova, J., 447
Koudstaal, P. J., 348, 349, 351, 352, 356, 357
Koudy Williams, J., 390, 391

Kouro, T., 122
Kouskoff, V., 169
Koutmani, Y., 285
Koutnikova, H., 120
Koutsari, C., 122, 127
Kouwenhoven, E., 489, 491
Kovacheva, E. L., 73, 74, 75, 76
Kovacs, A., 120, 218, 226
Kovacs, A. L., 10, 207
Kovacs, J., 308
Kovacs, S. J., 453, 454, 456, 457
Koval, J. J., 439
Kovalev, G. L., 378
Kowall, N. W., 190
Kowlessur, D., 48, 52
Koya-Miyata, S., 180, 182, 184
Kozak, E., 4, 5, 7, 10
Kozlowski, M. T., 204
Kozlowski, P., 357
Kozma, S. C., 205, 231
Kozy, H. M., 191
Kraal, G., 279
Krabbe, K. S., 286
Kraemer, W. J., 65, 94, 185
Kraft, P., 148
Kraft, R., 302
Kraig, E., 392
Krajcik, R., 205
Krajewska, B., 95
Krajewski, K. M., 327
Krajnak, K., 84, 85
Krakower, G. R., 121
Kral, J. G., 120, 122
Kralisch, S., 129
Kramer, D. M., 51, 55, 56
Kramer, J. M., 224
Kräuchi, K., 468
Krause, D. J., 67, 69, 70
Krause, D. N., 473
Krause, D. S., 190, 191
Krauskopf, A., 69
Kreppel, F., 262
Krestin, G. P., 341
Kretschmann, H. J., 336
Krewski, D., 286
Krieg, S., 437
Krieger, M., 258, 259, 260
Krishnakumar, R., 249, 256, 261
Krishnamurthy, G., 72, 73, 74, 75, 76, 79, 80, 84, 85, 86, 87, 89, 90, 94
Krishnamurthy, J., 378
Krishnaswami, S., 123
Kristiansen, M., 488
Kritchevsky, D., 26
Kritchevsky, S. B., 77, 78, 119, 123, 360
Kroemer, G., 68, 71, 83, 308
Kromhout, D., 359
Kronenberg, G., 408
Kronenberg, R. S., 425, 437
Krönke, G., 283
Kronmal, R. A., 168, 169, 433, 435
Kropsky, B., 326
Krsnik, Z., 65
Krtolica, A., 146, 167, 282
Kruck, T. P., 359
Krueger, J. M., 26
Kruger, E., 302
Kruger, R. G., 245
Krumholz, R. A., 427, 437, 438
Krumpe, P. E., 439
Kruse, J. P., 250, 255, 256
Krust, A., 388
Krustrup, P., 67
Kruth, H. S., 283
Kruuk, L. E., 486
Kruys, V., 260, 261
Ku, H. H., 54
Ku, M., 144
Kubo, M., 279, 281
Kubo, Y., 256
Kubota, H., 227
Kubota, S., 260
Kuczera, K., 82
Kudej, A. B., 392, 395
Kudej, R. K., 387, 392, 394, 395
Kudelski, A., 203, 226
Kudin, A. P., 69
Kudo, M., 412
Kufareva, I., 262
Kuhn, H. G., 154
Kuijpers, T. W., 71
Kuipers, J., 153
Kujala, P., 153
Kujoth, G. C., 55, 69, 70, 74, 92, 98, 489, 491
Kuk, J., 119, 120, 122, 123
Kulaots, I., 13
Kulkarni, S., 78
Kulkarni, S. K., 360
Kuller, L. H., 123
Kullmann, R., 466
Kulminski, A., 282
Kulminski, A. M., 282, 284
Kuma, A., 306
Kumar, A., 307
Kumar, C., 254
Kumar, R., 482, 487, 488
Kumar, S., 120
Kundtz, A., 357
Kung, A., 359
Kunieda, T., 125, 285
Kunisada, T., 300
Kuniyasu, H., 282
Kuno, S., 4, 5, 7
Kuntz, K. M., 123
Kunz, G. A., 169
Kunz, W. S., 69
Künzli, N., 286
Kuo, C. J., 65, 149, 151, 154
Kuo, C.-S., 433
Kuo, D. H., 251
Kuo, P. H., 328
Kupfer, D. J., 473
Kuranaga, E., 303
Kurapati, R., 300, 302
Kureishi, Y., 357
Kurihara, T., 260
Kurimoto, M., 180, 182, 184
Kuroda, M., 301
Kuroda-Kawaguchi, T., 388
Kuroki, H., 464
Kuro-o, M., 412
Kurosu, H., 224
Kurtev, M., 245, 252
Kurtoglu, S., 286
Kuruvilla, F. G., 204
Kurz, A., 470
Kurz, C. L., 16, 286
Kurzman, I., 391
Kushmerick, M. J., 68
Kushnareva, Y., 48
Kushwaha, R. S., 390
Kuslansky, G., 353
Kussmaul, L., 49
Kusuda, S., 483
Kuulasmaa, K., 386, 387
Kuznicki, J., 66, 82, 83
Kuzon, W. M., Jr., 84, 95
Kwak, D. W., 16
Kwak, H. B., 73, 74, 76, 85, 86, 87, 89, 92, 93, 95, 96, 98, 125
Kwak, M. J., 30
Kwolc, T., 324
Kwon, D. Y., 168
Kwon, H., 288
Kwon, H. S., 261
Kwon, O. S., 30
Kwon, S. H., 300
Kwon, T., 168
Kwong, L. K., 51
Kyle, U. G., 119, 122, 123
Kyo, S., 168
Kypreos, K. E., 129
Kyvik, K. O., 168

L

LaBarge, M. A., 145
Labat, C., 169
Labina, N., 205, 206, 207, 208
Labinskyy, N., 14, 16, 29, 96, 125, 248, 251, 259, 260, 261, 283, 287
Labrousse, A., 16
Labugger, R., 411
Lacaud, G., 169
Lachance, P. E., 205
Lachmansingh, E., 29
Lachowska-Kotowska, P., 436
Lack, L., 468
Lack, L. C., 473
Lacreuse, A., 391, 392

Author Index

LaCroix, A. Z., 323
Ladd, W., 250, 251
Ladelund, S., 77, 78
Ladhoff, A., 302, 304
Ladiges, W., 489, 491
Ladiges, W. C., 187, 188, 189
Laessig, T. A., 68
Lafontaine, J. S., 256
Lagaay, A. M., 353
Lagerstedt, A., 180, 184
Lago, F., 121
Lago, R., 121
Lagouge, M., 72, 224, 230, 248, 251, 252, 258, 261, 263
Laharrague, P., 125
Lahat, N., 358
Lai, C. Y., 217, 220, 300
Lai, K. N., 283
Lajemi, M., 168
Lajmanovich, A., 81
Lakatta, E. G., 96, 97, 98, 277, 281, 347, 349, 392, 393, 424
Lakowski, B., 4, 7, 9, 10, 12, 207, 208, 218, 219, 228, 229
Lalier, L., 71
Lam, C. S., 427
Lam, M. F., 283
LaManna, J. C., 358, 359
Lamantia, A. S., 338
Lamark, T., 306
Lamb, D., 428
Lamb, K. J., 170, 171
Lambacher, N., 16
Lambe, T., 152
Lambert, A. J., 49, 50, 125
Lambert, M., 327
Lambert, P. D., 230, 244, 248, 250, 251, 262, 263
Lambeth, J. D., 52
Lambie, E. J., 10
Lamming, D. W., 12, 16, 206, 207, 229, 230, 247, 250
Lamping, K. G., 413
Lancaster, J., 337
Lancrin, C., 169
Land, S. J., 341
Landar, A., 168, 169
Landi, F., 32
Landini, L., Jr., 286
Landis, G. N., 286
Landis, J., 5
Landis, L. S., 391
Landry, J., 224, 245
Lane, M. A., 27, 32, 35, 125, 391, 392, 449, 450
Lane, M. D., 129
Lane, R. M., 357
Lang, M. R., 428
Langa, K. M., 374
Lange, L. A., 285
Langelier, Y., 127
Langenberg, P., 324
Langer, M. R., 245
Langheinrich, A. C., 281
Langin, D., 123
Langlois, M., 168
Lango, H., 378
Langston, J. W., 388
Lanni, C., 280, 281
Lanning, N. J., 25
Lans, H., 219
Lansdorp, P. M., 151
Lanza, I. R., 85
Lanzrein, A. S., 280
Lapalombella, R., 300
Lapchak, P. A., 30
Lapin, B. A., 392
Lapointe, J., 28
Lapointe, T. K., 282
Lappan, S., 488
Lappe-Siefke, C., 340
Lara, E., 250
Larbi, A., 149, 279
Lardeux, B. R., 306
Lardy, H., 469
Largo, R., 484
Largrelius, A., 391
Larkin, L. M., 127, 327
La Rocca, N., 54
LaRocca, T., 412
Laroche, T., 244
Larochette, N., 71
Larroque, B., 483
Larsen, E., 193, 194
Larsen, P. L., 222
Larson, D. E., 468
Larson, V. G., 379
Larson-Meyer, D. E., 30, 258, 453, 456
Larsson, H. B., 351
Larsson, L., 246
Lartigue, L., 69
Lascelles, D., 281
Lascola, C. D., 285
Lasko, P. E., 205
Laskowitz, D. T., 285
Lasley, B. L., 390
Lassen, N. A., 351
Latch, S. S., 437
Latini, R., 413
Latorre-Esteves, M., 12, 229, 249, 261
Lau, C., 249
Lau, E., 283
Lau, L. F., 85
Lauer, T. W., 262
Laun, P., 250
Launer, L. J., 321, 359
Lauper, S., 71
Laurent, G. J., 428
Laurenzi, I. J., 388
Lauretani, F., 278
Laursen, H., 351
Lautenschlager, G., 339
Lavi, R., 349
Lavi, S., 349
Lavie, C. J., 285
Laville, M., 121, 122
Lavu, S., 16, 230, 244, 247, 249, 250, 251, 255, 256, 261, 262, 263
Law, G. L., 206
Lawitts, J. A., 123
Lawler, D. F., 447
Lawler, G., 52
Lawler, J. M., 73, 74, 76, 85, 86, 87, 89, 92, 93, 95, 96, 98, 125, 185
Lawrence, D., 32
Lawrence, R. A., 34
Laws, J. W., 437
Lawson, B., 301
Lawson, B. R., 412
Lawson, J. A., 388
Lazarus, S. A., 359
Lazennec, G., 282
Le, L., 258, 260
Le, S. S., 68
Leahy, D. J., 28, 164, 285
Leal, A. D., 357
Lean, M. E., 123
Leaper, S. A., 353
Leathers, C. W., 247
Le Bacquer, O., 231
Lebecque, P., 283
Leber, B., 83
Leblanc, P., 424, 433, 436, 437
LeBoeuf, R. C., 124
Le Bourg, E., 221
Lebovitz, H. E., 122
Lebovitz, R. M., 55, 191
Lebowitz, M. D., 424, 429, 435, 436
LeCapitaine, N., 99
Lechel, A., 168
Lechleitner, M., 120
Lecka-Czernik, R. E., 145, 149
Lecours, A. R., 334, 336, 337
Lecrux, C., 412
Lee, A. H., 209
Lee, A. T., 283, 284
Lee, B. C., 193
Lee, B. H., 152
Lee, C. K., 93, 98, 99, 125, 180, 184, 185, 301, 302
Lee, C.-K., 10
Lee, C. M., 392
Lee, D., 70
Lee, D. E., 285
Lee, D. H., 78
Lee, D. T., 469
Lee, E. K., 96, 260, 280
Lee, G., 299
Lee, G. D., 4, 5, 7, 9, 10, 221, 228, 229
Lee, H., 16, 285

Author Index

Lee, H. C., 70
Lee, H. W., 166
Lee, I., 68
Lee, I.-H., 254, 302
Lee, I.-M., 178, 179, 324
Lee, J., 16, 72
Lee, J. H., 170, 171
Lee, J. S., 490
Lee, J. Y., 358
Lee, L., 10, 25, 222
Lee, M., 65, 149, 151, 154, 285
Lee, M. S., 285
Lee, M. Y., 410
Lee, R. Y., 220, 228
Lee, S., 34, 254, 307, 413
Lee, S. C., 64
Lee, S. J., 4, 7, 9, 12, 16, 205, 206, 207, 208, 217, 227, 229, 230
Lee, S. M., 261, 263
Lee, S. R., 359
Lee, S. S., 220, 228, 286
Lee, S. W., 324
Lee, S. Y., 30
Lee, T. H., 16, 190, 191
Lee, W. L., 97
Lee, W. Y., 70
Lee, Y., 92, 121, 122
Lee, Y. C., 348
Lee, Y. H., 127
Lee, Y. J., 78
Lees, H. A., 64, 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 86, 87, 89, 90, 91, 95, 96
Lees, S. J., 64, 84, 424
Leeuwenburgh, C., 47, 55, 64, 66, 69, 70, 71, 72, 74, 75, 76, 77, 78, 79, 80, 83, 84, 86, 87, 88, 89, 90, 91, 92, 94, 95, 96, 98, 125, 260, 282, 308
Leevers, S. J., 8, 120, 205, 217, 224
Lefebvre, A. M., 121, 122
Lefebvre, M., 453, 456
Leff, T., 120, 121, 122, 128
Lefrak, S. S., 440
Leger, C., 51
Le Greves, M., 31
Le Greves, P., 31
Lehman, S., 122, 123, 469
Lehmann, K. A., 220
Lehninger, A. L., 50
Lehrer-Graiwer, J., 220, 223, 228
Lei, K., 205
Lei, S. F., 119
Leibel, R. L., 120, 124
Leick, L., 64, 96
Leido, P., 216
Leikert, J. F., 360
Leininger, J. R., 35
Leith, D. E., 433, 435
Leitner, J. M., 169
Leiva-Salcedo, E., 281
Leivo, I., 64
Le Marchand-Brustel, Y., 231
Lemasters, J. J., 68
Lemieux, M., 224, 261, 392
Lemmon, H. A., 353
Lemon, J. A., 179, 183, 195
Lenaerts, I., 4, 5, 7, 17
Lenaz, G., 50, 67
Lenburg, M. E., 119, 122, 123, 124, 126, 128, 129
Lenchik, L., 360
Lendeckel, U., 412
Lener, B., 55
Leneuve, P., 29, 217, 224, 485
Leng, S., 327
Leng, S. X., 328
Lennon, L., 123, 324
Lenroot, R., 334, 336
Lentz, S. R., 410, 411, 413, 415
Leo, O., 249
Leo, S., 219
Leonard, W. J., 32
Leone, A., 286
Leoni, S., 49, 50
Leopold, J. A., 411
Le Page, F., 194
Leprince, D., 248
Lerch, J., 334, 336
Leri, A., 96, 97
Lerin, C., 13, 224, 230, 247, 248, 250, 251, 259, 261, 263
Lerner, R. A., 304
Leroi, A. M., 13, 205
LeRoith, D., 28, 34
Le Roux, A., 357
Lescai, F., 286
Lesnefsky, E. J., 70
Lesniak, M. A., 30
Lesniewski, L., 412
Lesueur-Ginot, L., 285
Leung, J. C., 283, 324
Leung, P. C., 324
Leung, P. Y., 183
Leung, S. W., 358
Leutzinger, Y., 205
Leveille, S. G., 353
Leverenz, J. B., 357
Levin, D. L., 437
Levine, B., 4, 7, 12, 208, 226, 230, 305, 306, 308
Levine, J., 122, 468
Levine, R. L., 302
Levinson, Y., 325
Levitzky, M. G., 429, 434
Levy, A. P., 358
Levy, A. S., 259, 411, 413
Levy, C., 352
Levy, D., 96, 169, 347, 349, 392, 393
Levy, J. A., 5
Levy, R., 357
Lew, C. M., 34, 56, 190
Lewandowska, A., 299
Lewén, A., 194
Lewis, C. E., 169, 352
Lewis, K. N., 14, 16, 35, 248, 251, 259, 283
Lewis, L., 303
Lewis, S., 322
Lewis, S. E., 128
Lexell, J., 148
Ley, K., 281
Lezza, A., 67
L'Heurex, P. O., 437
Li, B., 96, 97, 98
Li, C., 229, 256, 257
Li, C. Y., 71
Li, D. Q., 375
Li, E., 66
Li, F., 82, 249, 302
Li, F. F., 283
Li, G. J., 302, 308
Li, H., 66, 258, 259, 397
Li, J., 33, 256, 302
Li, L., 10, 12, 190, 191, 204
Li, L. M., 207
Li, L. Y., 71
Li, N., 52
Li, P., 96, 97, 98, 306
Li, Q., 178
Li, S., 26, 166, 169
Li, X., 33, 258, 259, 260, 261, 263
Li, Y., 95, 190, 191, 192, 194, 225, 230, 248, 375, 378, 408, 411, 412
Li, Y. S., 26, 29
Li, Z., 97, 248
Liang, C. P., 259
Liang, G., 169
Liang, H., 67, 189, 190, 192, 194, 195
Liang, R., 280
Liang, X., 258, 260
Liao, C. Y., 284
Liao, J. W., 120
Liaudet, L., 100
Libby, P., 168, 169, 413
Libera, L. D., 95, 96
Liberek, K., 299
Libert, S., 15, 16, 222, 286
Libina, N., 4, 7, 9, 12, 217, 222, 227, 229, 230
Lichtenwalner, R. J., 31
Licinio, J., 471
Lick, S., 194
Lidell, M. E., 469
Lieber, S. C., 396, 397
Lieberburg, I., 391
Lieberman, E., 144
Liedo, P., 479, 485, 486, 489
Lietz, U., 120
Liggett, S. B., 394
Lightfoot, R. T., 301
Ligon, K. L., 378

Author Index

Liguori, R., 472
Lijnen, H. R., 125
Liker, A., 488
Lim, B., 306
Lim, C. S., 250, 259
Lim, F. C., 33
Lim, H., 97
Lim, K. O., 334, 337, 341
Lima, J., 353
Liminana, P., 406, 407
Lin, A., 354
Lin, J. N., 251
Lin, J. X., 351, 352
Lin, K., 10, 25, 222
Lin, L., 249, 261, 277
Lin, L. R., 191, 195
Lin, M., 73, 74, 79, 80, 86, 87, 89, 90, 91, 95, 260
Lin, S., 204
Lin, S. J., 3, 12, 97, 206, 219, 220, 229, 230, 247, 258
Lin, S. K., 16
Lin, S. S., 12, 83, 237, 249
Lin, V. C., 251
Lin, W. D., 127, 406
Lin, X.-Z., 328
Lin, Y., 10, 224, 248, 255, 256, 261, 262, 306
Lin, Y. H., 278
Linares, O. A., 382
Lindahl, T., 190, 193
Lindenberger, U., 339, 341
Linderman, J. K., 72, 78
Lindquist, R. A., 204
Lindquist, S., 4, 5, 7, 9, 10, 11, 16, 230, 247, 248
Lindsey, M. L., 29
Lindsten, T., 69
Lindstrom, J., 374
Linette, G. P., 71
Linford, N. J., 56, 188, 189, 218
Ling, P. R., 285
Lingard, S., 485
Lingard, S. J., 14, 30, 120, 208, 209, 217, 224, 381
Lingdren, C. M., 378
Link, A., 169
Link, C. D., 4, 5, 7, 11
Linke, A., 95, 96
Linseman, D. A., 68
Lintl, P., 337
Linton, M. F., 178
Linton, P. J., 150
Lio, D., 285
Liou, G. G., 245
Liou, L. L., 218, 220
Lip, G. Y., 281
Lipman, R. D., 179, 180, 181, 182, 185, 304, 322
Lippincott-Schwartz, J., 306
Lippl, S., 192
Lippman, S., 12, 204, 205, 207, 217, 227
Lips, P., 32
Lipschitz, D. A., 145, 149
Lipsett, M., 229
Lipsitz, L. A., 349, 350, 351, 352, 359
Lipton, B. H., 148
Lipton, R. B., 285, 353
Lipton, S. A., 71
List, E. O., 130, 224
Listenberger, L. L., 128
Listi, F., 387
Listner, K. M., 221
Liszt, G., 245, 247
L'Italien, G. J., 258
Lithell, H., 360
Lithgow, G. J., 205, 207, 208, 222, 227, 300
Litoshenko, A. Y., 221
Little, J. B., 432, 433
Little, R. R., 283, 373, 374
Littlefield, J. M., 325
Liu, B., 28, 164, 246, 285
Liu, C., 152
Liu, G., 259
Liu, G. L., 97
Liu, H., 78, 334
Liu, H. Y., 357
Liu, J., 67, 69, 120, 121, 122, 125, 254, 278, 302, 357
Liu, J. L., 27, 28, 34, 229
Liu, K., 169
Liu, L., 92, 93, 99, 166, 324
Liu, M. Y., 119
Liu, N., 278
Liu, P., 92, 93, 98
Liu, S., 11, 286
Liu, W., 217, 219, 220, 228
Liu, X., 69, 218
Liu, Y., 56, 67, 96, 97, 190, 191, 193, 194, 195, 222, 302
Liu, Y. L., 28
Liu, Z., 4, 5, 7, 10
Li Xue, Q., 324
li-Youcef, N., 72
Ljubicic, V., 64, 68, 92, 98, 99, 100
Ljubuncic, P., 165
Lloberas, J., 124
Lloret, A., 68, 388, 489, 490, 491
Llovera, M., 77
Lloyd, B. D., 64
Lloyd-Jones, D., 386, 387
Lo, E. H., 408
Locantore, J. K., 350
Lo Celso, C., 151
Locher, J. L., 321
Locke, M., 167, 300
Lockhart, D. J., 304
Lockshon, D., 85
Lockyer, J. M., 224
Lockyer, P., 300, 301
Loeb, J., 221
Loeffler, L., 82
Loeschcke, H. H., 438
Loeschcke, V., 221, 224
Loeser, R. F., 27, 31, 392
Loewith, R., 120, 204, 207, 226, 230, 231
Loewy, A. D., 473
Logan, G. D., 337, 338, 339
Logan, R., 387
Logue, S. E., 72, 82, 83
Loiseau, A., 427, 437, 438
Lokhandwala, M. F., 285
Lomb, D. J., 244, 255
Lombard, D. B., 225, 248, 254, 255, 256, 258, 302
Lombardi, A., 469
Lomecky, M. Z., 32
London, R. E., 388
Long, J. M., 154
Long, S. Y., 377
Long, X., 10, 97, 204, 208
Longenberger, A., 355
Longmate, J. A., 192
Longo, D. L., 328
Longo, V. D., 12, 207, 216, 217, 220, 225, 230, 248, 278, 280
Longstreth, W. T., Jr., 348, 353, 355, 356
Lonn, E., 178, 179
Lonnqvist, F., 120, 121
Lönnrot, K., 180, 184
Loomis, J. L., 471, 472
Lopatynski, J., 436
Lopes-Cardozo, M., 340
Lopez, M., 451
Lopez, M. E., 392
Lopez-Doriga, P., 405, 410, 411, 413, 414
Lopez-Lluch, G., 32, 125
Lopez-Nieva, P., 250
Lopez-Onieva, L., 143
Lopez-Soriano, F. J., 77, 78
Lorberg, A., 204
Lord, J. M., 281
Lord, R. V., 121, 122
Lorenz, J. N., 394
Lorenzo, B. J., 52
Lores, V., 425
Loria, C. M., 169
Lorusso, R., 95
Loscalzo, J., 411
Loskutoff, D. J., 123
Losonczy, G., 96, 125, 260, 261, 287
Losonczy, K. G., 324
Losordo, D. W., 358
Lott, J. M., 306
Lou, Z., 146, 248, 250, 255, 256
Louden, C., 97
Louie, J. L., 302

Louis, T. A., 423, 424, 435
Louisiana Healthy Aging Study, 33
Louvard, D., 153
Lovelock, C. E., 405
Lowe, D. A., 84, 94
Lowe, V. J., 341
Lowell, B., 121
Lowell, B. B., 123, 129, 253
Lozykowski, M., 229
Lu, A., 333, 334
Lu, C. C., 358
Lu, C. Z., 352
Lu, D., 13
Lu, J., 407, 473
Lu, K. H., 389
Lu, N., 55, 191
Lu, P., 278
Lu, P. H., 333, 334, 335, 336, 337, 338, 339, 340, 341
Lu, T., 145, 308
Lu, T. A., 13
Lu, X., 166, 169, 170
Lu, Y., 28
Lu, Z. D., 127
Lubonia, M., 338, 340
Luc, G., 278
Lucas, A. J., 336, 337, 339, 340, 341
Lucas, J. J., 299
Lucassen, P. J., 65
Lucero, J., 388, 489, 491
Luchsinger, J. A., 356
Lucius, R., 360
Luckinbill, I. S., 221
Lugaresi, E., 472
Luhasen, T., 248, 251
Luheshi, G. N., 277
Luiten, P. G., 357
Luiten, P. G. M., 405, 406, 407, 408
Lukaszuk, K., 279
Lulli, P., 32
Luna-Chavez, C., 51
Lund, D. D., 410, 411, 412
Lund-Anderson, H., 374
Lundberg, K. C., 304
Lundervold, A., 337
Lundgren, P. R., 221
Lunec, J., 182, 183
Lunell, N. O., 391
Luo, J., 13, 224, 225, 246, 254, 255, 256
Luo, S., 5, 307
Luo, X., 71
Luo, Z., 128, 357
Luscher, T. F., 358
Lushaj, E. B., 324
Lushington, K., 473
Lustgarten, M. S., 56, 67, 187, 188, 193, 195
Luttikholt, V., 471, 472
Lutz, R. J., 71
Lutz, W., 119
Lv, J. H., 119
Ly, D. H., 304
Lydersen, S., 324
Lydum, B. T., 4, 15, 217, 218, 219, 221
Lyle, A. N., 413
Lynch, A. V., 250
Lynch, C. D., 31, 457
Lynch, C. M., 410, 411, 412, 413, 415
Lynd, F. T., 14, 120, 231, 252, 322
Lynn, D. J., 277
Lynn, S., 193
Lyons, D., 31
Lyssenko, V., 378
Lythgoe, D. J., 351

M

Ma, J., 82
Ma, L.-J., 412
Ma, R., 286
Ma, W., 191, 258
Ma, X., 130
Ma, X. L., 97
Ma, X. M., 205
Ma, Y., 248
Ma, Y. S., 70
MacCallum, R. C., 32
MacCallum, T., 453, 455
Macchi, M., 468
MacCoss, M. J., 56
Macdonald, D. C., 82, 83
MacDonald, I. C., 466, 470
Macdonald, S. J., 286
MacDougall, J. D., 94, 96
MacEneaney, O. J., 168, 169
Macera, C. A., 421, 424
MacFarlane, M., 66
Machado de Oliveira, R., 252
Machann, J., 122, 244, 253
Machicao, F., 244, 253
Machida, S., 73, 83, 84, 148
Macho, A., 51
MacIntosh, C. G., 325
MacIntyre, A., 170, 171
Macintyre, S., 484
Macius, A., 94
Mack, I., 124
Mack, T. G., 262
MacKay, V. L., 12, 206, 207, 227
Mackenzie, L., 167
MacKenzie, M. A., 469, 470
Macklem, P. T., 430, 432, 433, 434, 435, 436, 437
MacLean, M., 220
Macleod, K., 244
Maclure, M., 359
MacMahon, S., 360
Macova, M., 360
MacRae, K. D., 423, 435, 439
Macri, M. A., 358
Madamanchi, N. R., 53
Madams, J., 324
Madan, A. K., 26
Madans, J., 321
Madar, Z., 127
Madden, D. J., 341
Madden, R., 216
Madeo, F., 220
Mader, M., 181, 185
Madesh, M., 52
Madia, F., 207
Madjid, M., 281
Maeda, H., 322, 325
Maeda, M., 204
Maeda, N., 408
Maeda, S., 358
Maedler, K., 377
Maehara, K., 97
Maeseneer, M. D., 95, 96
Maffucci, J. A., 389
Magaziner, J., 324
Magen, I., 14
Maggio, M., 328
Magnotta, V., 337
Maheshwari, H. G., 229
Mahley, R. W., 282, 285, 287
Mahotka, C., 72
Mai, A., 250
Maianski, N. A., 71
Maier, C. S., 68
Maile, L. A., 130
Mair, W., 3, 4, 5, 7, 8, 9, 10, 13, 205, 208, 221, 229
Maitland-Toolan, K. A., 16, 251
Maiuri, M. C., 308
Majander, A., 49
Majka, S. M., 127, 128
Majno, G., 276
Majumdar, R. A., 29
Makki, M., 407
Maklakov, A., 485
Maklashina, E., 51
Makrides, L., 439
Makris, T. K., 360
Malarkey, W. B., 32, 468
Malavolta, M., 300
Malayappan, B., 453, 456
Malcolm, D., 408
Maldener, C., 220
Malech, H. L., 358
Malhotra, A., 99, 259
Mali, W. P., 351
Malik, R., 250
Malik, S., 258
Malinska, D., 69
Mallat, Z., 413
Malloy, V., 205
Malluche, H., 390
Malspeis, S. M., 324
Maltepe, E., 52
Maly, F. E., 285

Author Index

Manabe, I., 120, 121, 125
Manabe, Y., 248, 254, 260
Mancevski, B., 338, 341
Manchester, J. K., 12
Mancinelli, R., 83
Mancini, A. M., 428
Mancini, E., 151
Mancini, F. P., 169
Mandarino, L. J., 27
Mandell, G. L., 121
Manders, M., 326
Maneen, M. J., 411
Mango, S. E., 10, 208
Maniero, A. L., 49, 50
Manini, T., 95, 96
Manis, J. P., 282
Mann, A. G., 278
Mann, G. E., 277
Mann, V., 490
Manning, B. D., 205, 209
Mannon, L. J., 337
Manns, M. P., 168
Mano, T., 472
Manolio, T. A., 353, 433
Manon, S., 71
Mansfield, K. G., 482
Mansilla, A., 65, 66
Manson, J. E., 123, 178, 179, 389
Mansouri, A., 147, 191, 193
Mantha, S., 191
Manton, K., 298
Manton, K. G., 484
Mantz, S. L., 447
Mantzaros- Flier, E., 121
Mantzoros, C., 121
Manzo, G., 360
Mao, C. J., 65, 66
Mao, D., 28
Mao, Z., 306
Maqani, N., 229
Maquoi, E., 125
Maraini, G., 304
Maras, A. H., 491
Maraver, A., 166
Marazzini, L., 430, 435
Marban, E., 52
Marcel, T., 125
Marcelo, C. L., 84, 95
Marcelo Arellano, E., 230
Marchbank, K., 306
Marchegiani, F., 286
Marchetti, C., 152
Marchetti, P., 51
Marcheva, B., 249, 253, 262
Marchi, S., 99
Marchington, J. M., 390
Marcinek, D. J., 67, 68, 70, 94
Marciniak, S. J., 82
Marck, B. T., 325
Marcovecchio, M. L., 27
Marcus, J., 340
Marden, J. H., 221
Marder, K., 391
Mardis, E. R., 390, 448
Mardon, J., 283
Margalit, I., 122
Marguerie, R., 286
Mari, B., 123, 125
Mari, V., 388
Marigo, V., 84
Marin, J., 405
Marini, M., 300
Marino, A., 258
Markaki, M., 308
Markel, T. A., 277
Markesbery, W. R., 302, 304, 356
Markides, K. S., 323, 353
Marklund, S. L., 195
Markou, V., 281
Markoulaki, S., 142
Markowska, A. L., 41
Marks, J. D., 48, 52
Marks, T., 185
Markus, H. S., 351, 352
Marlowe, F., 487
Marner, L., 338
Marques, C., 300
Marr, M. T., 224
Marrades, R. M., 437, 438
Marroquin, O. C., 169
Marsh, H., 485
Marshall, B. L., 246, 258, 261
Marshall, K. A., 185
Marshman, E., 143, 153
Marstrand, J. R., 351
Martelli, E., 258
Martens, G., 483
Martens, U. M., 165
Martens-Lobenhoffer, J., 412
Marthan, R., 437
Marthol, H., 349, 352
Martin, C., 68
Martin, C. J., 428
Martin, C. K., 30, 453, 456
Martin, D. E., 205, 226
Martin, F. P., 27, 30, 392, 451
Martin, G. M., 30, 56, 188, 189, 204, 207, 218, 222
Martin, H. L., 471
Martin, J.-F., 328
Martin, K., 153, 280
Martín, M. G., 192
Martin, R. R., 430
Martin-Aparicio, E., 299
Martín-Caballero, J., 166
Martindale, J. L., 302
Martinet, W., 281
Martinez, C., 124, 128
Martinez, M., 325
Martinez, O. I., 375
Martinez, P., 221
Martinez-Caballero, S., 68, 69
Martinez-Chantar, M. L., 250
Martinez-Diaz, P., 13
Martin-Gronert, M. S., 455
Martinko, J. M., 33
Martin-Marcos, P., 227
Martinou, J. C., 71
Martin-Ruiz, C. M., 170, 171
Martins, L. M., 71
Martorana, D., 304
Martyn, J. K., 72, 84, 85, 95
Maruff, P., 32
Maruyama, T., 192
Maruyama, Y., 97
Marygold, S. J., 205
Marzani, B., 83
Marzella, L., 306
Marzetti, E., 64, 67, 68, 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 86, 87, 89, 90, 91, 95, 96, 281, 282, 284, 360
Mascarucci, P., 32
Masdeu, J. C., 355
Maslov, A. Y., 257
Mason, J., 32, 282
Masoro, E. J., 3, 14, 32, 33, 35, 120, 125, 126, 203, 205, 207, 216, 222, 231, 247, 252, 256, 257, 322, 324, 325, 326, 327, 381, 421, 423, 424, 447, 448, 450, 452
Massa, S., 250
Massafra, C., 168
Massaro, D., 439
Massaro, G. D., 439
Massaro, J., 349
Masse, A., 221
Masse-Biron, J., 439
Massey, A. C., 307, 309
Massie, H. R., 179, 181, 183, 185
Massoudi, A., 121, 124, 128
Master, M., 301
Masternak, M. M., 14, 26, 27, 28, 29, 33, 229, 301
Masuda, K., 283
Masutani, H., 189
Maswood, N., 3, 32
Matas, J., 95
Matecic, M., 220, 229
Mates, J. M., 29
Mathalon, D. H., 334
Mathews, B. M., 205
Mathews, L. S., 27, 34
Mathieu, C. E., 52
Mathieu, P., 277
Mathis, C. A., 357
Mathur, N., 285
Mathys, J., 220
Matias, J. R., 13, 14, 205, 222
Matoba, S., 255
Matsuda, H., 71
Matsuda, M., 249, 358
Matsui, M., 192, 306, 309

Matsui, T., 97, 248, 249, 254
Matsui, Y., 360
Matsukawa, N., 414
Matsukawa, T., 470
Matsumoto, A. M., 325
Matsumoto, I., 379
Matsumoto, M., 225, 248, 251, 256
Matsunaga, T., 412
Matsuo, A., 352
Matsuo, M., 16
Matsuo, T., 219
Matsuoka, S., 147, 152
Matsuse, T., 430, 439
Matsuya, Y., 251
Matsuyama, S., 71
Matsuzawa, T., 351
Matsuzawa, Y., 123, 125
Matta, M. J., 251, 259, 282
Mattacks, C. A., 390
Mattagajasingh, I., 259
Mattar, E. H., 29
Matteini, A. M., 285
Matthews, F., 352
Matthews, F. E., 484
Mattison, J., 29, 33, 34, 392, 450
Mattison, J. A., 26, 28, 32, 229, 392, 449, 450
Mattson, M. P., 32, 125
Matveyenko, A., 376, 377
Maty, S. C., 374
Matz, P., 194
Maubaret, C. G., 284
Maurer, U., 66
Mauro, A., 147, 148
Maury, E., 285
May, L., 168
Mayack, S. R., 151, 156
Mayat, E., 71
Mayer, L. S., 340
Mayeux, R., 170, 171, 356
Mayhan, W. G., 349, 406, 410, 411, 413
Mayhew, D. L., 94
Maynard, L., 3, 14
Maynard, L. A., 27, 120
Mayot, G., 328
Mayr, A., 169, 170
Mayr, F. B., 169
Mayr, N., 192
Mazarati, J. B., 55
Mazumder, B., 286
Mazzelli, G., 324
McArdle, A., 70
McArthur, M. J., 257
McBride, S. M., 387
McBurney, M. W., 225, 230, 248, 251, 258
McBurnie, M. A., 323
McCaffery, J. M., 254
McCamant, L. E., 424
McCann, D., 120, 124
McCann, S. M., 471
McCarren, B., 437
McCarroll, S. A., 155, 206
McCarter, R., 27
McCarter, R. J., 327
McCartney, N., 439
McCauley, W. C., 425
McCay, C., 3, 14
McCay, C. M., 27, 120, 228
McClaran, S. R., 439
McClearn, G. E., 170
McClelland, R. L., 169
McClure, J. M., 220, 249
McColl, G., 222
McComas, A. J., 94, 96, 434
McConnell, A. K., 439
McCord, R. A., 244, 245, 254, 258
McCormack, W. C., 325
McCormick, K., 360
McCormick, K. M., 94
McCormick, W., 357
McCrea, K., 32
McCulloch, D. K., 376
McCulloch, E. A., 143
McCullogh, E. A., 143
McCullough, A. K., 194
McCullough, K. D., 302
McCullough, R. E., 425
McCusker, R. H., 32
McDaniel, L., 283
McDonagh, T., 16, 217, 218, 222, 230, 250, 251, 256
McDonald, R. B., 123, 325, 468, 470
McDonnell, M. E., 121
McElhaney, J. E., 279
McElmurry, R. T., 406, 412
McElroy, M. R., 119
McElvaney, G., 433
McEvoy, L., 94
McEwan, D. G., 304
McEwen, B. S., 280
McFadden, S. L., 190, 195
McFaline, J. L., 193
McFall, R., 32
McGarvey, K. M., 255
McGaugh, J. L., 30
McGeer, E. G., 280
McGeer, P. L., 280
McGehee, R. E., 145, 149
McGill, H. C., Jr., 390
McGinn, A. P., 353
McGrath, J. C., 360, 412
McGrew, K., 220, 225, 247
McGue, M., 287
McGuiness, B., 282
McHugh, D., 119
McIntosh, A. R., 350
McIntosh, C. H., 375
McKay, J., 151
McKee, A. C., 357
McKeel, D., 280
McKennie, D., 324
McKenzie, D., 55, 67
McKiernan, S., 67
McKiernan, S. H., 55, 99, 451
McKinnon, J., 408
McKnight, S. L., 253
McKolanis, T. M., 123
McLachlan, D. R., 359
McLean, R. R., 283
McMahan, C. A., 3, 14, 34, 35, 203, 207, 222, 322, 324, 325, 326, 327, 448
McMahon, A. D., 169
McMillan, D. R., 301
McMillen, T. S., 124
McMullen, C. A., 64, 73, 74, 79, 83
McMullen, G. L., 50
McMurray, M. A., 220
McStay, G., 66
McTiernan, A., 285
McVay, M., 256, 257
McVey, M., 217, 220, 224, 225, 246
Mead, J., 424, 428, 429, 430, 431, 432, 433, 435, 436, 439
Meador, B. P., 178
Meadows, K. A., 78
Mechkati, R., 427, 437
Medema, R. H., 224
Medhurst, A. D., 302
Medical Research Council Cognitive Function and Ageing Study, 356
Medina, K. L., 122
Medina, L., 341
Medvedik, O., 12, 206, 207, 216, 229, 237, 247, 249, 258
Meeker, H. C., 406
Meflah, K., 71
Megalou, E., 308
Meggs, L. G., 96, 98
Meguro, K., 351
Meh, D., 464, 466, 467
Mehrizi, A., 53
Mehta, J. L., 92
Mehta, L. H., 120
Mehta, P. K., 169, 413
Mehta, R., 4, 7, 11
Mehta, S. Q., 262
Mei, Y., 85
Meier, D. E., 326
Meier, E., 337
Meier, H., 34
Meier, J. L., 375
Meier, W. A., 33
Meier-Ruge, W., 337
Meigs, J. B., 283, 284, 324
Meij, H. J., 168
Meinzer, K., 349
Meissner, C., 69, 70
Mejia, W., 27, 34
Meka, C. S., 122
Mekjavic, I. B., 423, 426

Melby, C. L., 468
Mele, J., 28, 56, 182, 184, 187, 188, 189, 190, 192, 195, 196, 489, 490
Melendez, A., 226, 308
Melendez, A. J., 287
Melendez, J. A., 52
Meliska, C. J., 26, 29, 30, 31, 224
Melissari, M., 96
Mellinghoff, I. K., 208
Melov, S., 167, 191, 222, 300
Melton, D. A., 153, 375
Melton, L. J., 283
Mena, M., 33
Menaker, M., 185
Mendelsohn, M. E., 387, 388
Mendenhall, L. A., 68
Mendez, M. F., 337, 339, 340
Meneilly, G. S., 380
Menge, C., 128
Menghini, R., 258
Mennella, S., 360
Menuz, V., 218
Menzies, F. M., 65, 66
Menzies, K. J., 64, 68, 92, 98, 99, 100
Mercer, J. B., 470
Mercer, R. R., 94
Mercier, S., 283
Mercuro, G., 387
Merker, K., 298, 304
Merrifield, B., 470
Merrill, A. H., 123, 124, 125
Merry, B. J., 32, 125
Mertens, I., 244, 253, 258
Mervis, R. F., 31
Mery, A., 204
Mery, D. F., 204
Messaoudi, I., 33, 450
Messing, S., 379
Messner, K. R., 51
Mestler, G. E., 490
Mesulam, M. M., 338
Metcalfe, J. A., 151
Metcalfe, T. A., 178
Mets,T., 327
Metter, E. J., 64
Metz, S. A., 375
Meunier, P., 122
Meusser, B., 301
Meydani, M., 359
Meydani, S. N., 454, 456, 457
Meyer, A., 335
Meyer, A. S., 299, 300
Meyer, J. S., 351
Meyer, M.-E., 407
Meyer, M. M., 34
Meyer, T. E., 125, 453, 454, 456, 457
Meziane, H., 230, 248, 261, 263
Mi, L. J., 301
Mi, Q., 277
Mi, W., 262
Miake-Lye, S., 151
Miao, W., 97
Miard, S., 127
Micciolo, R., 120
Michalski, A. I., 222
Michan, S., 225, 244, 248, 254, 256, 257, 260
Michaud, S. E., 170
Michel, J.-B., 413
Michel, J. P., 149
Michelakis, E. D., 358
Michelon, E., 324
Michels, K. B., 3
Michelsson, J. E., 64
Michishita, E., 244, 245, 254, 258, 260
Mieczkowska, J., 436
Mieno, H., 282
Migheli, A., 95
Migliaccio, E., 69, 99, 100
Mihaylova, M. M., 204
Mijalski, T., 181, 185
Mikhailidis, D. P., 283
Mikhailov, V., 70, 71
Mikhailova, M., 70, 71
Mikkelsen, T. S., 144
Milani, R. V., 285
Milberg, W., 349
Milberg, W. P., 350
Milbrandt, J., 16, 251, 261, 262
Mildaziene, V., 48, 49
Mildner-Rihm, C., 358
Milic-Emili, J., 424, 425, 433, 434, 435, 436, 437
Millard, F. J. C., 424, 430
Millen, J., 250
Miller, A. A., 410, 413
Miller, A. K., 333, 335, 336, 337, 339, 358
Miller, C., 99, 247, 248, 250, 255, 256, 259, 261, 262
Miller, D. B., 29, 30, 34, 151
Miller, E. A., 299
Miller, E. E., 325
Miller, E. R. III, 183
Miller, F. D., 145
Miller, G. R., 94
Miller, G. S., 249
Miller, J. D., 412, 439
Miller, J. P., 207
Miller, R., 84, 85
Miller, R. A., 14, 26, 27, 28, 29, 30, 33, 34, 35, 181, 186, 205, 209, 222, 229, 301, 487, 491
Miller, R. M., 437
Milliman, C. L., 98
Millman, R. P., 425, 437, 439
Milloul, V., 349
Mills, C. D., 285
Milne, A. C., 326
Milne, G. L., 194
Milne, J. C., 230, 248, 250, 251, 252, 258, 260, 261, 262, 263
Milner, J., 255, 256, 258
Milner, T. A., 360, 413
Milner, Y., 302, 304
Milstien, S., 48, 52
Milton, S., 26
Min, H., 150
Min, J. N., 300, 301
Min, K. J., 13, 447
Minakami, S., 184
Minami, Y., 170
Minamino, T., 125, 285
Mingorance, C., 410
Minisini, R., 285
Minois, N., 217
Minors, D., 468
Minowa, O., 193
Minshall, C., 33
Minson, C. T., 471, 472
Minter-Dykhouse, K., 248, 256
Mintz, J., 333, 334, 335, 336, 337, 338, 339, 340, 341
Minx, P. J., 388
Miquel, J., 221
Miquet, J. G., 26
Mirchell, J., 32
Miron, M., 205
Misek, D. E., 34
Mishima, K., 359
Mishima, M., 428
Mishkin, A. D., 387
Mishkin, M., 391
Mishmar, D., 164, 170, 171
Mistry, N., 182, 183
Mistry, P., 182, 183
Mitarai, S., 394
Mitchell, D. H., 5
Mitchell, G., 126
Mitchell, P., 50
Mitchell, R. S., 427, 429, 436, 438
Mitchell-Felton, H., 83
Mitchell-Raymundo, F., 282
Mitcheltree, C., 33
Mithjell, K., 324
Mitic, L. L., 4, 7, 12, 206, 208, 226, 230, 308
Mitrofanis, J., 389
Mitrushina, M., 339
Mitsui, A., 189, 219
Mitsunaga, jF., 390
Mittleman, M. A., 359
Mittman, C., 430, 431, 434, 435, 436, 437
Mitzner, W., 428, 433
Miura, M., 70
Miwa, S., 51
Miyamoto, K., 147, 152
Miyao, K., 387
Miyashita, K., 360
Miyasu, T., 128

Miyauchi, T., 358
Miyokawa, K., 128
Miyoshi, H., 51
Miyoshi, K., 34
Miyoshi, T., 351
Mizuarai, S., 128
Mizumoto, K., 410
Mizuno, M., 433
Mizushima, N., 305, 306, 308
Mnuskin, A., 70
Moazed, D., 245, 249, 250
Mobbs, C. V., 17
Mobius, W., 340
Mobius-Winkler, S., 95
Mocchegiani, E., 286, 300
Mochimaru, H., 260
Mock, D., 453, 455
Mock, O. B., 35
Mockett, R. J., 247
Modrick, M. L., 410, 411, 412, 413, 415
Moe-Behrens, G., 145
Mohamed, S. A., 69, 70
Mohamed-Ali, V., 124
Mohammad, H. P., 255
Mohassel, P., 341
Mohlke, K. L., 378
Moir, R. D., 249
Molina, E. J., 95
Moline, M. L., 473
Moller, D. E., 412, 413
Moller, H., 483
Mollerstrom G., 391
Mombouli, J. V., 349
Monaco, M. R., 360
Monaghan, P. A., 486
Monchamp, T., 375
Monden, Y., 193
Monet-Lepretre, M., 407
Monette, J. S., 68
Mongini, T., 95
Monier-Faugere, M. C., 390
Monk, T. H., 468, 473
Monnier, V., 27
Monsen, A., 97
Montagna, W., 466, 472
Montagne, J., 204, 205
Montague, C. T., 139
Montanari, L., 14
Montanya, E., 375
Montecino-Rodriguez, E., 33, 150, 156
Monteiro, E. C., 425, 426
Montesanto, A., 388
Montessuit, S., 71
Montfort, W. R., 189
Montgomery, C. A., Jr., 257
Montgomery, R. R., 278, 281
Monthouel, M. N., 123, 125
Monti, D., 281, 300, 328
Monticone, R., 413
Montooth, K. L., 221
Moochhala, S., 407
Mooijaart, S. P., 28
Moon, S. K., 53
Mooney, M., 41
Mooney, M. P., 283
Mooradian, A. D., 168
Moore, F., 359
Moore, J. H., 389
Moore, J. S., 169
Moore, L., 152
Moore, S. A., 300, 301, 407
Moore, X. L., 221
Moorhouse, M. J., 280, 284
Moraes, C. T., 52
Morales, M., 360
Moreau, C., 71
Moreau, P., 358
Moreau, R. F., 185
Moreno, P. R., 358
Moreno, S. G., 192
Morgan, A. A., 155, 229
Morgan, A. L., 471, 472
Morgan, D. G., 71
Morgan, D. W., 433
Morgan, J. E., 147
Morgan, T. E., 278, 280
Morganroth, G. S., 375
Mori, A., 181, 186
Mori, M., 82, 83
Mori, T. A., 183
Moriguchi, M., 168
Morimoto, R. I., 11, 222, 297, 299, 300, 301
Morin, C. L., 123, 124, 125, 126, 129
Morin, G. B., 151, 152
Morinaga, S., 337
Morinari, M., 360
Morisco, C., 97
Morishima, N., 66
Morita, S., 84, 85
Morita, Y., 147, 150
Moritake, K., 406
Moriwaki, H., 360
Morley, A. A., 179, 182
Morley, J. E., 122, 324, 325
Morley, J. F., 222, 299, 301
Moroni, M., 69, 99, 100
Morris, A., 486
Morris, A. D., 284
Morris, A. H., 427, 437, 438
Morris, D. R., 206
Morris, J. C., 280
Morris, J. Z., 222
Morris, M., 468
Morris, S. N., 246
Morris, Z., 348
Morrison, J. H., 336, 337, 389, 431, 432
Morrison, M., 254
Morrison, N. J., 433
Morrison, S. F., 473
Morrison, S. J., 145, 150, 155, 168, 377
Morrow, G., 300, 302
Morrow, L. A., 375, 382
Morselli, E., 308
Morshead, C. M., 154
Mortamet, B., 406
Mortensen, E. L., 351
Mortimer, J. A., 356
Mortimer, R. K., 216
Mortimore, G. E., 306
Morton, J. F., 56, 188
Moscrip, T., 450
Moscrip, T. D., 450
Moseley, P. L., 300
Moser, B., 277
Moser, C. C., 50
Moser, D. J., 352
Moskovitz, J., 190, 192, 193
Mosoni, L., 66, 68, 148
Moss, M. B., 336, 337, 391
Mostoslavsky, R., 224, 225, 244, 245, 254, 255, 256, 257, 258, 302
Mote, P. L., 16, 29, 301
Motohashi, K., 359
Motta, M. C., 13, 224, 225, 247, 252, 261
Mouly, V., 84, 148
Mountz, J. D., 33
Mouradian, M. M., 299
Mourikis, P., 153
Mouritsen, A., 483
Moustaid-Moussa, N., 124
Movva, N. R., 204, 226
Moy, V. N., 218, 220
Moya-Rull, D., 230
Moynihan, K. A., 252
Moynihan, S., 32
Moyssakis, I., 360
Mozaffari, M., 69, 70, 77, 78
Mozzhukhina, T. G., 221
Mracek, T., 51
Mucci, L. A., 170
Mueller, K., 195
Mueller, M., 64
Mueller, S. T., 357
Mueller-Steiner, S., 288
Muhammad, S., 224
Muhammad, Z. S., 379
Muiesan, G., 427, 437, 438
Mukai, S., 349
Mukamal, K. J., 359
Mukattash, R., 71
Mukhopadhyay, D., 12, 204, 205, 207, 217, 227
Mukhopadhyay, P., 29, 52, 100
Mukke, V., 72, 78
Mulder, H., 26
Mulder, M. J., 169

Author Index

Mulders, A. J. M. J., 326
Muldoon-Jacobs, K., 258
Mulks, M. H., 249
Mullan, M., 357
Mullen, R. T., 306
Muller, B., 360
Muller, D. P., 178, 321
Muller, F., 10, 51, 204, 207, 208, 218, 226
Muller, F. L., 55, 56, 67, 191, 193, 194, 195
Muller, H. G., 216
Muller, J. E., 359
Muller, W. J., 166
Muller-Delp, J. M., 405, 410
Muller-Hocker, J., 68
Muller-Sieburg, C. E., 150
Mullett, M. A., 325
Mullin, J. M., 280
Mulukutla, S. R., 169
Munaut, C., 125
Mundy, G. R., 283
Mungas, D., 349
Muñoz, D. P., 125, 146, 281, 282
Munoz, M. C., 26
Munoz, M. J., 217
Munoz, O., 121
Muntner, P., 387
Murakami, C. J., 220, 222
Murakami, K., 194
Murakami, M., 204
Murakami, S., 29
Murakami, T., 218, 302
Muranaka, S., 49
Murano, I., 126
Murao, K., 256
Muraoka, I., 73, 83, 84
Murashev, A. N., 285
Murata, I., 14, 231, 322, 325
Murata, S., 300, 302, 303, 306, 309
Muresanu, D. F., 406, 407
Murlasits, Z., 64, 76, 85, 89, 94
Murlasits, Z. S., 72, 85
Muro, S., 428
Murohara, T., 358
Murphy, A. J., 244, 245, 254, 255
Murphy, A. N., 48
Murphy, C., 170
Murphy, C. T., 4, 5, 7, 11, 155, 206, 222, 300
Murphy, D. G., 349
Murphy, E. A., 359, 388
Murphy, H. M., 473
Murphy, M., 224
Murphy, M. P., 48, 51, 52, 54, 184
Murphy, S., 407
Murphy, S. L., 484
Murphy, T. J., 56
Murphy, W. J., 32
Murr, M. M., 258, 260
Murray, A. D., 353
Murray, A. J., 69, 70
Murray, A. M., 360
Murray, B. E., 16
Murray, H. M., 278, 352
Murray, J. D., 65
Murray, J. F., 438
Murtagh, K. N., 484
Murtagh-Mark, C., 325
Murtaugh, L. C., 153
Musch, D. C., 191
Musso, E., 97
Musso, F., 341
Musters, M., 167
Musters, M. W., 167
Muth, T., 431
Muthukumar, A. R., 34
Muzumdar, R., 130
Muzzioli, M., 286
Myliwska, J., 279
Mylonakis, E., 16
Mylotte, L. A., 83

N

Naar, E. M., 29
Naaz, A., 120, 121, 125
Nabeshima, Y., 145
Nacher, V., 375
Nadon, N. L., 388, 390
Nadzam, G. R., 473
Nagahama, Y., 351
Nagai, T., 184
Nagakane, Y., 360
Nagamachi, S., 357
Nagano, K., 351
Nagao, T., 407
Nagaoka, S., 65
Nagaraju, K., 82
Nagasaki, M., 120, 121, 125
Nagasawa, S., 406
Nagase, T., 430, 439
Nagashima, K., 473
Nagashima, T., 184
Nagata, T., 34
Nagin, D., 338
Nagin, D. S., 338
Nagy, Z., 352
Nahhas, F. A., 258
Naidoo, N., 301
Nair, K. S., 68, 85
Nair, R. N., 349
Nair, S., 122, 125, 127
Nair, U., 301, 302
Naito, H., 248, 249
Najjar, S. S., 281
Naka, K., 152
Nakagaki, I., 70
Nakagawa, T., 66, 71, 145, 244, 255
Nakagawa, Y., 191
Nakahara, Y., 306, 309
Nakahata, Y., 248, 249, 253, 262
Nakai, S., 193
Nakajima, T., 168, 412
Nakamaru, Y., 251, 261
Nakamaru-Ogiso, E., 48
Nakamoto, H., 302
Nakamura, E., 387, 392
Nakamura, H., 189, 219
Nakamura, K., 48, 52, 306, 309
Nakamura, M., 170
Nakamura, N., 302
Nakamura, S., 97, 351, 352
Nakamura, T., 483
Nakamura, Y., 254
Nakano, M., 379
Nakashima, E., 122, 123
Nakashima, Y., 279, 281
Nakatsuru, Y., 193
Nakauchi, H., 147, 150
Nakaya, H., 306
Nakazawa, S., 28
Nam, M. H., 30
Namba, Y., 219
Nandi, A., 224
Napoli, C., 169
Napoli, E., 302
Naqvi, A., 259
Narala, S. R., 251
Narita, M., 71, 351
Naritomi, H., 360
Narkar, V. A., 16
Narula, J., 97
Narula, N., 97
Naruo, Y., 406
Nash, R. K., 392
Nasmyth, K. A., 244
Nasrin, N., 11
Natarajan, S. K., 193
Nathans, D., 85
Natividad, F., 392, 395, 397
Natividad, F. F., 387, 389, 390, 392, 393, 395, 396, 397, 398, 413
Naumann, E., 407
Naumova, A. K., 488
Nauseef, W., 52
Navarro, A., 67, 179, 182, 195
Navon, A., 302
Nawrot, T. S., 168, 169
Nayagam, V. M., 250, 256, 258, 260
Nayak, L., 281
Nayer, A., 120, 121, 125
Naylor, R. J., 360
Nazir, M., 302
Neal, B., 360
Nechushtan, A., 71
Nedelkina, S., 71
Nedergaard, J., 123
Nedergaard, M., 405, 407
Neel, J. V., 481
Neermann, G., 464
Negrel, R., 120
Neider, M. W., 392

Nelson, C. B., 338
Nelson, G., 281
Nelson, J. F., 120, 209, 218, 226, 277, 282, 284, 309, 310, 381, 389, 390
Nelson, L. E., 251
Nemoto, S., 99, 100, 224, 247, 248
Nepomnyashchikh, L. M., 96
Neretti, N., 13
Neubauer, I., 351
Neuchterlein, K. H., 337, 340
Neufeld, T. P., 204, 226, 230
Neuhuber, W. L., 465
Neumann, C. A., 190, 191
Nevitt, M., 119, 123
Newbold, K. B., 286
Newcomb, T. G., 187, 188, 189
Newcomer, B. R., 122, 123, 453, 456
Newman, A. B., 77, 78, 119, 123, 321, 323, 354, 355, 360
Newswanger, D. L., 471, 472
Newton, I., 488
Newton, W., 451
Nezis, I. P., 303
Ng, A. H., 34
Ng, C. K. Y., 429, 430
Ng, J. C., 204
Ng, R., 191, 193
Ng, S. C., 258
Ng, T., 250, 256, 258, 260
Ng, Y. C., 73, 74, 75, 76, 83
Ngai, A. C., 408
Ng Eaton, E., 13, 224
Nghiem, P., 205
Ngo, A., 50
Nguyen, D. D., 187
Nguyen, D. K., 387
Nguyen, K., 97
Nguyen, M. D., 251, 262
Nguyen, M. V., 359
Ni, C. T., 82, 83
Nicholls, D. G., 48, 52
Nichols, S., 337, 338, 339
Nicholson, J. K., 27, 30, 392, 451
Nickening, G., 169
Nicks, C. R., 433
Nickson, P., 78
Nicod, L. P., 427
Nicolakakis, N., 412
Nicoloy, K., 337
Nie, J., 144
Nie, Y., 251
Niederlander, N. J., 125
Nielsen, M. L., 254
Nieman, D. C., 359
Niemann, C., 145
Niemi, T., 469
Niesler, U., 169
Niessner, A., 170
Nievergelt, C., 285
Niewoehner, D. E., 427, 432, 433, 435
Nijnik, A., 152
Nikolaev, A. Y., 13, 224, 254, 255
Nikolaus, S., 28, 156, 285
Nikolich-Zugich, J., 33
Nilsen, J.-A., 324
Nilsen, S. P., 13
Nilsen, T. I. L., 324
Nilsson, H., 387
Nilsson, R., 277
Nishida, E., 206, 208
Nishida, J., 128
Nishikawa, S., 169
Nishikawa, T., 168
Nishimoto, S., 4, 5, 7
Nishimura, M., 352
Nishimura, N., 190, 191
Nishimura, S., 120, 121, 125, 193
Nishimura, Y., 360
Nishino, J., 155
Nishino, K., 70
Nishino, M., 83
Nishio, I., 170
Nishiyama, S., 408
Nishizawa, M., 249
Nishizawa, S., 351
Nisoli, E., 247, 259
Nitahara, J. A., 96, 97, 98
Nitta, R. T., 178, 179, 183
Nitta, T., 407
Nitti, D., 282
Niu, S. L., 31
Niv, H., 81
Njemini, R., 327
Noble, B., 490
Nochlin, D., 357
Noda, K., 260
Noda, S., 195
Noda, T., 305
Noguchi, T., 128
Nojiri, H., 191
Noll, G., 358
Nomoto, K., 72
Nonn, L., 56, 192
Nopoulos, P., 337
Noppinger, P. R., 388, 389
Norddahl, G. L., 152
Nordenbrand, K., 184
Noriega, L., 251
Norling, B., 184
Norman, D. C., 464
Norman, D. J., 26
Norris, A. H., 430, 431, 434, 435, 436, 437
Norris, A. W., 129
Norris, E. H., 405, 413
North, B. J., 244, 246, 254, 255, 258, 261
North, C. M., 124
Northrop, J. H., 221
Norton, J. D., 85
Norton, W. T., 333
Notte, A., 97
Novak, I., 304, 306
Novak, K. F., 392
Novakofski, J., 33
Novoa, I., 82
Novoselov, S. V., 193
Novoseltsev, V. N., 222
Novosyadlyy, R., 34
Nowak, J. E., 258
Nowell, T. R., Jr., 302, 304
Nozaki, M., 390
Nuechterlein, K. H., 333, 334, 335, 336, 337, 338, 339
Nui, S., 11
Nukina, N., 307
Nunez, G., 72
Nunez, N., 34
Nunn, J. F., 437
Nuremberg, P., 122
Nursten, H., 283
Nurzynska, D., 97
Nuse, B., 286
Nuss, J. E., 301
Nussbacher, A., 98
Nussenzweig, A., 147, 151, 152
Nussey, D. H., 486
Nuystrom, T., 220
Nyberg, F., 31
Nyengaard, J. R., 338
Nygren, J. M., 152
Nystrom, T., 216, 246

O

Oakes, J. A., 230
Oakes, S. A., 83
Oakhill, J. S., 285
Oates, J. A., 178
Oatley, J. M., 145
Obal, F., Jr., 26
Obata, T., 227
Oberdoerffer, P., 225, 256, 257
Oberg, A. L., 379
Oberhollenzer, F., 169, 170
Oberholzer, J., 377
Oberholzer, M., 427, 437
Oberley, L. W., 300
Oberley, T. D., 186, 187, 191, 195, 300
Obeso, A., 425, 426
Obled, C., 328
Obreztchikova, M., 99
O'Brien, E., 169, 170, 171
O'Brien, J. T., 357
O'Brien, K. K., 466
O'Brien, M., 121, 127
O'Brien, P., 122, 127
O'Brien, R., 466
O'Brien, T., 83

O'Brien, W., 167
O'Cain, C., 429, 436
O'Callaghan, J. P., 29, 30, 34
O'Connor, F. C., 98
O'Connor, G., 4, 7, 9, 16, 222
O'Connor, J. C., 32
O'Connor, J. P., 99, 100
O'Connor, P. M., 282
O'Connor, T. P., 196
Odejinmi, S., 250, 251
Odell, I., 217, 219, 220, 228
Odewale, I., 258, 259
O'Donnell, C. J., 169
O'Dorisio, T. M., 468
Oelsner, E., 125, 169
Oeppen, J., 164, 165, 480, 481
Oeseburg, H., 170
O'Fallon, W. M., 356
Ofstedal, M. B., 323, 374
Ogata, J., 336
Ogata, T., 73, 83, 84
Ogawa, K., 97, 410
Ogawa, Y., 128
Ogburn, C. E., 56, 151, 187, 188, 189, 218
Ogg, S., 10, 11, 25, 222
Ogino, S., 225, 256, 257
O'Gorman, J. T., 471
Ogston, S. A., 284
Ogunleye, A. A., 284
Ogura, M., 254
Oguro, H., 147
Oh, S. W., 225, 246
O'Hagan, H. M., 255
Ohira, Y., 65
Ohkuma, S., 302
Ohkura, C., 219
Ohm, J. E., 255
Ohnishi, S. T., 49
Ohnishi, T., 48, 49, 50, 357
Ohshima, S., 68
Ohsugi, M., 120, 121, 125
Ohsumi, Y., 305, 306
Ohta, M., 325
Ohtani, R., 351
Ohuchi, Y., 219
Oishi, Y., 73, 83, 84
Ojala, J., 260
Ojcius, D. M., 64
Oka, S., 189
Okada, H., 165
Okada, K., 304
Okada, S., 130
Okamoto, M., 351
Okamura, A., 406
Okamura, T., 408
Okazaki, M., 127
O'Kroy, J. A., 433
Okuda, K., 168, 169
Okudera, T., 351
O'Kusky, J., 336, 337
Olashaw, N., 261, 263
Oldendorf, W. H., 337
Oldendorf, W., Jr., 337
Oldham, S., 204, 229
O'Leary, M. F., 64, 67, 68, 85, 95
Oling, D., 246
Oliver, J., 96
Oliver, L., 71
Olivetti, G., 96, 97, 98
Olivieri, F., 281
Olliver, V., 413
Olofsson, B., 360
Olovnikov, A. M., 165
Olsen, A., 205, 207, 208, 227
Olshansky, S. J., 483
Olson, J. L., 191
Oltvai, Z. N., 71, 98
Oluwadara, B., 336, 337, 339, 340, 341
O'Mahoney, M. E., 83
Onder, G., 32, 360
Onder, T. T., 193
O'Neill, J., 341
Ongali, B., 412
Onken, B., 4, 7, 12, 16, 206, 208, 226, 230, 308
Ono, S., 351
Onyango, P., 254
Ooka, H., 14, 222
Oorschot, V., 231
Oostra, B. A., 244, 253
op den Buijs, J., 167
Opferman, J. T., 83
Oppenheimer, N. J., 249
Oppong, Y., 82
Orava, J., 469
Orbe, J., 124, 128
Orcel, B., 437, 438
Ordenes, G., 281
Orentreich, N., 13, 14, 205, 222
Orgren, K. I., 407
Orimi, H., 430, 439
Orimo, M., 125, 285
Orito, K., 359
Orjalo, A. V., 280
Orosz, L., 10, 207, 218, 226
Orosz, Z., 260
O'Rourke, B., 52
Oroz, L. G., 307
Orozco, D., 479, 485, 486
Orr, W. C., 218, 247
Orrenius, S., 66
Orsini, F., 69, 99, 100
Ortiz-Alonso, F. J., 382
Ortiz-Vega, S., 10
Ortmeyer, H. K., 127, 449
Orus, J., 360
Ory, D. S., 128
Osaki, M., 245
Osawa, T., 181, 185, 186, 304
Oscar-Berman, M., 391
Oshima, H., 192
Oshima, M., 192
Oshiro, N., 204
Oskvig, R. M., 434
Ostegaard, L., 351
Osterod, M., 190, 193, 194
Ostir, G. V., 323, 353
Ostrowski, S., 436
O'Sullivan, M., 351, 352
Osyczka, A., 50, 51
Ota, H., 256
Ota, K., 227
Otis, A. B., 426, 432, 435
Ott, M., 244, 254
Ottenbacher, K. J., 323
Ottinger, M. A., 389, 390
Ouaissi, M., 256
Oudit, G. Y., 71
Oudot, A., 68
Oury, T. D., 55, 56, 187, 188
Outeiro, T. E., 262
Outzen, H., 306
Ouyang, J., 72, 85
Ovaa, H., 302
Overvad, K., 123
Overvatn, A., 306
Ovodov, N. D., 486
Owens, D. M., 145
Owens, J., 34
Owles, W. H., 432
Oyadomari, S., 82, 83
Ozaki, M., 470
Ozanne, S. E., 455
Ozawa, T., 55, 70
Ozawa, Y., 128, 260
Ozbilge, H., 121
Ozcan, L., 209
Ozcan, U., 209
Ozdelen, E., 209
Ozturk, M. A., 286

P

Pache, J. C., 427
Pacher, P., 52
Pacholec, M., 250, 251
Pacini, G., 379
Pack, A. I., 301, 425, 426, 437, 439
Packard, C., 123
Packer, C., 482, 491
Packer, L., 70, 181, 184, 186
Padala,K. P., 326
Paddon, C. J., 227
Padgett, R. C., 412
Padilla-Nash, H., 244, 245, 254, 258
Padin-Iruegas, M. E., 99
Padmanabhan, N., 360
Pae, M., 123, 124, 125
Paez, P. M., 338
Paffenbarger, R. S., 421, 424
Paffenbarger, R. S., Jr., 324

Pafliassotti, M. J., 125
Pagans, S., 261
Page, G. P., 125, 280, 284
Paglialunga, S., 231
Pagliassotti, M. J., 123, 124, 126, 129
Pahlavani, M. A., 33, 190, 300, 301
Pahor, M., 64, 67, 77, 78, 123, 360
Paik, J. H., 259
Paik, M., 356
Pain, J., 396
Paintal, A. S., 466
Pak, J. W., 67
Pakkenberg, B., 336, 338
Palacios, O. M., 248, 254, 260
Palackal, T., 96, 98
Palikaras, K., 308
Pallardo, F. V., 68, 70, 388, 489, 490, 491
Pallottini, V., 14
Palm, W., 163
Palma, J., 95
Palmer, A. J., 130
Palmer, H. J., 302
Palmer, J., 327
Palmer, L. A., 358
Palmer, M., 217, 224
Palmer, W. H., 437
Palmiter, R., 29, 34
Palmiter, R. D., 27, 34
Paloma, M. A., 32
Palomero, J., 70
Palter, J. E., 205, 207, 208, 220, 227
Paltser, G., 120, 121, 125
Palumbo, G., 169
Pamenter, R. W., 26, 29
Pamir, N., 124
Pamplona, R., 168
Pan, C., 302, 308
Pan, D., 10
Pan, G., 144
Pan, K. Z., 205, 207, 208, 220, 227
Pan, W. T., 121, 122
Pan, Y., 230
Panaretou, B., 220
Panczel, G., 352
Panda, A., 278, 281
Pandey, M., 123
Pandey, P., 97
Pandita, T. K., 13, 224, 255, 256, 261
Pandolf, K. B., 472
Pang, B., 193
Pang, H., 146
Pang, W. W., 225, 255, 258
Pani, L. N., 283, 284
Panici, J. A., 26, 29, 31, 229
Panigrahi, S., 143
Pankov, Y. A., 32
Pankratz, M., 13
Pannell, C., 357
Panoutsakopoulou, V., 69
Panowski, S. H., 4, 7, 8, 9, 10, 15, 146, 208, 221, 229
Pant, R., 259
Pantages, J., 336, 337, 339, 340, 341
Pantano, M. M., 325
Pantoni, L., 351, 355, 356
Panuk, E. R., 324
Panzer, K., 55, 69, 70, 74, 92, 98
Paolisso, G., 28, 32
Paoloni-Giacobino, A., 119, 123
Paolucci, D., 69
Papaconstantino, J., 29
Papaconstantinou, J., 26, 27, 29, 30, 33, 34, 35, 224, 301
Papadakis, M. A., 32
Papadopoulos, D. P., 360
Papageorgiou, K., 341
Papaioannou, A., 283
Papatheodorou, P., 254, 255
Papavasiliou, F. N., 277
Papet, I., 83
Pappas, B. A., 357
Paradis, S., 10, 25, 218, 222
Parameswaran, V., 283
Parashar, V., 247
Pardy, R. L., 433
Pare, G., 388
Parenti, C. G., 67
Parenti Castelli, G., 49
Parhami, F., 283
Paris, D., 357
Parise, G., 94
Park, B., 33, 450
Park, D. C., 339
Park, G. A., 357
Park, H. J., 30
Park, I. K., 147, 377
Park, J. H., 204
Park, J. Y., 68
Park, K., 286
Park, L., 405, 407, 408, 410, 411, 413
Park, O. J., 16
Park, S. K., 4, 5, 7, 11, 99, 256, 257, 277, 280, 284, 412
Park, S. Y., 30, 359
Park, Y., 413
Parker, B. A., 387
Parker, R., 277
Parker, T., 82, 357
Parkhill, J., 151
Parlow, A. F., 34
Parmar, K., 352
Parreira, V. F., 433
Parrinello, S., 167
Parry, D. J., 94
Parry, E. M., 166
Parsons, G., 439
Parsons, J. A., 27
Parthasarathy, S., 205
Partilla, J. S., 127
Partiman, T. S., 473
Partridge, L., 3, 8, 12, 13, 14, 15, 16, 27, 51, 120, 205, 216, 217, 221, 222, 224, 229, 230, 251, 253, 261
Partridge, T. A., 145, 147
Parving, H. H., 374
Pasarica, M., 248, 249
Pasinetti, G. M., 280
Pasparaki, A., 308
Pasquali, A., 392
Pasquini, J. M., 338
Passananti, H. B., 300, 302
Passarino, G., 254, 388
Passino, C., 349, 352
Passmore, P., 282
Passos, J. E., 281
Pastor, J. V., 224
Pastukh, V., 69, 70, 77, 78
Pasupuleti, R., 250, 256
Patabendige, A. A. K., 406, 408
Patel, D. A., 285
Patel, K., 258
Patel, M. S., 52
Patel, N. V., 32, 282, 284
Patel, S., 169, 170, 357
Paterno, R., 410
Paterson, D. H., 425, 439
Path, M. C., 424, 430
Pathare, P., 148
Patil, C. K., 125, 146, 280, 281, 282
Patino, W. D., 255
Paton, V., 97
Patrick, J. M., 425
Patrick, J. S., 27
Patronek, G. J., 26
Patterson, C., 53, 194, 297, 298, 300, 301
Patterson, G. I., 10, 25, 222
Pattwell, D., 70
Paturi, S., 92
Paul, G., 216
Pavlidis, I., 468
Pawelczyk, J. A., 471, 472
Pawelec, G., 33, 149, 279
Payette, H., 321, 324
Paz Gavilan, M., 301
Pearce, B., 251, 258
Pearson, J., 143
Pearson, K. J., 13, 14, 16, 35, 230, 248, 250, 251, 259, 283
Pechenino, A. S., 490
Pedal, A., 261
Pedersen, A. N., 77, 78
Pedersen, B. K., 77, 78, 123, 124, 125, 285, 286
Pedersen, M., 77, 78, 123, 124, 125
Pedersen, O., 374
Pederson, R. A., 375
Pediaditakis, P., 68
Pedruzzi, I., 12
Peek, M. K., 323
Peeters, A. V., 244, 253, 258

Author Index

Pegelow, D. F., 424, 432, 433, 436, 439
Peh, B. K., 256
Pelberg, J. A., 387
Pelham, C. J., 412
Pelicci, P., 99
Pellegrinet, L., 153
Pelliniemi, L. J., 483
Peltonen, M., 374
Pemberton, A. J., 303
Pemberton, J. M., 486
Pena, J. M., 325
Pende, M., 231
Pendergast, A. M., 149
Pendergrass, W. R., 4, 7, 11, 29, 216, 231
Penfield, W., 464
Peng, H. B., 413
Peng, M. T., 473
Peng, Q., 204
Peng, S. L., 249, 261
Penicaud, L., 121, 124, 125, 128
Penn, L. Z., 83
Penner, K. M., 124
Pennington, J. D., 258
Penninx, B. W., 360
Penumathsa, S. V., 259
Penzo, D., 69
Peppa, M., 285
Peppas, A., 92, 98, 387, 393, 394, 395, 396, 397, 398
Pequignot, J. M., 359
Perander, M., 306
Peraud, A. L., 245
Percudani, R., 304
Pereira, P., 300
Pérez, C., 281
Perez, E., 14, 35, 248, 249, 254
Perez, V. I., 28, 29, 56, 182, 186, 187, 188, 189, 190, 192, 193, 194, 195, 196, 489, 490
Perez-Gomez, C., 29
Perez-Perez, M. E., 208
Perez-Terzic, C., 125
Perez-Villa, F., 360
Periasamy, M., 82, 251, 259, 282
Pericak-Vance, M., 357
Perkins, M. W., 78
Perlman, R. A., 325
Permana, P. A., 125, 128
Perrod, S., 244
Perruchoud, A., 427, 437
Perry, R., 220, 356
Perry, V. H., 261
Persson, H., 338
Pert, C. B., 30
Pervaiz, S., 64, 287
Peshkin, L., 14, 16, 248, 251, 259, 283
Peshock, R., 94
Pessin, J. E., 30
Pestka, J. J., 124
Peten, E. P., 34
Peterlin, B. M., 261
Peters, A., 336, 337, 338, 339, 340, 391
Peters, D. G., 337, 339
Peters, E. A., 302, 304
Peters, J. F., 481
Petersen, H. C., 170, 171
Petersen, K. F., 68
Petersen, O. W., 145
Petersen, P. H., 388
Peterson, B. S., 334, 337
Peterson, C. A., 145, 149, 327
Peterson, D. D., 425, 426, 437, 439
Peterson, T. C., 387
Peterson, T. R., 204
Petherwick, A., 216
Petidier, R., 405, 410, 411, 413, 414
Petronilli, V., 68, 69
Petropoulos, I., 302, 304
Petroulakis, E., 231
Petrovic, K., 351
Petrovic, N., 55
Petschow, B. W., 121
Peyster, R. G., 337
Pezzi, R., 250
Pfefferbaum, A., 334
Pfizenmaier, K., 77
Pfluger, P. T., 13, 248, 251, 256, 260, 261
Pfrieger, F. W., 333
Phan, T., 353, 355, 356
Phaneuf, S., 66, 67, 92, 94, 98
Phelan, J. N., 65, 94
Phelps, R. A., 68
Philips, J. L., 457
Phillips, C. L., 355, 356
Phillips, D., 169
Phillips, K. M., 424
Phillips, R. J., 127, 128
Phillips, S. M., 94
Phillips, T., 77, 79, 80, 90, 91, 96, 260, 491
Piatyszek, M. A., 165, 166
Picard, F., 120, 127, 205, 209, 231, 252
Piccin, A., 219
Piccini, D., 99, 100
Picciolini, E., 168
Pichard, C., 122
Pichiule, P., 358
Pickering, J. G., 258, 259
Pieke-Dahl, S., 191, 195
Pieper, C. F., 353, 357
Pierce, A., 196
Pierce, G. L., 411, 413
Pierce, J. A., 427, 428, 429, 430, 433, 435
Pierce, W. M., 411
Pierri, M. K., 97
Pierzga, J. M., 471, 472
Pietrangeli, P., 54
Pietrangelo, T., 83
Pietrelli, A., 413
Pietrobelli, A., 324
Pietrokovski, S., 81
Pietrzyk, U., 351
Pietschmann, P., 387
Pietu, G., 148
Pihalja, M., 147
Pilegaard, H., 64, 96
Pilgaard, K., 382
Pilgrim, D. M., 360
Pillai, J. B., 260
Pillai, V. B., 254, 260
Pillus, L., 224, 244
Pineyro, M. A., 127
Pinheiro, J., 391
Pinkert, C. A., 27, 34
Pinkney, J., 124
Pinn, V. W., 484
Pinswasdi, C., 218
Pintado, C., 301
Pinto, D. W., 248
Pinto, J. T., 96, 125, 260, 261, 282, 287
Pinton, P., 99
Pintus, G., 413
Pio, J. R., 258
Piper, J., 438
Piper, M. D., 3, 12, 13, 14, 15, 30, 205, 221, 224, 447
Piper, P. W., 220
Piras, B. A., 84, 85
Pirolli, T., 97
Pirozzolo, F., 339
Pirtskhalava, T., 119, 122, 123, 124, 125, 126, 127, 128, 129
Pischon, T., 123
Piskunova, T. S., 16
Pistelli, F., 437
Pistilli, E. E., 64, 67, 68, 70, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 84, 85, 86, 87, 88, 89, 90, 91, 94, 95
Pi-Sunyer, F. X., 324
Pitas, R. E., 340
Pitel, K., 125
Pitossi, F., 277
Pitstick, R., 405, 413
Pittas, A. G., 453, 454, 456, 457
Piva, R., 95
Placzek, A., 357
Planat-Benard, V, 125
Plant, C., 306
Plant, P. J., 95
Plass, A., 285
Plass, J. R., 357
Platt, F. M., 307
Plazzi, G., 472
Pleiner, J., 170

Pletcher, S. D., 8, 12, 13, 15, 16, 72, 205, 206, 207, 216, 217, 222, 286
Pletjushkinda, O . Y., 68
Pletnev, A. A., 250
Pluck, R. A., 424, 436
Plueger, M. M., 252
Pluijm, S. M., 32, 77, 78
Pocar, P., 143
Podhorska-Okolow, M., 85, 95
Podlutsky, A., 29, 259, 260, 261, 302
Podlutsky, A. J., 486
Podmore, I. D., 182, 183
Poeggeler, B., 185
Poehlman, E. T., 468
Pogacnik, T., 349, 352
Poggesi, A., 355
Pogosova, G. V., 360
Pogue, J., 178, 179
Pohjasvaara, T., 356
Poirier, J., 282, 340
Pokorski, M., 425, 426
Polak, P., 231
Polito, L., 282
Pollack, M., 32, 94
Pollock, M. R., 322
Polster, B. M., 52
Polvikoski, T., 356
Pond, C. M., 390
Pond-Tor, S., 128
Ponesse, J. S., 337, 338, 339
Ponnappan, S., 302
Ponnappan, U., 302
Ponsot, E., 148
Ponto, R. A., 437
Poole, S., 277
Poon, H. F., 411
Poon, P. C., 13
Poon, S. S., 165
Pope, C. A. III, 286
Popescu, B. O., 406, 407
Popescu, L. M., 406, 407
Popovich, I. G., 16
Poppas, A., 352
Porta, E. A., 178, 179, 183
Portbury, A. L., 297, 298, 300, 301
Porte, D., Jr., 375, 379
Porter, C. J., 358
Portnoy, D. A., 277
Posakony, J., 258
Poslutsky, A., 54
Posner, B. I., 26
Possemiers, N., 95, 96
Post, J. A., 167
Post, W., 169
Postel-Vinay, M. C., 33
Postiglione, A., 169
Potente, M., 256, 258, 259
Potten, C. S., 143, 153
Potter, J., 326
Potter, J. F., 326
Potthoff, S. A., 412
Pouilly, L., 412
Poulain-Godefroy, O., 121
Poulin, F., 231
Poulin, M. J., 425, 439
Poulsen, A., 250, 256, 258, 260
Poulsen, P., 382
Poulton, J., 55
Pousette, A., 391
Pouvreau, S., 359
Powe, N. R., 353
Powell, C. C., 379
Powell, J. M., 411
Powers, R. W. III, 12, 206, 207, 217, 218, 220, 226, 227, 228, 230
Powers, S., 92
Powers, S. K., 67, 68, 69, 70
Powis, G., 56, 189, 192
Pozza, F., 12, 207, 216, 217
Pozzan, T., 83
Prabakaran, D., 121
Prabhu, S. D., 52
Prado, L. F., 433
Prasad, A., 437
Prasad, P., 251
Prats, C., 55
Pratz, J., 34
Preacher, K. J., 32
Precht, T. A., 68
Prefaut, C., 439
Pregla, R., 388, 389
Premachandra, B. N., 453, 454, 456, 457
Prentki, M., 127
Presty, S. K., 391
Prevot, P. P., 260, 261
Price, D. A., 169
Price, D. L., 391
Price, L. B., 94
Price, N. L., 13, 14, 16, 35, 230, 248, 250, 251, 259, 283
Price, S. R., 306
Price, T. R., 353
Pride, N. B., 429, 431, 436
Prigeon, R. L., 376
Priller, J., 408
Primeau, A. J., 64
Principe, N., 358
Prins, J. B., 139
Prins, N. B., 357
Prior, R., 179, 180, 181, 182, 185
Privitera, G., 67
Procaccio, V., 164, 170, 171
Proctor, C. J., 167, 281
Proctor, D. N., 387
Prolla, T. A., 10, 55, 69, 70, 93, 98, 99, 125, 190, 192, 193, 280, 284, 301, 302
Promislow, D. E. L., 485, 487, 488, 491
Pronk, C. J., 152
Prospective Cohort Study on Myocardial Infarction Study Group, 278
Provinciali, M., 286
Prowse, D. M., 145
Prowse, K. R., 166
Pruitt, K., 255
Prusty, R., 220
Przerneck, G. K., 192
Psaty, B. M., 168, 353
Pu, F., 359
Puddey, I. B., 183
Puebla-Osorio, N., 146
Pugh, K. G., 349, 352
Pugh, T. D., 55, 69, 70, 74, 92, 98, 125, 180, 184, 185
Puglielli, C., 83
Puhl, S. M., 471, 472
Puig, O., 224
Puigserver, P., 224, 247
Pulkrabek, D. J., 70, 71
Pump, K. K., 427, 428, 436, 439
Pun, P. M. L., 407
Purser, C. B., 392
Purushotham, A., 258, 261
Pusey, A., 482
Pusey, A. E., 491
Pushparaj, P. N., 287
Puska, P., 491
Puzianowska-Kuznicka, M., 66, 82, 83
Pye, D., 70
Pyka, G., 94
Pyzik, R., 285

Q

Qi, F., 282
Qi, H., 308
Qi, W., 189, 190, 191, 192, 194, 195, 196, 490
Qian, D., 147
Qiao, L., 252
Qin, J., 250, 255, 256
Qin, M., 302
Qin, W., 3, 262, 282
Qin, X., 16
Qiu, H., 387, 389, 390, 395, 396, 397, 398
Qiu, H. L., 357, 393, 396, 397, 398
Qiu, W., 78
Qu, D., 121
Qu, T., 328
Quadrilatero, J., 259
Quagliato, R., 429, 436
Quaife, C., 29, 34
Quaife, C. J., 27, 34
Quaini, F., 97
Quick, K. L., 181, 186, 388, 489, 491
Quigg, R. J., 406
Quindry, J., 92

Quintanilha, A. T., 70
Quirion, R., 30
Quissac, A., 216
Quyyumi, A. A., 169, 349

R

Rabek, J. P., 29, 301
Raber, J., 340, 411
Rabin, B. E., 286
Rabin, M. L., 337
Rabinovitch, P., 489, 491
Rabkin, S. W., 122
Rabol, R., 55
Rabold, R., 428, 433
Racchi, M., 280, 281
Racette, F. G., 249
Racette, S. B., 453, 455, 456
Rackl, A., 351
Rader, D. J., 168, 388
Radermacher, M., 48
Radhakrishnan, K., 170, 171
Radimerski, T., 10, 204, 224
Rae, M. J., 277
Raes, M., 302, 304
Rafalski, V.A., 155
Raff, H. V., 33
Raff, M. C., 261
Raffai, R. L., 285
Rafty, L. A., 245
Ragan, C. I., 49
Rage, F., 472
Raghavakaimal, S., 55, 68, 85
Raghavendra, V., 360
Ragheb, K., 52
Ragno, R., 250
Raguso, C. A., 119, 123
Rahman, I., 260, 261, 262
Rahman, O., 484
Rahn, H., 432, 435
Rai, P., 193
Raices, M., 146
Raikhel, A. S., 204
Raine, J. M., 437
Raineri, I., 186, 187
Rainey, W. E., 165, 166
Rainone, A. C., 360
Raja, S. N., 464, 466, 469, 470
Rajamohan, S. B., 254, 260
Rajan, S., 325
Rajavashisth, T. B., 413
Rajendrasozhan, S., 261
Rajesh, M., 52, 100
Rajewsky, K., 97
Raji, M. A., 323
Rajwa, B., 52
Rajwani, A., 170
Raker, V. A., 99, 100
Rakic, P., 334, 338
Rakoczy, S. G., 28, 29, 31, 67
Rakugi, H., 414
Ramaiah, K. V., 301
Raman, A., 451
Ramasamy, R., 277, 283
Ramírez, T., 425
Ramos, B., 301
Ramos, F. J., 4, 7, 11
Ramp, U., 72
Ramsey, C. S., 256, 260
Ramsey, J. J., 9, 391, 449, 450, 451
Ramsey, K. M., 249, 253, 262
Ramsey, M. M., 34
Ramsey, M. R., 378
Ramzan, R., 68, 69
Ran, Q., 28, 182, 184, 187, 189, 190, 191, 192, 196, 489, 490
Rana, S. R., 169
Randall, P. K., 389
Randerath, W., 431
Rando, T. A., 65, 145, 147, 148, 149, 151, 154, 156, 186
Rane, A., 52
Rane, S., 259
Ranjan, P., 277
Rankin, E. B., 11
Rankin, M., 375, 377
Rao, L., 191
Rapino, C., 99, 358
Rapino, M., 99, 358
Rapp, P. R., 389, 391
Rashedi, I., 143
Rasmussen, H. N., 67
Rasmussen, L. K., 470
Rasmussen, U. F., 67
Rasola, A., 68, 69
Ratan, R. R., 358, 359
Rathbone, C. R., 84
Rathel, T. R., 360
Rathinam, C., 147, 151, 170
Ratner, R. E., 374
Rattan, S. I., 221, 224, 247
Ratti, G., 360
Rau, K. M., 251
Rau, R., 164, 484
Rauchova, H., 51
Rauner, M., 387
Rautenberg, W., 466
Ravagnan, L., 71
Ravara, B., 85, 95, 96
Ravasi, T., 277
Ravid, T., 302
Ravikumar, B., 299, 307
Ravussin, E., 453, 456, 468
Rawles, J. M., 334
Rawson, E. J., 26
Rayasam, G. V., 250
Raymann, R. J. E. M., 464, 466, 472
Raz, N., 341, 466
Raza, S. R., 146, 281
Razzouk, L., 387
Rea, S. L., 286, 489
Read, V., 450
Reaper, P. M., 146
Reaume, A. G., 190, 195
Reaven, G. M., 27
Reaven, P. D., 128
Rebetz, J., 151
Rebrin, I., 96, 180, 184, 185, 186
Recchia, F. A., 29
Rechnitzer, P. A., 439
Reckelhoff, J. F., 168
Reda, T., 48
Reddan, W. G., 424, 433, 439
Redden, D. T., 178
Reddi, A. S., 34
Reddy, E. P., 78
Reddy, K. G., 349
Reddy, V. N., 191, 195
Redelman, D., 32
Redfield, M. M., 427
Redinger, R. N., 285
Redman, L., 453, 456
Redman, L. M., 30, 453, 456
Reed, B. R., 349, 357
Reed, J. C., 71, 72
Reed, M., 229
Reenan, R. A., 13
Regan, B., 30
Regitz-Zagrosek, V., 388, 389
Regrigny, O., 406, 407
Rehman, M., 254
Reichs, K. J., 392
Reick, M., 253
Reid, D., 327
Reid, L., 424, 430
Reijerkerk, A., 407
Reik, W., 144
Reiling, J. H., 205
Reimann-Philipp, U., 357
Reinberg, D., 254
Reinhard, C., 204
Reinhardt, J., 244, 253
Reinhardt, R. R., 30
Reinheckel, T., 304
Reinke, H., 262
Reinke, V., 286
Reinvang, I., 337
Reis, S. E., 169
Reiser, K., 439
Reiser, K. M., 35
Reiss, A. L., 334
Reissmann, S., 299, 300
Reiter, R. J., 185
Reitzschel, E. R., 168
Remahl, S., 338
Remington, L., 257
Remmen, H. V., 28, 489, 490
Remotti, H., 301
Remuzzi, G., 413
Ren, J., 70, 387
Ren, Z., 123, 124, 125
Renaud, S., 359
Renauld, H., 244

Renault, V., 84, 148
Renault, V. M., 155
Rendell, M., 472
Renken, C., 85, 95
Rennels, E. G., 30, 34
Renquist, D. M., 450
Repnevskaya, M. V., 12, 206, 220, 229
Requena, J., 190, 192, 193
Resnick, H., 123
Resnick, H. E., 373, 374
Resuello, R. G., 387, 389, 390, 392, 393, 395, 396, 397, 398, 413
Resuello, R. R., 397
Rettorri, V., 471
Retzlaff, B. M., 125, 376, 379
Revollo, J. R., 248, 249
Rex, T. S., 262
Reyes, A., 55
Reynisdottir, I., 378
Reynisdottir, S., 120, 121
Reynolds, B. A., 154
Reynolds, M., 324
Reynolds, M. A., 13, 392
Reynolds, T. H., 127
Reynolds, T. H. IV, 327
Reznick, A. Z., 165
Reznick, D. N, 485
Rezvani, K., 169
Rezzi, S., 27, 30, 392, 451
Rhee, J. G., 78
Rhee, S. G., 191
Rhodes, D. J., 387
Ribeiro, A. A., 167
Ribish, S., 250
Ricci, C., 69, 70, 77, 78
Ricci, T., 360
Riccio, O., 153
Rice, K. M., 73, 74, 76, 92
Rice, K. S., 390
Rich, G., 407
Richard, A., 337, 339
Richard, D., 231
Richards, J. B., 169
Richardson, A., 29, 56, 182, 186, 187, 189, 191, 193, 194, 300, 301, 490
Richardson, D., 169
Richardson, G. S., 473
Richardson, J. A., 301
Richey, H. K., 470
Richey, M. L., 470
Richie, J. P., Jr., 205
Richmond, A., 282
Richter, B., 170
Richter, T., 281
Rickert, W. S., 428
Ricklefs, R. E., 485, 486
Riddle, D. L., 204, 206, 207, 217, 223, 224, 226
Riddle, D. R., 31, 407
Rider, D. A., 304
Riders, M. E., 224
Ridker, P. M., 178, 179, 278, 282
Riekse, R. G., 357
Rier, S. E., 392
Riesen, M., 229
Riezman, I., 218
Rifai, N., 168, 169
Rigamonti, A. E., 325
Riggs, B. L., 283
Rigor, D. L., 72
Rigoulet, M., 220, 246
Rigual, R., 425, 426
Rikke, B. A., 284
Riley, K. P., 356
Rimessi, A., 99
Rincon, M., 224, 285
Rine, J., 224, 244
Ring, P., 351
Ringelstein, E. B., 349
Ringman, J., 341
Ringqvist, I., 387
Rink, L., 149, 281
Rinn, J. L., 388
Rinna, A., 300
Rintelen, F., 224
Riondino, M., 32
Riordan, M. M., 456
Rios, M., 125
Rios, R. M., 301
Riou, J. P., 121, 122
Rippe, C., 412
Riquelme, D. M., 281
Ris, F., 375
Risch, N. J., 340
Ristow, M., 16
Ritchie, C. S., 321
Ritz, B. W., 33
Ritz, P., 468
Ritzel, R., 373, 379
Rivadeneira, F., 244, 253
Rivard, A., 170, 358
Rivera, A., 260
Rivett, A. J., 303, 304
Rivetti, C., 304
Rizza, R. A., 27, 122, 127, 373, 379
Rizza, S., 258
Rizzato, G., 430, 435
Rizzi, C., 85, 95
Rizzo, G., 219
Roach, R., 349, 352
Robb, R. A., 283
Robbins, J. A., 353
Robbins, S. H., 392
Roberson, P. K., 324
Roberts, C., 408, 411
Roberts, C. M., 423, 435, 439
Roberts, J. A., 390, 392
Roberts, L. J., 490
Roberts, L. J. II, 29, 178, 189, 190, 192, 194
Roberts, S. A., 153
Roberts, S. B., 325, 454, 456, 457
Robertson, K., 229
Robertson, P. C., 433
Robertson, R. P., 375
Robin, D. A., 337
Robine, S., 153
Robinson, A., 13, 225, 247, 252
Robinson, A. R., 280, 284
Robinson, I. C., 27
Robinson, K. C., 145
Robinson, K. G., 97
Robinson, R. D., 439
Robinson, S., 439
Robitaille, P. M., 95
Roca, J., 437, 438
Roca, P., 96
Roca, V., 277
Rocca, W. A., 387
Roccella, E. J., 347
Rocha, J. S., 14, 26, 28, 29, 229
Rocheleau, J. V., 248
Rocher, A. B., 3, 282
Rockwood, K., 323
Rod, J. L., 322
Rodan, A., 10, 25, 222
Rodarte, J. R., 424, 433
Rodeheffer, R. J., 427
Roderick, H. L., 82
Rodgers, J. B., 390
Rodgers, J. T., 224, 247, 251, 262
Rodier, F., 125, 146, 280, 281, 282
Rodman, D., 122, 123, 469
Rodrigue, K. M., 341
Rodriguez, A., 195
Rodriguez, J. A., 124, 128
Rodriguez, M., 56, 190
Rodriguez, P., 168, 169
Rodriguez, Y., 341
Rodriguez-Borlado, L., 217
Rodriguez-Manas, L., 405, 410, 411, 413, 414
Rodriguez-Rodriguez, R., 410
Rodriguez-Roisin, R., 437, 438
Roebuck, S. J., 48, 51, 52
Roecker, E. B., 391, 392, 448, 450, 451, 489
Roevens, P., 244, 253, 258
Roewer, N., 407
Roff, D., 485
Rogers, A., 205, 206
Rogers, A. N., 13, 203, 205, 207, 208, 209, 227
Rogers, C. Q., 258, 260
Rogers, J., 390, 448
Rogina, B., 13, 16, 205, 217, 221, 224, 225, 229, 230, 246, 247, 250, 251
Rohrbach, S., 96
Rohrig, K., 129

Roig, E., 360
Rojas-Rios, P., 143
Rojeski, M., 30
Rojo, B., 425
Rokeach, L. A., 216
Rollo, C. D., 26, 28, 29, 30, 179, 183, 195, 491
Roman, G., 15, 16
Roman, R. J., 407
Roman, W. J., 94
Roman-Blas, J. A., 484
Romanic, A. M., 97
Romanick, M. A., 29
Romero, L., 433
Ron, D., 209, 301
Roncari, A. K., 129
Rondini, E. A., 281
Rongvaux, A., 249
Rood, J., 30, 258, 453, 456
Rood, J. C., 453, 456
Rooke, G. A., 471
Rooman, I., 375
Roos, D., 71
Roos, R. A., 353
Roosen, J., 12
Rooyackers, O., 68
Rooyackers, O. E., 68
Ropele, S., 351
Rosa-Neto, P., 412
Rosano, C., 354, 355, 356
Rose, A. B., 244
Rose, A. M., 204, 208
Rose, F., 358
Rose, G., 254
Rose, M. R., 13, 166, 205, 221, 300, 302
Rose, R. P., 30
Rose, S., 302
Rosen, A., 82
Rosen, C. J., 28
Rosen, E. D., 129
Rosenbaum, D. M., 353
Rosenbaum, M., 120, 124
Rosenbaum, S., 351
Rosenberg, I., 325
Rosenblatt, J. D., 94
Rosenbluth, J., 340
Rosene, D. L., 336, 337, 391
Rosenfeld, M. G., 26
Rosengren, A. H., 375
Rosenman, R. H., 119
Rosenstiel, P., 360
Rosentreich, D., 124
Roses, A. D., 282
Rosewell, I., 193
Rosin, A., 482, 487, 488
Rosner, L., 349
Rosoklija, G., 338, 341
Ross, A. J., 69
Ross, J., Jr., 97
Ross, J. L., 334
Ross, R., 119, 120, 122, 123, 169, 281
Ross, R. R., 433
Ross, W. R. D., 433
Rossetti, L., 225, 248, 251, 252, 256
Rossi, A., 439, 440
Rossi, D. J., 150
Rossi, F., 92, 98, 387, 389, 390, 393, 394, 395, 396, 397, 398
Rossi, S. R., 147, 151, 152
Rossig, L., 169, 256, 258, 358
Rossini, K., 95
Rossini, P. M., 352
Rossios, C., 251, 261
Rossiter, C. E., 424
Rossouw, J. E., 387
Rostami, A., 262
Rostrup, E., 351
Roszman, T. L., 33
Rota, M., 97, 99
Roth, D. L., 321
Roth, G. S., 28, 32, 34, 120, 229, 392, 449, 450
Roth, J., 30
Roth, J. A., 33
Roth, K. A., 78
Roth, S., 191
Roth, S. S., 450
Rother, J., 351
Rothman, D. L., 68
Rothrock-Christian, T. R., 357
Rothwell, P. M., 405
Roth-Yousey, L., 325
Rotnitzky, A., 123
Roubenoff, R., 122, 323, 327
Rouger, H., 285
Rous, P., 3
Roussou, I., 207
Roux, A. E., 216
Rovio, A. T., 55
Rovira, J., 230
Rowe, L., 485
Rowson, J., 166, 167
Roy, A., 277
Roy, N., 72
Roy, R. R., 65, 72, 78, 84, 94
Roy, S., 65, 149, 151, 154
Roynette, C., 119, 123
Rozovsky, I., 278, 280
Rozowsky, J. ., 388
Ruan, H., 219
Ruan, R., 304
Rubin, B. S., 389
Rubin, C. S., 129
Rubin, J., 277
Rubin, S., 425, 426
Rubin, S. M., 119, 123
Rubinsztein, D. C., 65, 66, 299, 307
Ruckenstuhl, C., 310
Rudin, E., 124, 224
Rudling, M., 413
Rudner, A., 217, 221, 222
Rudnicki, M. A., 147
Rudolph, K. L., 166, 168
Ruegg, M. A., 204, 231
Ruehle, K. H., 431
Ruff, F., 424, 433, 436, 437
Ruggeri, B. A., 26
Ruhf, M. L., 224
Ruitenberg, A., 356, 357
Ruiz-Pesini, E., 164, 170, 171
Rujescu, D., 181, 185
Rumpel, C., 321
Rumpler, W. V., 27, 450
Ruotolo, R., 304
Ruotti, V., 144
Rush, J. W., 259
Russell, E. S., 287
Russell, J. D., 358
Russell, M. J., 412
Russell, R. L., 223
Russo, A., 32
Rutherford, J. C., 219
Ruts, E., 119, 123
Rutter, J., 253
Ruvkun, G., 207, 208, 217, 218, 219, 220, 222, 223, 228
Ryan, D. H., 120, 121, 127, 128
Ryan, M. J., 67, 70, 72, 75, 85, 88, 89, 94, 413
Ryan, N. A., 149
Ryan, S. F., 427, 429, 436, 438
Ryazanov, A., 308
Rychlik, M., 436
Ryder, S. D., 285
Rydstrom, J., 53, 58

S

Saarinen, M., 286
Saavedra, J. M., 360
Sabatini, D. M., 10, 14, 30, 35, 204, 207
Sabbadini, R., 85, 95
Sabia, M. R., 29
Sablitzky, F., 85
Sabourin, G., 248
Sabourin, L. A., 147
Saccani, S., 32
Sacchi, N., 387
Sacco, A., 148
Sachdev, P., 355
Sachithanandan, T., 437
Sack, M. N., 168
Sacks, D. B., 283
Sadek, H. A., 304
Sadoshima, J., 99, 258, 259, 392
Saez-Cassanelli, J. L., 32
Safieh, B., 33
Sager, J., 4, 15, 217, 218, 219, 221
Sager, R., 167
Sagnella, G. A., 169

Author Index

Saha, A. K., 251
Saha, M., 170
Sahakian, B. J., 357
Sahar, S., 248, 249, 253, 262
Saher, G., 340
Sahin, M., 209
Saikumar, P., 70, 71
Saillan-Barreau, C., 121, 124, 128
Saisho, Y., 375
Saito, K., 181, 186
Saito, S., 225
Sakahira, H., 298, 300
Sakai, Y., 305
Sakano, Y., 221
Sakharov, D. V., 68
Sakiyama, N., 405, 410
Salas-Salvadó, J., 285
Salat, D. H., 349
Salazar, V., 32
Sale, D. G., 94, 96
Saleem, A., 68
Saleh, M., 66
Salem, N., Jr., 31
Salford, L. G., 151
Salgin, B., 27
Saliba, S., 261
Salih, D. A., 155
Salio, M., 413
Salive, M. E., 353
Salloway, S., 357
Sallstrom, J., 357
Salminen, A., 260
Salmon, A., 29
Salmon, A. B., 29, 56, 187, 188, 190, 193, 195
Salmon, M., 281
Salpea, K. D., 284
Salsgiver, W. J., 27
Salthouse, T. A., 338, 339
Salto-Tellez, M., 256
Saltzman, E., 453, 454, 456, 457
Salvador, C., 191
Salvador, N., 306
Salvesen, G. S., 71, 72
Salvi, M., 54
Salvi, R. J., 190, 195
Salvioli, S., 280, 281
Samad, F., 123
Samali, A., 82, 83
Samani, N. J., 169
Samaniotto, S., 33
Samant, S. A., 254, 260
Samaras, K., 121, 122
Samaras, T. T., 26, 491
Sambasivan, R., 145
Sambhara, S., 277
Samper, E., 166, 167
Samson, M. M., 32
Samuelsson, B., 194
Sanadi, D. R., 5
Sananbesesi, F., 251, 262
Sancak, Y., 204
Sanchez-Pino, M. J., 179, 182, 195
Sanden, S. K., 34
Sanders, L., 139
Sanders, L. K., 85
Sanderson, W., 119
Sandig, G., 298
Sandmand, M., 77, 78
Sandoval, I. V., 305
Sandri, M., 85, 95
Sandy, M. S., 388
Sang, Y., 144
Sanges, D., 84
Sanjay, A., 301
Sanna, M. G., 72
Sannikov, I., 222
Sano, M., 356
Santana, L. F., 56
Santelli, J., 5
Santos, J. L., 283
Santos, R. A. S., 413
Sanz, A., 29, 489, 491
Sanz, P. R., 358
Sapey, E., 278, 281
Sapin, V., 13, 205, 206, 217, 218, 226
Sapolsky, R. M., 285
Saprin, A. N., 53
Sarangarajan, R., 96, 97, 98
Sarbassov, D. D., 10, 204
Sarewicz, M., 51
Sarge, K. D., 300
Saris, N. E., 82
Sarkar, S., 307
Sarwar, N., 278
Sasahara, T. H., 167
Sasaki, H., 283, 407
Sasaki, K., 259
Sasaki, R., 128
Sasaki, S., 70
Sasaki, T., 227
Sasaki, Y., 261, 262
Sass, M., 230
Sassone-Corsi, P., 248, 249, 253, 262
Sastre, B., 256
Sastre, J., 68, 70, 388, 489, 490, 491
Sathiavageeswaran, M., 32
Satinoff, E., 472
Sato, K., 121, 251, 408
Sato, M., 33
Sato, N., 392
Sato, S., 194
Sato, T., 71
Satofuka, S., 260
Satoh, M., 170
Satoh, T., 351
Sattar, N., 278
Satz, P., 339
Saucedo, L. J., 10
Saucer, B., 27
Saumon, G., 427, 437, 438
Saunders, A. M., 282, 340, 357
Saunders, T. J., 119, 120, 122, 123
Saupe, K. W., 440
Saurbier, B., 405, 410
Sautet, A., 148
Sauve, A. A., 245, 249, 250
Savage, M. V., 471
Savage, P. J., 353
Savino, W., 33
Savinova, O. V., 68
Savoia, C., 413
Sawada, T., 379
Sawar, A., 357
Sawaya, G., 391
Sawyer, P., 321
Saxena, R., 378
Saxman, J. H., 387
Sayed, D., 259
Scadden, D. T., 168
Scaffidi, C., 82
Scalbert, E., 406, 407
Scalera, F., 412
Scanniffio, D., 325
Scarani, P., 428
Schaap, L. A., 77, 78
Schachar, R., 337, 338, 339
Schachar, R. J., 337, 338, 339
Schächter, F., 285
Schadt, E. E., 171, 388
Schaeverbeke, J., 34
Schafer, F. Q., 53
Schafer, M. R., 341
Schaffer, S. W., 69, 70, 77, 78
Schaie, K. W., 339
Schally, A. V., 28
Schambelan, M., 32
Scharenberg, A. M., 245
Schauer, A., 310
Schechter, C. B., 285
Schechtman, K. B., 453, 455, 456
Scheer, B. T., 183
Scheffler, J., 392
Scheid, P., 438
Scheller, M., 151
Scheltens, P., 337
Schenk, S., 230, 248, 250, 251, 258, 260, 262, 263
Schenke, W. H., 169
Schenker, M., 435
Schenker, M. B., 433
Schenker, N., 333, 334
Schenkman, K. A., 70
Scheper, W., 299, 301
Scher, M. B., 254
Scherbov, S., 119
Scherder, E. J. A., 464, 466
Scherer, P. E., 285
Scherrer, M., 437
Schett, G., 283
Scheurich, P., 77
Schick, F., 244, 253

Schiegl, T., 169
Schiffrin, E. L., 412, 413
Schilder, D. P., 435
Schindelhoz, B., 10
Schisler, J. C., 297, 298
Schlattner, U., 194
Schlauch, K., 119, 122, 123, 124, 126
Schlicker, C., 254, 255
Schlittler, D. L., 491
Schmechel, D. E., 340, 357
Schmeissner, P. J., 221
Schmidt, A., 204
Schmidt, A. M., 283
Schmidt, D. H., 322
Schmidt, H., 304
Schmidt, H. H. H. W., 410, 413
Schmidt, M. T., 245, 249, 302
Schmidt, P. J., 389
Schmidt, R., 337, 351
Schmidt, S., 407
Schmidt-Glenewinkel, T., 304
Schmidt-Lucke, C., 169
Schmitt, E. M., 257
Schmitt, F. A., 357
Schmitz, H. H., 359
Schmutz, S., 64
Schneider, E. L., 300
Schneider, M., 191, 192
Schneider, P., 72
Schneiderbanger, K., 68
Schnellmann, R. G., 250, 251
Schnoedt, B., 122
Schnolzer, M., 261
Schnyer, D. M., 350
Schoefiled, K., 391
Schoeller, D. A., 451
Schoenberg, J. B., 435
Schoenemann, P. T., 333, 334
Schofield, B., 428, 433
Schokrpur, S., 280
Schoonjans, K., 72, 224
Schorderet, P., 142
Schork, N. J., 168
Schorr, A. B., 122
Schrader, L. I., 411, 412, 413
Schrag, M., 31
Schrager, M., 64
Schramm, V. L., 245, 249
Schrantz, N., 72
Schrattenholz, A., 298
Schrauwen, P., 127
Schreibelt, G., 407
Schreiber, S. L., 204, 205
Schreiner, P. J., 169
Schriner, S. E., 56, 187, 188, 189, 218
Schröder, A. K., 281
Schroder, M., 83
Schroeck, H., 408
Schroeder, I., 51
Schroeder, T., 169
Schroll, M., 77, 78
Schubert, A., 69
Schubert, R., 195
Schug, T. T., 258, 261
Schuler, A. D., 258
Schuler, G., 96
Schulman, S. P., 98
Schulthess, F., 377
Schultz, E., 94, 148
Schultz, P. G., 304
Schultzberg, M., 406, 407
Schultz-Darken, N. J., 482
Schulz, A., 69, 70
Schulz, T. J., 16
Schulz, V. P., 151
Schulze, K. L., 262
Schulze, M. B., 123
Schulze, P. C., 96
Schumacher, B., 280, 284
Schumacker, P. T., 52, 258
Schumann, D. M., 377
Schummer, M., 206
Schupf, N., 170, 171
Schuurman, T., 357
Schwab, C., 280
Schwark, H. D., 464
Schwartz, M. W., 124, 376
Schwartz, R. S., 324, 379
Schwartz, S., 392
Schwartz, S. M., 388
Schwarz, M., 81
Schwarze, S. R., 392
Schwer, B., 244, 248, 254, 255, 256, 258
Schwerzmann, J. W., 277
Sciartilli, A., 358
Scola, L., 285
Scorrano, L., 68, 69, 83
Scott, D. K., 33
Scott, G. K., 280
Scott, L. J., 378
Scozzafava, J., 30
Scrofano, M. M., 304
Seale, P., 147
Seals, D. R., 412, 413, 434, 439
Seaman, M., 226, 308
Sebastian, C., 124
Sebban, C., 392
Sedivy, J., 64
Sedivy, J. M., 125
Seed, W. A., 423, 435, 439
Seeger, W., 358
Segall, P. E., 14, 222
Segawa, K., 249
Segre, J. A., 143, 145
Sehgal, S. N., 203, 226
Sehl, M., 222
Seidinger, D., 170
Seidman, A., 97
Seifert, A., 143
Seifert, E. L., 248
Seiler, A., 191
Seita, J., 147, 151, 152
Seki, H., 128
Seki, T., 128, 154
Sekiguchi, C., 65
Sekimoto, H., 128
Sekoguchi, S., 168
Selkoe, D. J., 356
Sell, C., 26
Sell, D. R., 35
Sell, H., 124
Selman, C., 14, 30, 67, 120, 208, 209, 217, 224, 381, 485, 491
Selmer, R. M., 491
Seluanov, A., 167
Semba, R. D., 83
Sembai, R., 324
Semeins, C. M., 122
Semendeferi, K., 333, 334
Semenza, G. L., 208, 358
Semigran, M., 97
Semmens, M., 427
Sempos, C. T., 123
Sempowski, G. D., 33, 279
Senawong, T., 252
Sengupta, K., 256, 257
Sengupta, M., 484
Senjem, M. L., 341
Senokuchi, T., 259
Senoo-Matsuda, N., 72
Senson, B., 482
Sentman, M. L., 195
Seo, A. Y., 68, 86, 87, 95, 96, 281, 284, 489, 491
Seo, E. J., 3, 14, 203, 207, 222
Seo, Y., 407
Seow, C. K., 432, 433
Seow, K. C., 424, 433, 439
Sepe, A., 124, 129
Seplaki, C. L., 278
Seppala-Lindroos, A., 123
Sepulcre, J., 355
Sequeira, J., 261
Sercombe, R., 349, 352
Serdula, M. K., 324
Seremet, T., 33
Sergent, S., 353
Sergent, S. M., 353
Serikawa, K. A., 206
Seror, I., 3, 282
Seroude, L., 72
Serrador, J. M., 349, 350
Serrano, M., 13, 248, 251, 256, 260, 261
Serrano, R., 124, 128
Serrill, L., 437
Servais, S., 68
Seso-Simic, D., 65
Sessler, D. I., 470
Sethares, C., 336, 337, 338, 339, 340

Setser, J., 34
Sevini, F., 281
Sevransky, J. E., 440
Seweryniak, K., 260, 261, 262
Sexton, V. L., 392
Seylaz, J., 349, 352
Seymour, F., 244
Sfeir, A., 165
Sgaramella, G., 97
Shabalina, I. G., 55
Shackelford, D. B., 204
Shackleton, M., 144
Shadel, G. S., 230
Shadrach, J. L., 151, 156
Shaffer, M. L., 359
Shafi, N. I., 408, 409, 410
Shah, S., 359
Shak, S., 97
Shaltouki, A., 85
Sham, A., 324
Shama, S., 300
Shamieh, L. S., 4, 7, 11, 226, 227, 230
Shamji, A. F., 204, 205
Shan, Y., 302
Shang, F., 302, 304
Shankland, E. G., 68
Shanley, D. P., 243
Shanmuganayagam, D., 27, 30, 392, 451
Shannon, K. M., 360
Shannon, S., 261, 263
Shao, J., 252
Shaoguang, J., 306
Shaper, A. G., 123, 324
Shapiro, S., 358
Shapses, S. A., 450
Shargill, N. S., 14, 125
Sharifi-Azad, S., 95
Sharma, P., 250
Sharma, S., 31
Sharp, F. R., 358
Sharp, Z. D., 120, 209, 218, 226, 309, 310, 381
Sharpe, G. M., 349, 410, 411, 413
Sharpless, N. E., 163, 300, 301
Shaul, Y. D., 204
Shaw, C. A., 150, 151
Shaw, L. K., 169
Shaw, P., 334, 336
Shaw, P. X., 277
Shaw, R. J., 4, 7, 8, 9, 10, 208, 221, 229
Shaw, S., 358
Shaw, W. M., 5
Shay, J. W., 163, 165, 166, 167
Shay, K. P., 185
Shea, R. J., 249
Sheaffer, K. L., 10, 208
Shedden, K. A., 34
Sheehan, A. E., 262
Sheehan, H. M., 349
Sheehan, M. J., 333, 334
Shefer, G., 149
Sheffield-Moore, M., 122, 123
Sheikh, K. H., 457
Shen, J., 149, 156
Shen, Q., 411
Shen, Y. T., 92, 98, 392, 393, 395, 396, 397, 398
Shen, Z., 258, 260
Shepherd, J., 169
Sheridan, P., 178, 179
Sherman, J. M., 244
Sherman, P. W., 485
Sherrill, D. L., 436
Sherwin, B. B., 389, 391
Sherwood, J. B., 359
Shesely, E. G., 408
Shetty, A. K., 155
Shetty, P. S., 183
Shi, J. J., 65, 66
Shi, T., 254
Shi, X., 251
Shi, Y., 71, 72, 81, 186, 187, 286, 326
Shibasaki, H., 352
Shibata, D., 154
Shibata, M., 308, 351
Shibatani, T., 191, 302
Shideler, S. E., 390
Shieh, J. C., 251
Shieh, P. C., 251
Shiesh, S.-C., 328
Shigenaga, M. K., 67, 194
Shih, H. C., 358
Shih, V. F., 68
Shih, W. J., 357
Shilatifard, A., 220
Shiloh, A., 13, 224, 254, 255
Shimazu, T., 248, 256
Shimizu, I., 125, 285
Shimizu, K., 390
Shimizu, S., 71
Shimizu, T., 121, 191
Shimomura, I., 123, 125
Shimosato, T., 408
Shimosegawa, E., 351
Shimoyama, T., 128
Shin, H. K., 407, 408
Shindler, K. S., 262
Shindo, M., 217, 219, 220, 228
Shinobu, L. A., 194
Shinoda, K., 195
Shiozaki, E. N., 71
Shiozaki, T., 351
Shiraishi, I., 97
Shirayoshi, Y., 258
Shiromani, P., 473
Shively, C. A., 389, 390
Shnier, R., 355
Shock, N. W., 430, 431, 434, 435, 436, 437
Shoemaker, J. E., 4, 7, 9
Shofer, J. B., 376, 379
Shoffner, J. M., 67
Shoji, W., 190, 191
Shore, D., 244
Short, D. K., 85
Short, K. R., 55, 68, 85
Short, R. A., 392
Shoubridge, E., 218
Shoyama, S., 466, 470
Shroff, S., 340
Shroyer, T., 217, 222
Shuldiner, A. R., 285
Shulman, G. I., 251, 253
Shulmeister, V. M., 51
Shumaker, S. A., 389
Shuster, L. T., 387
Shuster, M. W., 327
Sibley, C. G., 448
Sicinska, E., 377
Sick, A., 262
Siddals, K. W., 128
Siddiq, A., 359
Siddle, K., 30
Sidney, S., 169
Sidorov, I., 168
Sieburg, H. B., 150
Siegel, P., 166
Siegmund, K. D., 154
Sielezneff, I., 256
Siems, J. E., 349
Siems, J. W., 406, 412
Sierra, A., 280
Sierra-Dominguez, S. A., 32
Sies, H., 185
Sievers, C., 52
Sifri, C. D., 16, 286
Siggeirsdottir, K., 355, 356
Sigler, R., 14, 205, 222
Sigmond, T., 208, 230, 308
Sigmund, C. D., 407, 408, 410, 411, 412, 413, 415
Signer, R. A., 150, 156
Sigurdsson, S., 355, 356
Silage, D. A., 425, 426, 437, 439
Silberberg, R., 35
Silva, E., 298, 302
Silva, J. P., 55
Silver, A. E., 411, 413
Silver, I., 193
Silver, L. E., 405
Silver, M., 358
Silver, P. A., 220
Silvestre, R., 256
Simeoni, S., 250
Simic, G., 65
Simili, V., 67
Simillion, C., 281
Siminovitch, L., 143
Simmons, D. M., 26
Simoes e Silva, A. C., 413

Simon, E., 466
Simon, G., 424, 430
Simon, J., 32
Simon, M. C., 52
Simoneau, J. A., 123
Simons, B. D., 145
Simonsen, A., 308, 309
Simonsick, E. M., 77, 78, 123, 324, 353
Simpson, J. E., 352
Sims, C., 389
Sinbonmatsu, T., 388
Sinclair, A., 281
Sinclair, D., 244
Sinclair, D. A., 12, 206, 207, 216, 220, 225, 237, 244, 245, 246, 247, 249, 254, 255, 257, 258, 259, 261, 263
Singer, H. S., 334
Singer, T., 339
Singh, K. V., 16
Singh, N. A., 64
Singh, R., 55, 68, 300
Singh, R. K., 169
Sinha Hikim, A. P., 73, 74, 75, 76
Sinkey, C. A., 349
Sipos, W., 387
Sirfi, C. D., 16, 221
Sirota, N. P., 179, 183, 195
Sirotina-Meisher, A., 33
Sirry, B., 204
Sistonen, L., 300
Sitte, N., 302, 304
Siu, P. M., 64, 67, 68, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 84, 85, 86, 87, 88, 89, 90, 91, 94, 95, 100
Sivas, A., 298
Sivatchenko, A., 169, 170, 171
Siwek, D. F., 190
Sjoberg, M., 388
Skaletsky, H., 388
Skepper, J. N., 411
Skillington, J., 122
Skjaerven, R., 483
Skoog, C., 428, 430
Skoog, I., 360
Skorupa, D. A., 13, 15
Skovbro, M., 55
Skurnick, J., 167, 168
Skurnick, J. H., 168
Skvortsov, D., 286
Skytthe, A., 287, 488
Slagboom, P. E., 170
Slagle, B. L., 257
Slama, J. T., 245
Slatin, R. C., 424, 429
Slawik, M., 122
Sloan, L. A., 300
Sloane, J. A., 338, 340
Slomianny, C., 70
Slosman, D. O., 122
Slowing, K. V., 257
Smirne, C., 285
Smit, J. H., 282
Smith, A. C., 187, 188, 189
Smith, A. R., 185
Smith, B. C., 245, 249, 250
Smith, C., 453, 456
Smith, C. A., 72, 75, 76, 84, 85, 94
Smith, C. L., 71
Smith, D., 27
Smith, D. E., 179, 180, 181, 182, 185, 304
Smith, D. L., 229
Smith, D. L., Jr., 220, 247, 249
Smith, D. W. E., 487
Smith, E. D., 4, 5, 7, 9, 10, 11, 12, 15, 216, 217, 218, 219, 220, 221, 225, 226, 227, 228, 229, 231
Smith, E. E., 352
Smith, E. J., 185
Smith, E. K., 183
Smith, J., 408, 410
Smith, J. E., 473
Smith, J. J., 230, 248, 250, 251, 262, 263
Smith, J. M., 221
Smith, J. S., 220, 224, 229, 247, 249
Smith, J. V., 453, 456
Smith, K., 260
Smith, K. J., 338
Smith, K. R., 169, 170, 171
Smith, M. I., 64
Smith, M. J., 379, 380, 382
Smith, P. J., 129
Smith, R. A. J., 184
Smith, R. G., 32
Smith, S. A., 139
Smith, S. R., 125, 453, 456
Smith, S. Y., 391
Smith, U., 123, 125
Smith, W. L., 359
Smithmyer, S. L., 387
Smith-Wheelock, M., 14, 205, 222
Smolander, J., 468, 469
Smolen, J., 283
Smucny, D. A., 482
Smuga-Otto, K., 142
Smulders, N. M., 469
Smyth, E. M., 388
Smythe, G. M., 147, 149, 151
Snibson, K. J., 34
Snih, S. A., 323
Snijders, A. H., 353
Snitker, S., 468
Snook, J. T., 468
Snowdon, D. A., 356
Snow-Harter, C., 94
Snyder, M., 388
Sobering, A., 208
Sobey, C. G., 408, 410, 413
Sobin, S. S., 427, 428, 429, 436, 438, 439
Sobol, B. J., 427, 437
Socotch, T. M., 353
Soda, M., 122, 123
Sohal, B. H., 247, 284
Sohal, R. S., 51, 54, 96, 179, 180, 182, 184, 185, 186, 218, 247, 284, 490
Sokoloff, L., 358
Solaini, G., 410
Solana, R., 149
Solaro, R. J., 97
Soler, J., 375
Solerte, S. B., 325
Solfrizzi, V., 285
Solinas, E., 437, 438
Solis, J., 405, 410, 411, 413, 414
Solomon, E., 306
Solomon, J. M., 250, 256
Solt, A. C., 286
Somay, G., 277
Someya, S., 55, 69, 70, 74, 92, 98
Sommer, T., 301
Sonenberg, N., 204, 205
Song, S., 254
Song, W., 56, 73, 74, 76, 85, 86, 87, 89, 92, 93, 95, 96, 98, 185, 187, 188, 191, 195
Song, Y., 52
Song, Y. M., 491
Sonne, M. P., 382
Sonnenblick, E. H., 96, 98
Sonntag, W. E., 26, 27, 30, 31, 41, 407, 457
Sonoda, N., 251, 258, 260
Sonu, R., 150
Sonzogi, A., 413
Soong, W. E., 307
Sooparb, S., 306
Soos, M. A., 30
Sorbini, C. A., 427, 437, 438
Sorci, L., 261
Sorensen, T. I., 488
Sorenson, R. L., 27
Sornson, M. W., 26
Sorond, F., 349, 360
Sorond, F. A., 349, 350, 358, 359
Soro-Paaconen, A., 123
Soscia, S. J., 277
Soskic, V., 298
Sostin, C., 283
Sotelo, A. I., 26, 34
Soti, C., 301
Sotiropoulos, A., 208
Soulard, A., 12, 204, 205, 207, 217, 227
Sovak, G., 307, 309
Sovijarvi, A., 123
Sowell, E. R., 334, 337
Spadaccio, C., 64

Spalding, K. L., 126
Spangler, E. L., 32
Sparkman, N. L., 280
Sparla, F., 50
Sparrow, D., 434
Speakman, J. R., 54
Speck, N. A., 169
Spector, A., 191
Speer, D. L., 32
Speil, A. O., 169
Speizer, F. E., 423, 424, 435
Spelbrink, J. N., 55
Spencer, W. D., 466
Sperling, G., 3, 14
Spiegelman, B. M., 123, 124, 125, 224, 247
Spierings, D., 66
Spiess, C., 299, 300
Spiess, J. P., 437
Spina, M., 428
Spindler, S. R., 16, 29, 216, 222, 301
Spinetti, G., 413
Spiteller, G., 54
Splett, P. L., 325
Spradling, A., 144
Sprenger, M., 285
Sprick, M. R., 78, 81
Sproul, M. S., 95
Sprung, J., 434, 439
Spycher, C., 204, 208
Spyridopoulos, I., 281
Squier, T. C., 82
Squire, M., 244
Srikanth, V., 353, 355, 356
Srinivasan, A., 82
Srinivasan, S. R., 166, 168, 169
Srinivasula, S. M., 71, 72
Sroczynska, P., 169
Stacey, M., 151
Stacy, S., 392
Stadtman, E. R., 54, 190, 192, 193, 302, 304
Stadtman, T. C., 192
Staessen, J. A., 168, 169
Staff, R. T., 353
Stahl, W., 185
Stallard, E., 484
Stallock, J. P., 204
Stamatas, G., 221
Stanfel, M. N., 226, 227, 230
Stanford, C. B., 285
Stanford, W. L., 71
Stange, D. E., 153
Stanger, B. Z., 153
Stannard, B., 27
Stapleton, J., 353, 355, 356
Stappenbeck, T. S., 145
Starai, V. J., 224
Starcher, B. C., 428
Stark, D., 151
Stark, P. C., 453, 456, 457
Starkov, A. A., 52
Staron, R. S., 65
Starr, J. M., 170, 171, 353
Stassi, G., 97
Staten, N. R., 27
Statt, M., 94
Stauber, P. M., 119
Stauffer, B. L., 168, 169, 170
Staveley, B. E., 224
Stavinoha, W. B., 277
Stearns, S. C., 216, 286, 485
Stebbins, J., 224
Steegborn, C., 254, 255
Steele, A. D., 4, 5, 7, 9, 10, 11, 16, 230, 247, 248
Steenbergen, C., 388
Steensland, P., 31
Stefan, N., 122
Stefanadi, E., 281
Stefani, F. H., 68
Stefano, G. B., 357
Steffen, K. K., 4, 5, 7, 9, 10, 12, 206, 207, 216, 217, 218, 220, 226, 227, 228, 229, 230
Steffen, M. J., 392
Steger, R. W., 26, 29, 30, 31, 34
Steger-May, K., 453, 455, 456
Steinberg, G. R., 285
Steinberg, J. D., 473
Steinberg, S. F., 99
Steinbrich, W., 351
Steiner, S., 170
Steiner, V., 72
Steinkraus, K. A., 4, 7, 11, 216, 231
Steinthorsdottir, I., 378
Stek, M. L., 282
Stemper, B., 349, 352
Stenman, G., 167
Stennicke, H. R., 71, 72
Stephanou, N. C., 71
Stephens, J. W., 284, 286
Stephenson, C., 169
Steppan, C. M., 129
Stern, J. S., 34
Stern, N., 27
Stern, Y., 356, 391
Sternglanz, R., 224, 245
Stevens, S. M., Jr., 300
Stewart, A. S., 97
Stewart, C. E., 78
Stewart, E. J., 216
Stewart, J., 32
Stewart, J. A., 428
Stewart, M. J., 205
Stewart, S. B., 336, 337, 339, 340, 341
Stice, J. P., 490
Sticker, M., 120, 205, 209, 231
Stickland, M. K., 421, 433, 438, 439
Stienstra, C. M., 357
Stieren, E., 254
Stiles, J. W., 5
Stingl, J., 144
St Leger, A. S., 359
Stock Conference Working Group, 284, 285
Stocker, H., 10, 205, 224, 229
Stoclet, J. C., 360
Stoeter, P., 341
Stoffers, D. A., 375
Stoler, M. H., 358
Stone, D., 280
Stone, K. C., 323
Stopka, P., 51
Stoquart-El Sankari, S., 407
Storgaard, H., 382
Storm, T., 69, 70
Storms, L. H., 26, 491
St-Pierre, J., 48, 51, 52
Strasser, A., 78
Stratford, F. L., 303, 304
Strathearn, K. E., 262
Strathern, J. N., 244
Stratmann, M., 262
Strawford, A., 126
Stray-Gundersen, J., 94
Streeper, R. S., 254, 255
Strehle, A., 230, 251, 252, 258
Striet, J. B., 208
Strieter, R. M., 127, 128
Striker, G. E., 34, 277, 283, 285
Striker, L. J., 29, 34
Strindhall, J., 33, 77
Strittmatter, W. J., 340, 357
Strle, K., 32
Strom, A., 452
Strong, M. A., 165, 166
Strong, R., 120, 191, 195, 209, 218, 226, 300, 301, 309, 310, 381, 489, 491
Stroobant, N., 349
Strotman, B. A., 64, 71, 95
Struble, R. G., 391
Strum, J., 282
Stuart, J. A., 194
Stubbs, R. J., 468
Studenski, S. A., 323, 354, 355
Stunkel, W., 256
Sturgill, J. F., 224, 248, 255, 256, 261, 262
Sturgis, I., 52
Sturnvoll, M., 122
Sturtz, L. A., 12, 220
Su, F., 13, 224, 254, 255
Su, G., 357
Su, L., 378
Su, Y. D., 221
Suchankova, G., 251
Sudarsky, L., 353
Sudo, K., 150
Sueishi, K., 279, 281
Suenaga, T., 352

Suganami, T., 128
Sugawara, A., 412
Sugihara, T., 428
Sugiyama, S., 55
Sugiyama, Y., 185
Suh, H., 83, 145, 154
Suh, Y., 28, 164, 195, 257, 277, 280, 284, 285
Sulaiman, M., 251, 259, 282
Sullivan, D. H., 324
Sullivan, E. V., 334
Sullivan, P. M., 285
Sulston, J. E., 64
Sultzer, D., 337
Sumandea, M., 97
Sumien, N., 179, 180, 182, 184, 185
Sun, C., 251
Sun, D., 34
Sun, G., 219
Sun, H., 34, 218, 349, 410, 411, 413
Sun, J., 51
Sun, K., 83
Sun, L., 29
Sun, L. Y., 31
Sun, X. M., 66
Sun, Y., 32, 125, 256, 282, 411
Sun, Z. W., 357
Sundaram, M., 357
Sundaresan, N. R., 251, 254, 259, 260, 282
Sundermeyer, M. L., 222
Sundquist, A. R., 185
Sung, B., 96, 260, 412
Sung, J., 491
Sunga, J., 4, 5, 7, 10
Suo, Z., 357
Supiano, M. A., 127, 327, 382
Supinski, G. S., 430
Surapureddi, S., 258, 261
Surdelescu, G. L., 169
Susin, S. A., 71
Susulic, V. S., 123
Sutphin, G. L., 4, 5, 7, 11, 216, 220, 231
Sutter, N. B., 26
Sutton, A. J., 222, 224
Sutton, J. R., 94, 96
Suuronen, T., 260
Suzkuki, K., 16
Suzuki, K., 262
Suzuki, N., 184
Suzuki, Y., 71
Suzuki-Migishima, R., 306, 309
Svanborg, A., 405
Svensson, J., 194
Swaab, D. F., 464, 466, 472, 473
Swanson, L. W., 26
Swanson, S. C., 68
Swatkoski, S., 245
Sweeney, L. B., 224, 248, 255, 256, 261, 262
Sweeting, H., 484
Swihart, A., 339
Swindell, W. R., 29, 284, 301
Swinscoe, J. C., 34
Swisher, A. K., 85
Syed, G. M., 357
Symanski, M., 357
Syner, J. C., 423, 435, 436
Synetos, A., 281
Syntichaki, P., 208
Szabadkai, G., 219
Szabo, C., 411
Szabo, Z., 248, 249
Szakai, A. K., 277
Szalai, A. J., 277
Szary, A., 33
Szathmary, E., 391
Szczepaniak, L. S., 122
Sze, G., 337
Szegezdi, E., 82, 83
Szekely, T., 488
Szewczyk, N. J., 4, 5, 7, 10
Szmit, E., 279
Szoke, E., 379
Szweda, L. I., 298, 302, 304
Szweda, P. A., 304
Szymanski, M., 357

T

Taaffe, D. R., 77, 78
Tabtiang, R., 217, 221, 222
Tack, M., 425, 426
Taddei, F., 216
Tafesse, U., 336
Tafrov, S. T., 224
Tager, M., 412
Tagliamonte, M. R., 32
Taguchi, A., 217, 224
Taillandier, D., 66, 148
Tainsky, M. A., 258
Tajbakhsh, S., 145
Takacs, K., 51
Takacs-Vellai, K., 10, 207, 208, 218, 226, 230, 308
Takagi, G., 387, 392, 394, 395, 413
Takagi, M., 337
Takahashi, K., 128, 142
Takahashi, M., 122
Takahashi, R., 190, 191, 300, 301
Takahashi, S., 385
Takahashi, Y., 170
Takahashi-Niki, K., 71
Takahasi, R., 71
Takaku, K., 192
Takakura, M., 168
Takanashi, M., 358
Takanishi, C. L., 245
Takano, H., 190, 191
Takano, S., 302
Takao, M., 337
Takata, S., 407
Takata, T., 259
Takayama, M., 28
Takayanagi, R., 184
Takeshige, K., 184
Takeuchi, K., 412
Takono, S., 325
Takubo, K., 147, 152
Takubo, Y., 428
Tal, I., 122, 123, 469
Talbot, L. A., 64
Tall, A. R., 259
Tallal, P., 334
Talloczy, Z., 226, 308
Talmud, P. J., 284
Tam, W., 277
Tamaki, T., 84, 94
Tamame, M., 227
Tamminen, M., 123
Tan, G., 120, 124, 125
Tan, J. M., 307
Tan, K. T., 281
Tan, M. W., 286
Tan, Q., 300
Tan, Y. C., 250, 256, 258, 260
Tanabe, T., 358
Tanahashi, N., 337
Tanaka, D., 254
Tanaka, H., 165
Tanaka, K., 300, 302, 303, 304
Tanaka, M., 249, 299, 385, 473
Tanaka, N., 302
Tanaka, T., 33, 65
Tanaka, Y., 340, 359
Tandon, R., 349
Tang, M. X., 170, 171, 391
Tang, Q. Q., 129, 283
Tang, S. C., 283
Tang, T., 354
Tang, W., 169
Tang, X. D., 219
Tang, Y., 250, 255, 256, 338, 354
Tangen, C. M., 323
Tanguay, R. M., 300, 302
Tanida, I., 306, 309
Tanimoto-Koyama, K., 128
Tankersley, C. G., 428, 433, 471, 472
Tannenbaum, A., 231
Tanner, K. G., 224, 245
Tannock, R., 337, 338, 339
Tanny, J. C., 245, 249, 250
Tanriverdi, K., 121
Tansky, M., 121, 127
Tantucci, C., 439, 440
Tanzi, R. E., 287
Tapia-Arancibia, L., 472
Tardif, S. D., 390
Tarin, D., 195
Tarlton, A., 262
Tarnopolsky, M. A., 55, 67, 69, 70, 94

Tarry-Adkins, J. L., 455
Tasdemir, E., 308
Taskinen, M. R., 123
Tatar, M., 9, 12, 13, 16, 26, 28, 217, 218, 221, 224, 230, 250, 251, 300, 447, 479, 482, 485, 486, 489, 491
Tataranni, P. A., 468
Tate, D. F., 352
Tatemichi, T. K., 356
Tatsumi, N., 165
Taub, D., 32
Taub, D. D., 278
Taubert, S., 4, 7, 9, 12, 205, 206, 207, 208, 217, 227, 229, 230
Taupin, P., 154, 155
Tavare, S., 154
Tavares, R., 357
Tavernarakis, N., 12, 208, 308
Tawa, M., 408
Taylor, A., 300, 302, 304
Taylor, B. C., 323
Taylor, M. B., 464
Taylor, N. A. S., 423, 426, 429, 430, 431, 432, 433, 436, 439
Taylor, V., 145
Taylor-Jones, J. M., 145, 149
Tazawa, R., 340
Tchakmakjian, L., 166, 169
Tchkonia, T., 119, 121, 122, 123, 124, 125, 126, 127, 128, 129, 130
Tchoukalova, Y., 127, 128, 129
Technau-Ihling, K., 358
Tedesco, L., 247, 259
Tedesco, M. A., 360
Tedesco, P., 218
Teerlink, H. P. C., 468
Tegnér, J., 277
Teijido, O., 68
Tejada-Vera, B., 484
Tejera, A., 166
Tekeli, F., 298
Telci, A., 298
Teli, T., 285
ten Dam, V. H., 352
Tenney, S. M., 437
Tennison, C. G., 464
Tepe, N. M., 394
Teramoto, S., 430, 439
Terayama, Y., 351
Terborg, C., 351
Teri, L., 357
Terlecky, S., 306
Terman, A., 67, 68, 182, 307, 308
Ternes, T., 259
Terragno, N. A., 413
Terron, J. A., 360
Terry, R. D., 336, 337
Tertoolen, L. G., 224
Teruya-Feldstein, J., 208
Terzibasi, E., 16, 216, 230
Teschner, M., 96
Teshima-Kondo, S., 283
Teta, M., 377
Tew, J. G., 277
Tews, D. S., 64, 95
Thannickal, V. J., 413
Tharakan, B., 72
Tharmarajah, G., 72
Tharner, C., 122
Thebaud, B., 358
Thiele, C. J., 387
Thiels, E., 55, 56, 187, 188
Thinggaard, M., 286
Thireos, G., 207
Thirunavukkarasu, M., 259
Thomas, A. J., 430
Thomas, C. F., 257
Thomas, C. M., 471
Thomas, E. L., 13
Thomas, F., 168
Thomas, G., 14, 204, 205
Thomas, G. V., 208
Thomas, J. H., 4, 5, 7, 9, 10, 12, 218, 221, 228, 229
Thomas, M. M., 70
Thomas, S., 353
Thomas, S. C., 248, 249
Thomas, T. E., 151
Thome, M., 72
Thomou, T., 122, 124, 125, 128, 129
Thompson, C., 82, 348
Thompson, C. B., 64, 67, 71
Thompson, J. C., 301
Thompson, J. M., 77
Thompson, J. R., 169
Thompson, L. V., 67, 142, 147, 302, 304
Thompson, P. M., 334, 337
Thompson, T. J., 324
Thomson, K., 169
Thoreen, C. C., 204
Thorleifsson, G., 378
Thorne, A., 120, 121
Thornell, L. E., 84
Thorner, M. O., 30
Thornton, A., 82
Thornton, C., 94
Thornton, P., 31
Thornton, P. L., 31, 457
Thorpe, S. R., 190
Thun, M., 324
Thurlbeck, W. M., 427, 428, 429, 430, 436, 437, 438, 439
Thurston, H., 168, 169
Tian, B., 393, 396, 397, 398
Tian, J., 328
Tian, S., 142, 144
Tian, T., 72
Tian, Y., 92, 98, 393, 397
Tian, Z., 323
Tibaldi, E., 54
Tichansky, D. S., 26
Tiengo, A., 169, 170
Tieppo, C., 282
Tierney, M. J., 32
Tierney, T., 27
Tigges, J., 336, 337, 391
Tiikkainen, M., 123
Tikuisis, P., 468, 469
Till, J. E., 143
Tilmont, E., 3, 32, 450
Tilmont, E. M., 449, 450
Timiras, P. S., 14, 222
Timmons, L., 9, 12
Timsit, J., 33
Ting, C. T., 97
Tingus, K., 337, 339, 340
Tinkler, G. P., 391
Tinsley, F. C., 26
Tintut, Y., 283
Tirosh, O., 127
Tishler, T. A., 338
Tissenbaum, H. A., 4, 7, 9, 10, 25, 217, 222, 224, 225, 229, 246
Tiveron, C., 219
Tjian, R., 224
Tobin, J. D., 283, 321
Tochihara, Y., 466, 470, 471
Tocilescu, M. A., 48
Toda, T., 258
Todorich, B., 338
Todoriki, H., 455
Toescu, E. C., 406, 407
Toffin, E., 221
Toga, A. W., 334, 337
Toh, S., 387
Toivonen, J. M., 13, 222
Tojjar, D., 375
Toker, A., 218
Tokics, L., 438, 439
Tokinaga, Y., 410
Tokuda, M., 33
Tokunaga, E., 256
Tokunaga, K., 122
Tokura, H., 470
Toledano, M. B., 53
Toledo, J. B., 355
Tolep, K., 433
Tolkovsky, A. M., 306
Tomaselli, K. J., 82
Tomás-Loba, A., 166
Tomasson, R. F., 481
Tomazela, D. M., 56
Tombaugh, T. N., 339
Tomimoto, H., 351, 352
Tominaga, K., 33, 283
Tominaga, T., 122, 123
Tomioka, H., 121
Tomioka, T., 303
Toms, R., 78
Tonello, C., 247, 259
Tong, L., 245
Tong, Q., 254

Toniutto, P., 285
Tonkonogi, M., 68
Tonoki, A., 303
Tonouchi, M., 473
Topark-Ngarm, A., 252
Tordjman, K., 27
Torella, D., 97
Tornheim, K., 121
Torninaga, Y., 193
Tornqvist, H., 120
Tornroth, S., 51
Torres, C., 303
Torres, D., 95
Torres, I. J., 466
Torres, P., 437
Torres-Jardón, R., 286
Torrice, C., 378
Toscano, K., 308
Toshimori, T., 306
Toth, A., 78
Tóth, M., 308
Toth, M. L., 208, 230
Tothova, Z., 152
Touboul, P. J., 352
Toussaint, O., 302, 304
Toutouzas, K., 281
Tower, J., 286, 300, 302, 388
Town, T., 357
Townsend, C. M., Jr., 301
Toyoda, K., 407
Toyoda, Y., 340
Toyokuni, S., 304
Toyoshima, H., 351
Traber, J., 357
Traber, M. G., 178
Tracy, R. P., 124, 168, 323
Trainor, C., 33
Trainor, K. J., 179, 182
Tran, C., 208
Tran, G. T., 283
Tran, K. M., 464, 469, 470
Tran, T., 33
Tranh, M., 349
Trapp, C., 194
Traurig, M. T., 125
Travlos, G., 35
Treadway, J. L., 30
Trembath, D., 357
Tremblay, R. E., 338
Tremer, H. M., 427, 428, 429, 436, 438, 439
Trentalance, A., 14
Trent Hepatitis C Study Group, 285
Tresguerres, A. F., 32
Tresguerres, J. A., 32, 33
Tretter, L., 51
Treuting, P., 56, 189, 218, 489, 491
Treuting, P. M., 56, 188
Trifunovic, A., 55
Trivers, R. L., 487
Troemel, E. R., 286
Trop, D., 433, 437
Tropepe, V., 154
Trougakos, I. P., 303, 304
Troulinaki, K., 208
Trounce, I., 67
True, H. L., 299
Trujillo, M. E., 285
Trumpower, B. L., 50, 51
Trumpp, A., 145
Truong, D., 120, 121, 125
Trzeciakiewicz, A., 283
Trzonkowski, P., 279
Tsai, H., 120, 121, 125
Tsai, J. G., 72
Tsai, S. C., 358
Tsang, A. W., 245
Tsang, F. A., 12, 229
Tsao, C. S., 183
Tschen, S.-I., 375, 377
Tschop, M. H., 13, 248, 251, 256, 260, 261
Tschudi, M. R., 358
Tse, J. G., 258, 259, 260
Tseng, V., 31
Tsialogiannis, E., 283
Tsiaras, W. G., 130
Tsiridis, E., 283
Tsirigotis, M., 304
Tsuchiya, M., 4, 5, 7, 9, 10, 12, 29, 206, 207, 217, 218, 219, 220, 221, 226, 227, 228, 229, 230, 247
Tsuda, M., 219
Tsuji, Y., 33
Tsujimoto, Y., 71
Tsukada, H., 408
Tsukamoto, S., 304
Tsukiyama, T., 262
Tsuneyama, K., 251
Tsushima, Y., 348
Tsuzuki, T., 193
Tu, J. C., 374
Tu, M. P., 217, 224, 485
Tucker, S. M., 277
Tukhovskaya, E. A., 285
Tullberg, M., 349
Tullet, J. M., 11, 14, 120, 208, 209, 217
Tullet, J. M. A., 381
Tung, Y. F., 358
Tuomilehto, J., 491
Tupling, A. R., 70
Turcato, E., 120
Turley, S. D., 340
Turner, C. H., 391
Turner, D. L., 85
Turner, J. M., 429, 430, 431, 433, 435, 439
Turner, M. C., 286
Turner, N. L., 471, 472
Turner, S. L., 473
Turner, T. M., 432, 433
Turra, C. M., 278
Turrens, J. F., 50
Turtle, M., 333, 334, 337
Turturro, A., 322
Turyn, D., 26, 27, 34, 38
Tutt, S., 71
Tverdal, A., 491
Tylavsky, F., 123
Tyurin, V. A., 70
Tyurina, Y. Y., 70
Tzamarias, D., 207
Tzankoff, S. P., 439
Tzavelas, C., 303
Tziomaloes, K., 283
Tzourio, C., 352, 360

U

Uberti, D., 280, 281
Uchida, K., 304
Uchiyama, S., 84, 94, 195
Uchiyama, Y., 84, 94
Udayan, R., 283
Udeani, G. O., 257
Udranszky, I. A., 4, 5, 7, 10
Uematsu, H., 325
Uemura, K., 49
Ueyama, M., 221
Ugo, I., 95
Uguccioni, G., 64, 96
Ukraintseva, S. V., 282, 284
Ukropcova, B., 96, 247, 259, 453, 456
Ullrich, O., 304
Ulrich, J., 337
Ulrich, P., 284
Ulshafer, R. J., 392
Um, S. H., 120, 205, 209, 231
Um, S. J., 250
Umbehr, M., 285
Umberger, G., 32
Umeda-Kameyama, Y., 219
Unakar, N. J., 191
Unger, R. H., 121, 122
Unger, T., 360
Ungvari, Z., 260, 358, 410, 411, 413
United Nations Demographic Yearbook, 480, 481
Uno, H., 360, 392
Uno, M., 206, 208
Updike, D. L., 10, 208
Uranga, A. P., 122
Urban, J., 12, 204, 205, 207, 217, 227, 249
Urban, N., 16
Urbanchek, M. G., 84
Urbanski, H. F., 390, 450
Urbich, C., 259, 358
Uribarri, J., 285
Ursell, P. C., 191
Ursini, F., 189

Uruno, A., 412
U.S. Department of the Office of the Civil Administrator of the Ryukyu Islands, 452
US Census Bureau, 165
Ushio, S., 180, 182, 184
Usui, I., 251
Uthus, E. O., 29
Utsuyama, M., 32
Utzschneider, K. M., 125, 376, 379
Uysal, K. T., 123
Uzunkoy, A., 121

V

Vacher, C., 307
Vahsen, N., 71
Vaillant, F., 144
Vaina, S., 281
Vaiserman, A. M., 221
Vaishnav, R. A., 411
Valancuite, A., 406
Valcerde, C. R., 390
Valdes, A., 169
Valdes, A. M., 125
Valentine, J. S., 218, 220
Valenzano, D. R., 16, 216, 230
Valenzano, M. C., 280
Valenzuela, L., 227
Vallabhapurapu, S., 78
Vallejo, S., 405, 410, 411, 413, 414
Valles, S. L., 489
Vallette, F. M., 71
Van, A. T., 72
Van, R. H., 489, 490, 491
Van, R. M., 77, 78
van Beek, W., 353
Van Bodegom, D., 168
Vance, R. E., 277
Van Craenenbroeck, E. M., 170
VandeBerg, J. L., 390
van den Born, M., 153
van den Bosch, P. P., 167
van den Heuvel, D. M., 352
Van De Parre, T. J., 281
van de Poll-Franse, L. V., 360
van der Flier, L. G., 143, 153
van der Graaf, Y., 351
van der Grond, J., 351, 352
van der Harst, P., 169, 170
van der Horst, A., 224
van der Horst, G. T., 194, 195
van der Kooy, D., 154
Van der Kuip, D. A., 359
Van der Loo, B., 411
van der Pluijm, I., 280, 284
Van der Pol, S., 407
van der Steege, G., 169
Vandervoort, A. A., 434
van de Warrenburg, B. P., 353
van Dijk, E. J., 357
Van Dongen, H. P. A., 468
Van Doorn, R., 407
van Duijn, C. M., 357
van Eickels, M., 388
van Es, J. H., 153
van Exel, E., 282
Van Eygen, S., 4, 5, 7, 17
Vanfleteren, J. R., 4, 5, 7, 9, 10, 16, 208, 220, 228
van Gaal, L., 121, 122
van Gijn, M. E., 153
van Gilst, W. H., 170
Van Gool, F., 260, 261
van Gorp, W., 339
Van Guilder, G. P., 168, 169, 170
van Haeften, T., 379
Van Harmelen, V., 120, 121, 129
van Heemst, D., 28, 170, 171
Vanhommerig, J. W., 469
Vanhoutte, P. M., 349, 408, 409
van Huffelen, A. C., 352
VanItallie, J. B., 324
Van Langenhove, G., 281
van Lohuizen, M., 147
van Marken Lichtenbelt, W. D., 469
van Meurs, J. B., 244, 253
Vann, J. M., 14, 96, 99, 251, 256, 257
Van Nguyen, T., 146
Van Nostran, S., 65
Van Obberghen, E., 123, 125
Van Remmen, H., 56, 182, 184, 186, 187, 189, 190, 191, 192, 193, 194, 195, 196
van Riel, N., 167
van Riel, N. A., 167
Van Someren, E. J. W., 464, 465, 466, 472, 473
van Steeg, H., 195
van Swieten, J. C., 356, 357
Van Tendeloo, V. F., 170
van Veen, E., 13, 225, 247
Vanvoorhies, W., 15, 16
Van Zant, G., 152, 168
Vaquero, A., 254
Vara, E., 32
Varadhan, R., 83, 323, 328
Varga, A. W., 355
Vargas, M. A., 13, 205, 206
Vargo, T. M., 389
Varner, D. E., 392
Varricchio, M., 28
Vartiainen, E., 491
Varvis, C. J., 437
Vasa, M., 169, 358
Vasan, R. S., 168, 169
Vashistha, H., 99, 259
Vasilaki, A., 70
Vasquez, B. J., 30
Vasquez, D. S., 204
Vassar, R., 282
Vassilopoulos, A., 254
Vastro, S., 387
Vasunilashorn, S., 276, 286
Vata, K. C., 169
Vataja, R., 356
Vatner, D. E., 99, 100, 392, 413
Vatner, S. F., 92, 98, 99, 258, 387, 392, 393, 394, 395, 396, 397
Vatten, L. J., 483
Vaupel, J. W., 164, 165, 170, 171, 217, 287, 479, 480, 481, 484, 485, 486
Vavuranakis, M., 281
Vaziri, H., 13, 151, 224, 255, 256, 261
Vazquz, J. J., 325
Veale, W. L., 473
Vega, N., 121, 122
Vega, W. A., 338
Vegh, M., 224
Vehkavaara, S., 123
Veis-Novack, D. J., 71
Vela, J., 301
Velasco-Miguel, S., 13, 248, 251, 256, 260, 261
Velavicius, J., 406
Veldhuis, J., 376, 377
Veldhuis, J. D., 380
Velez de Mendizabal, N., 355
Velkoff, V. A., 484
Vellai, T., 10, 207, 218, 226
Venable, S., 29
Venien, A., 83
Venkatachalam, M. A., 70, 71
Ventadour, S., 66
Ventura, B., 49
Ventura, E., 262
Ventura, H. O., 285
Verbeke, P., 304
Verbrugge, L. M., 386, 387, 484
Verdery, R., 453, 455
Verdery, R. B., 392
Verdin, E., 244, 246, 254, 258
Verdin, R. O., 244, 254
Verdu, J., 205
Verghese, J., 353
Vergouwe, M. N., 279
Verhaar, H. J., 32
Verhaeghen, P., 338, 339
Verheye, S., 281
Vermeer, S. E., 348, 356
Vermeulen, A., 469
Vermeulen, K., 170
Vermeulen, T., 95, 96
Vermulst, M., 56
Vernace, V. A., 304
Vernieri, F., 352
Vernooij, M. W., 341
Verrecchio, J. J., 280
Verrijken, A., 244, 253, 258
Verrips, T., 167

Vescovo, G., 95
Vessby, B., 387
Vezina, C., 203, 226
Vidal, K., 328
Vidal-Puig, A. J., 122
Viegi, G., 437
Vieira, V. J., 285
Vielhaber, S., 69
Vig, J., 381
Vigna, C., 70
Vignon, G., 122
Vijg, J., 34, 56, 163, 182, 186, 187, 189, 195, 257
Vila, E., 412
Villablanca, P., 338
Villamor, A., 425
Villanua, M. A., 33
Villanueva, J. M., 16, 221, 286
Villareal, D., 453, 456
Villareal, D. T., 453, 454, 455, 456, 457
Villaret, A., 125
Villa-Verda, D. M., 33
Villen, J., 4, 5, 7, 9, 10, 206, 208, 229
Vina, J., 68, 70, 388, 489, 490, 491
Vincent, T. N., 427, 429, 436, 438
Vingerhoets, G., 349
Vining, M., 183
Vinke, R. S., 412
Vinti, G., 224
Viola, L., 353
Virchow, R., 276, 281
Virmani, R., 97
Virtanen, K. A., 469
Visser, M., 77, 78, 119, 123
Visvader, J. E., 145
Viswanathan, M., 16, 224, 225, 230, 250, 261
Vitaro, F., 338
Vitek, M. P., 285
Viteri, G., 298, 302
Vitez, T. S., 472
Vitorica, J., 301
Vitorovic, S., 148
Vitseva, O. I., 121
Vitullo, L., 408, 411
Vlassara, H., 277, 283, 285
Vockentanz, L., 151
Vodovotz, Y., 277
Vodyanik, M. A., 142
Vogel, H., 55, 191
Vogel, R., 53
Vogelzang, J. L., 325
Vogt, S., 68, 69
Vohra, B. P., 262
Voight, B. F., 378
Voigt, A., 16
Voitenko, V. P., 221
Vojtiskova, A., 51
Volanakis, J. E., 277
Volanen, I., 286
Volek, J. S., 185
Volk, B., 337
Volkov, M., 222
Vollmar, A. M., 360
Vollmer, W. M., 424
Volpato, S., 374
Volterrani, M., 95
vom Saal, F., 389, 390
von Eller-Eberstein, H., 28, 156, 285
von Euler, C., 425
von Zglinicki, T., 146, 170, 171, 302, 304
Voors, A. A., 169
Vorbrodt, A. W., 406
Vos, J. P., 340
Votteas, V. E., 360
Voytko, M. L., 391
Vrbacky, M., 51
Vrints, C. J., 170
Vrooman, H. A., 341
Vucurevic, G., 341
Vukmanovic-Stejic, M., 277
Vuppusetty, C., 251, 261
Vyas, M., 349, 360

W

Waack, B. J., 412
Wacholder, S., 124
Waclawiw, M. A., 169
Wada, H., 251, 261
Waddell, T. K., 387
Wadhwa, R., 302
Wagers, A. J., 148, 149, 150, 151, 154, 156
Wagner, D., 144, 262
Wagner, J. A., 439
Wagner, J. D., 390, 391, 449
Wagner, P. D., 437, 438, 439
Wagner, T. E., 26, 27, 29, 34
Waguri, S., 306, 309
Wahba, W. M., 437, 438
Wajant, H., 77
Wakabayashi, I., 405, 410
Wakasugi, C., 464
Wakita, H., 351, 352
Walczak, H., 78, 81
Waldemar, G., 351
Walden-Hanson, T., 327
Walenta, K., 169
Walford, R. L., 13, 26, 27, 32, 33, 35, 216, 231, 392, 447, 452, 453, 455
Walhout, A. J., 225, 246
Walhovd, K. B., 337
Walker, C., 281
Walker, D. W., 72
Walker, G. A., 13, 222
Walker, J., 130
Walker, L. C., 391
Walker, M., 324
Walker, M. L., 390
Walker, M. R., 145
Walker, V. R., 388
Wall, N. R., 99, 247, 248, 255, 259, 262
Wallace, B. A., 389
Wallace, D. C., 67, 70, 164, 170, 171, 253
Wallace, E. B., 324
Wallace, J. I., 324, 325
Wallace, R. B., 353
Waller, H., 183
Wallerath, T., 259
Wallimann, T., 194
Walls, K. C., 78
Walmsley, R. M., 216
Walsh, B., 68
Walsh, K., 262, 357
Walshe, T. M., 357
Walski, M., 425, 426
Walston, J., 83, 323, 327
Walston, J. D., 285, 328
Walter, C. A., 189, 407
Walter, P., 209, 301
Walter, P. B., 194
Walters, G. B., 378
Walther, T. C., 254
Waltregny, D., 254, 255
Walz, T., 245
Wan, C., 129
Wan, H., 484
Wanagat, J., 55, 99, 148, 392
Wandycz, A. M., 145, 150
Wane, V., 306
Wang, B., 221
Wang, C., 12, 178, 229, 248, 278, 281, 324
Wang, C. F., 325
Wang, D. H., 255, 256
Wang, D. Y., 229
Wang, E., 280
Wang, F., 254
Wang, H., 124, 388
Wang, J., 3, 14, 96, 99, 119, 123, 218, 251, 262, 282
Wang, M., 281, 392, 413
Wang, M. S., 261, 277
Wang, P., 78
Wang, P. S., 358
Wang, P. Y., 13
Wang, R. H., 248, 251, 256, 257
Wang, R. Y., 358
Wang, S., 388
Wang, S. W., 358
Wang, S. Y., 248
Wang, W., 67, 69, 258
Wang, X., 69, 71, 97, 250, 256, 258, 260, 286, 301, 302, 304
Wang, X. T., 302
Wang, X. Z., 301
Wang, Y., 4, 7, 9, 32, 225, 246, 251, 302, 306, 308

Wang, Y. C., 123
Wang, Y. X., 16, 388
Wang, Z., 259
Wang, Z. Q., 449
Wang, Z. W., 121, 122
Wangpoengtrakul, C., 304
Wanke, R., 34
Wanke, V., 12
Wannamethee, S. G., 123, 324
Ward, J. L. III, 248, 254, 260
Ward, W. F., 194, 302, 490
Warden, C. H., 469
Wardlaw, J. M., 406
Wardman, P., 52
Ware, J. H., 423, 424, 435
Warfield, S. K., 353, 354
Warner, D. F., 484
Warner, D. O., 434, 439
Warner, H. R., 487
Warner, J., 33, 450
Warner, L. A., 338
Warnk, H., 69, 70
Wartschow, L. M., 224, 377
Wasay, M., 353
Wasfy, G. W., 83
Washicosky, K. J., 277
Wassell, R., 71
Wasserman, J. D., 224
Watanabe, M., 120, 205, 209, 231, 262
Watenpaugh, D. E., 470
Waterhouse, J., 30, 468
Waterlow, J. C., 277
Waters, D. J., 26, 491
Waters, S., 306
Waterstrat, A., 168
Watkins, D. N., 255
Watkins, N. G., 27
Watson, J. D., 165
Watt, F. M., 145
Waugh, S., 84, 85
Wautier, J. L., 283
Wautier, M. P., 283
Waxman, D. J., 388
Waxman, S. G., 338
Weathers, S., 351
Webb, A. C., 178
Webb, A. E., 155
Webb, C. R., 34, 56, 190
Webb, J. R., 248, 251
Weber, D. K., 283
Weber, F., 348
Weber, R. V., 120
Weber, T., 483
Weed, J. L., 32, 450
Weedon, M. N., 378
Weeraratna, A. T., 32
Weger, S., 169, 170
Wegiel, J., 307
Wegner, F. H., 392
Wehr, C. M., 68
Wehr, M. C., 340
Wei, H., 144
Wei, J. Y., 92, 93, 99
Wei, M., 12, 207, 220, 225, 247
Wei, M. C., 69
Wei, X., 353, 354
Wei, Y. H., 47, 70
Weibel, E. R., 426, 427
Weigand, S. D., 341
Weigel, P. H., 357
Weigent, D. A., 32
Weigle, D. S., 327
Weil, B., 277
Weil, B. R., 277
Weil, J. V., 425
Weil, R. J., 231
Weill, H., 423, 429, 434, 435, 436
Weiller, C., 351
Weindruch, R., 10, 13, 26, 27, 30, 32, 33, 35, 93, 98, 99, 125, 178, 180, 184, 185, 193, 216, 231, 280, 284, 300, 301, 302, 324, 392, 447, 448, 450, 451, 452, 489, 490
Weinert, D., 30
Weingand, K. W., 391
Weinkove, D., 16, 230, 251, 253
Weinstein, F., 64
Weinstein, M., 278
Weinstock, G. M., 390, 448
Weinstock, K. G., 244
Weintraub, H., 85
Weis, N., 77, 78
Weisberg, S. P., 120, 124
Weisgraber, K. H., 282, 285, 340
Weisiger, R. A., 186
Weisleder, N., 82
Weiss, E. P., 453, 454, 455, 456, 457
Weiss, S., 154
Weiss, S. T., 434
Weissman, I. L., 145, 147, 148, 149, 150, 151, 152, 154, 156
Weissman, J., 299
Weissmann, N., 358
Weitzman, E. D., 473
Welcome, S. E., 334, 337
Welle, S., 94
Wellens, H. J., 388
Wells, J., 357
Wells, L., 32
Wells, T. B., 251
Welniak, L. A., 32
Welsbie, D. S., 208
Welsh, P., 278
Welsh-Bohmer, K., 357
Welton, K. L., 4, 5, 7, 9, 10, 12, 15, 217, 218, 219, 221, 228, 229
Wen, W., 355
Wencker, D., 97
Wendruch, R., 450
Wendtner, C. M., 387
Weng, Q. P., 204
Werner, J., 423, 426
Werner, N., 169
Wernig, M., 142
Wertheim, B., 13
West, J. B., 437
Westbrook, R., 14, 26, 29
Westenbrink, B. D., 170
Westendorp, R. G., 168, 170, 171, 352
Westerbacka, J., 123
Westergren, R., 469
Westerheide, S. D., 300
Westerkamp, L. M., 149
Westermark, P. O., 126
Westley, H. J., 33
Westman, E. A., 12, 206, 207, 217, 218, 220, 226, 227, 230, 247, 250, 251
Westphal, C. H., 258
Westwood, M., 128
Weyman, C. M., 85
Weyrich, P., 244, 253
Whaley, R. A., 300, 301
Wharram, B. L., 34
Whearcroft, S. B., 170
Wheelson, C. M., 34
Whelton, P., 347
Whincup, P. H., 123, 324
Whisnant, J. P., 356
Whitaker, R., 13
White, A., 128
White, C., 168, 169
White, C. W., 187
White, D. A., 256
White, E., 70, 71
White, L. E., 341
White, L. S., 71
White, M. F., 224
White, T. M., 222, 300
Whitehouse, C., 306
Whitelaw, W. A., 425, 437, 438
Whiteley, W. N., 348
Whitman, G. T., 354
Whitmore, A. V., 261
Whittaker, R. H., 203
Wicks, K. L., 68, 85, 95
Widegren, B., 151
Wideman, C. H., 473
Widlock, N. M., 324
Wieckiewicz, J., 279
Wiegraebe, W., 151
Wielopolski, P. A., 341
Wiesbrock, S. M., 123
Wieser, D., 14, 120, 208, 209, 217, 381
Wiesinger, H., 53
Wiggins, J. E., 34
Wigle, D. T., 286
Wijnen, H., 253
Wikstrom, M., 49
Wilcox, P. G., 433

Wilczynski, S., 192
Wilhelm, J., 51
Wilkinson, C. W., 285
Wilkinson, R., 339
Willard, H. F., 388
Willcox, B. J., 28, 156, 455
Willcox, D. C., 455
Willer, C. J., 378
Willerson, J. T., 281
Willett, W. C., 123, 324
Williams, B., 168, 169
Williams, B. O., 257
Williams, B. R., 337, 338, 339
Williams, D. D., 392
Williams, D. S., 16
Williams, G., 122, 123, 469
Williams, G. C., 210, 485
Williams, G. T., 64
Williams, J., 482
Williams, L. E., 437
Williams, M. D., 191, 194
Williams, P. D., 485
Williams, R. M., 27
Williams, R. R., 56, 192
Williams, S. C., 351
Williams, S. M., 389
Williams, T. N., 164
Williams, W. J., 426
Williams, W. M., 190, 192, 193
Williams-Blangero, S., 390, 482
Williamson, C. L., 64
Williamson, D. A., 30, 453, 456
Williamson, D. F., 324
Williamson, D. L., 72, 85, 100
Williamson, D.A., 64
Williamson, J., 374
Willis, I. M., 249
Willis, M. S., 297, 298
Willis, S. L., 339
Willoughby, D. A., 280
Wilmont, E. M., 27
Wilms, H., 360
Wilson, A., 145
Wilson, B. A., 99
Wilson, J. F., 323
Wilson, M. A., 4, 5, 7, 9, 10, 221, 228, 229
Wilson, M.-M. G., 325
Wilson, R., 35
Wimmer, M., 349, 352
Winderickx, J., 12
Windmiller, D., 123, 124, 126, 129
Winer, S., 120, 121, 125
Wing, R. R., 123
Wingard, D. L., 324, 484
Winn, H. R., 408
Winning, A. J., 423, 435, 439
Winsloe, C., 283
Winter, B., 408
Winterer, G., 341
Winters, T. A., 26
Wintzen, A. R., 353
Wirth, E. K., 191
Wirtz, K. W., 68
Wirz-Justice, A., 468
Wise, B., 124, 129
Wise, L. S., 129
Wise, P. M., 390
Wissing, S., 220
Wiste, H. J., 341
Wiswell, R. A., 94
Withers, R. T., 435
Witt, E., 302
Witt, H., 388, 389
Witt, K. A., 468
Witt, W. W., 322
Witta, J., 279
Witte, K., 259
Witteman, J. C., 359
Witter, D., 178
Witters, L. A., 31
Wittwer, F., 10
Wittwer, M., 64
Witzenbichler, B., 357
Witztum, J. L., 169
Wladkowski, S. L., 471, 472
Wleklinski-Lee, M., 149
Woerle, H. J., 379
Wohl, M. E., 429, 430, 431, 433, 435, 439
Wohlfart, P., 360
Wohlgemuth, S. E., 55, 64, 67, 68, 69, 70, 71, 73, 74, 75, 76, 77, 78, 79, 80, 86, 87, 89, 90, 91, 92, 95, 96, 98, 282, 308
Wolberger, C., 245, 249
Wolden-Hanson, T., 325
Wolf, B. B., 66
Wolf, E., 34
Wolf, N. S., 29, 190
Wolf, P. A., 347
Wolfberger, C., 245
Wolff, S., 4, 7, 9, 10, 15, 208
Wolin, M. S., 259, 260, 261
Wolkow, C. A., 4, 5, 7, 9, 10, 217, 221, 228, 229
Wollschlager, H., 349
Wolter, K. G., 71
Wong, E. S., 307
Wong, G., 221
Wong, J., 120, 121, 125
Wong, K. S., 352
Wong, M. L., 471
Wong, N. D., 169, 258
Wong, R., 13, 221
Wong, S., 128
Wong, S. N., 12, 229
Woo, J., 283, 324
Woo, M., 95
Wood, A., 278
Wood, C. E., 390
Wood, J. G., 16, 230, 237, 246, 247, 249, 250, 251
Wood, M., 122
Woodall, A. A., 194
Woodbine, L., 152
Woodbury, M. A., 484
Woods, C. E., 392
Woods, J. A., 285
Woods, N. F., 323
Woods, S. C., 285
Woodward, M., 360
Woodward, P. M., 466, 470
Woodworth, K. A., 285
Working Group on Functional Outcome Measures for Clinical Trials, 326
World Population Data Sheet, 169
Wouters, B. G., 304
Wouters-Wesseling, W., 326
Wozniak, D., 181, 186
Wozniak, J. R., 337, 341
Wozniak, M. A., 285
Wragg, A., 255
Wrangham, R., 482
Wray, L. A., 374
Wredenberg, A., 55
Wright, C., 29
Wright, H. R., 473
Wright, J., 260, 261, 262
Wright, J. C., 28, 34, 229
Wright, N. A., 145
Wright, R. R., 428, 433
Wright, W. E., 163, 165, 166, 167
Wu, B., 300, 301
Wu, D., 123, 124, 125, 179, 180, 181, 182, 185
Wu, G., 55, 56, 185
Wu, G. Y., 187, 188
Wu, I.-C., 328
Wu, J., 389, 390, 412, 413
Wu, J. W., 151, 281
Wu, L. C., 253
Wu, M., 92
Wu, M. F., 144
Wu, P. K., 466, 470
Wu, Q., 33
Wu, S. B., 70
Wu, W., 26
Wu, X., 185
Wu, Y., 28, 34, 277
Wu, Y. Y., 120
Wu, Z., 72, 262, 357
Wuisman, P. I., 122
Wullschleger, S., 120, 204, 207, 226, 231
Wyllie, A. H., 64

X

Xi, L., 194, 195
Xi, X. G., 71

Xia, Y., 254
Xiang, Y., 306
Xiao, C., 256, 257
Xiao, Q., 169, 170
Xiao, X, 301
Xiao, Y., 259
Xie, H., 248, 249
Xie, J., 484
Xie, W., 122, 128
Xie, Y., 121, 122, 151
Xie, Z., 278, 280
Xin, H. B., 388
Xingziang, W., 413
Xiong, C., 181, 186, 221, 388, 489, 491
Xiong, Y., 191
Xu, B., 92, 93, 98
Xu, B. C., 229
Xu, C. J., 66
Xu, G., 375
Xu, H., 120, 124, 125, 282
Xu, J., 66, 68, 387, 484
Xu, L., 250, 256, 300
Xu, P. T., 357
Xu, Q., 258, 261
Xu, S., 16, 251
Xu, W., 225, 230, 248
Xu, X., 245, 248, 251, 423, 424, 435
Xu, X. L., 206
Xudong, X., 413
Xue, Q. L., 323
Xue, Q.-L., 323, 328

Y

Yablonka-Reuveni, Z., 145, 149
Yadav, V. S., 99
Yadava, N., 52
Yadoguchi, I., 466, 470
Yaffe, K., 341, 391
Yagi, K., 29
Yahata, N., 262
Yakar, S., 27, 28, 34
Yakovlev, G., 48
Yakovlev, P. I., 334, 336, 337
Yakovskaya, V., 51
Yamada, H., 245
Yamada, M., 33
Yamada, Y., 34
Yamaguchi, M., 195
Yamaguchi, O., 191
Yamaguchi, S., 128, 245
Yamaguchi, T., 351
Yamakawa, H., 360
Yamamori, T., 259
Yamamoto, A., 299, 306, 309
Yamamoto, H., 283
Yamamoto, M., 224
Yamamoto, M. E., 482
Yamamoto, R., 13
Yamamoto, T., 206, 208
Yamamoto, Y., 129
Yamanaka, S., 142
Yamashita, H., 120, 121, 125
Yamauchi, H., 351
Yamauchi, S., 351
Yamazaki, S., 152
Yamazaki, Y., 251
Yan, F., 411
Yan, L., 99, 100, 392, 397
Yan, S. F., 277, 283
Yan, T., 251
Yan, X., 145
Yanase-Fujiwara, M., 473
Yanez, D., 321, 353
Yáñez, M., 283
Yang, C., 144
Yang, D., 120, 124, 125
Yang, F., 65, 66, 349
Yang, G. P., 392, 395
Yang, H., 32, 191, 248, 249, 254, 282, 327, 328
Yang, H.-C., 412
Yang, J., 69, 286, 413, 414
Yang, Q., 120, 124, 125, 286, 306
Yang, S., 26
Yang, S. R., 260, 261, 262
Yang, T., 54, 262
Yang, X., 71, 123, 248, 251, 388
Yang, Y., 277
Yang, Y. P., 65, 66
Yang, Z., 301, 302
Yankner, B. A., 64, 66
Yano, K., 28, 156, 455
Yano, M., 99
Yano, Y., 357
Yanrong, L., 413
Yant, L. J., 191
Yaoita, H., 97
Yarasheski, K. E., 94
Yarbrough, C. J., 448, 451, 452
Yashin, A., 168
Yashin, A. I., 222, 282, 284
Yashiroda, H., 302
Yasojima, K., 280
Yasuda,M., 392
Yasui, W., 65, 282
Yasumura, K., 29
Yasunami, M., 190
Yatabe, Y., 154
Yau, J., 33
Yazdani, B., 392
Ye, H., 71
Yeh, J. I., 51
Yeh, S.-H., 328
Yeh, S.-S., 327
Yen, K., 17
Yen, R. C., 255, 256
Yen, S. S., 389
Yeo, H. C., 194
Yerg, J. E., 434, 439
Yernault, J.-C., 430
Yeung, F., 256, 260
Yeung, V. T., 283
Yi, S., 288
Yikilmaz, A., 286
Yilmaz, E., 209
Yin, C. M., 217, 224
Yin, D., 82
Yin, T., 151
Yin, X. M., 71
Yip, A. G., 357
Yoder, M. C., 169
Yodoi, J., 189, 192
Yokomizo, T., 169
Yokosawa, H., 304
Yokota, T., 122
Yokoyama, K., 218, 302
Yokoyama, M., 125, 285
Yokozawa, T., 181, 185, 186
Yonezawa, K., 10, 204
Yong, A., 285
Yong, D., 94
Yong, S., 261, 263
Yorimitsu, T., 301, 302
York-Crowe, E., 453, 456
Yoshida, S., 122, 145
Yoshida, T., 189, 280
Yoshikawa, K., 250
Yoshikawa, T. T., 464
Yoshimori, T., 306, 308
Yoshimoto, M., 169
Yoshimura, S., 72, 84, 94
Yoshino, J., 249, 253, 262
Yoshioka, H., 181, 186
Yoshioka, S., 127
Yoshizaki, T., 251, 258, 260
You, L., 251
You, S., 300
Youle, F. J., 71
Youle, R. J., 71
Youm, Y. H., 32
Young, A. J., 469
Young, H., 357
Young, J., 3, 32, 392
Young, J. D., 64
Young, J. J., 193
Young, M., 183
Young, N. S., 170
Yousef, M. K., 472
Yowe, D. L., 68
Yu, A. Y., 358
Yu, B. P., 3, 14, 96, 120, 126, 203, 207, 222, 231, 252, 280, 322, 325, 392, 412
Yu, G.-Q., 411
Yu, J., 78, 95, 121, 122, 141, 142
Yu, J. W., 72, 81
Yu, R., 28
Yu, R. T., 16
Yu, S. L., 190, 191
Yu, W., 258
Yu, W. H., 307, 471

Yu, X., 99, 100, 258, 259
Yuan, J., 64, 66, 71, 248, 256
Yuan, J. Y., 64
Yuan, M., 193
Yuan, X., 128
Yuan, Z., 251
Yuasa, S., 262
Yudkin, J. S., 285
Yudkin, P. L., 392
Yue, Q., 353
Yue, T. L., 97
Yui, J., 165
Yukawa, M., 325, 327
Yukin, A. Y., 285
Yun, C. Y., 82
Yun, J. S., 26, 27, 29, 34
Yurochko, A. D., 281
Yusof, S. R., 406, 408
Yusta, B., 375
Yusuf, S., 178, 179

Z

Zabel, M., 95
Zabezhinski, M. A., 16
Zablocki, D., 99, 100, 258, 259
Zabrocki, P., 220
Zacharek, A., 408, 411
Zachary, J. F., 33
Zackert, W. E., 490
Zahn, J. M., 150
Zajitschek, F., 485
Zak, D. E., 277
Zakian, V. A., 151, 220, 224
Zaletel, M., 349, 352
Zalewski, P. D., 97
Zalos, G., 169
Zaltsman, Y., 81
Zaman, V., 155
Zambetti, G. P., 78
Zamboni, M., 120, 124, 129, 324, 325
Zammit, P. S., 145
Zampa, C. C., 433
Zampieri, S., 95
Zamzami, N., 51, 71, 83
Zandi, P. P., 340
Zang, M., 16
Zanjani, H., 280
Zannoli, R., 324
Zantopf, D., 302
Zappasodi, F., 352
Zarember, K. A., 358
Zarse, K., 16
Zbinden, R., 170
Zeballos, L., 283
Zeger, S. L., 323
Zeggini, E., 378
Zeigler, B. M., 169
Zeiher, A. M., 168, 281, 349, 358, 405, 410
Zeitlin, J., 483
Zeleznik, J., 423
Zelinksi, M. B., 389, 390
Zeller, C., 119
Zeng, B. Y., 302
Zeng, D., 406
Zeng, M., 307, 309
Zeng, S., 277
Zeng, Y., 484
Zennaro, R., 95
Zervos, A. S., 71
Zha, H., 71
Zha, J. M., 121, 122
Zhai, P., 99, 100, 258, 259
Zhai, R. G., 262
Zhan, L., 259
Zhang, B., 258
Zhang, C., 259, 260, 261, 307, 309, 310, 408, 411
Zhang, F., 251
Zhang, G., 251
Zhang, H., 50, 55, 191, 204, 256, 412, 413
Zhang, H. H., 120
Zhang, H. J., 379
Zhang, J., 120, 121, 125, 225, 261, 263, 281, 413
Zhang, J. W., 129
Zhang, L., 78, 302
Zhang, M., 4, 7, 9, 11, 258, 261, 263, 304
Zhang, M. Z., 251
Zhang, P., 300, 302
Zhang, Q., 248, 282
Zhang, Q. J., 259
Zhang, R., 437
Zhang, S., 258, 259, 260
Zhang, T., 249, 256, 261
Zhang, W., 67, 69
Zhang, X., 92, 93, 99
Zhang, X. H., 285
Zhang, X. P., 92, 98, 393, 397
Zhang, Y., 10, 82, 190, 191, 192, 207, 216, 218, 226, 245, 258, 261, 263, 300
Zhang, Y. H., 473
Zhang, Z., 3, 32, 51, 71
Zhao, B., 412
Zhao, C., 154, 285
Zhao, C. X., 151
Zhao, H., 190, 193
Zhao, L. P., 206
Zhao, Q., 70
Zhao, W., 250, 255, 256, 262
Zhao, X., 82
Zhao, Z., 262, 285
Zheng, F., 277, 285
Zheng, J., 72
Zheng, W., 259
Zheng, Y., 248, 251
Zhidko, N. L., 360
Zhong, J., 279
Zhong, W., 388
Zhou, D., 306
Zhou, H., 191
Zhou, J., 360
Zhou, J. N., 357
Zhou, P., 360, 405, 407, 408, 410, 411, 413
Zhou, Q., 72
Zhou, S., 259
Zhou, Y., 229, 262, 288
Zhou, Y. T., 121, 122
Zhu, C., 146
Zhu, D., 302, 308
Zhu, H., 66
Zhu, L., 277, 285
Zhu, M., 4, 5, 7, 9, 10, 129, 221, 228, 229
Zhu, T., 14, 121, 122, 204
Zhu, W., 83
Zhu, X. Q., 357
Zhu, Y., 301
Zhu, Y. X., 357
Zhuang, J., 66
Zickermann, V., 48
Zickmantel, R., 423, 435
Zid, B. M., 13, 205, 206, 217, 218, 226, 230
Ziegler, M., 249
Ziegler, S., 170
Ziegler, T. E., 390
Ziemann, J., 169
Zietkiewicz, S., 299
Zijdenbos, A., 334
Zijlmans, J. M., 165
Zillikens, M. C., 244, 253
Zimber-Strobl, U., 153
Zimbric, M. L., 392
Zimmerman, E. A., 349
Zimmerman, J. A., 13, 14, 205, 222
Zimmerman, J. C., 473
Zimmerman, R. A., 349
Zimmermann, C., 349, 352
Zimmet, P. Z., 120, 122, 123, 124, 321
Zinn, R. L., 255
Zinszner, H., 301
Zipser, B., 357
Zipursky, R. B., 334
Zivony, D., 468
Zlokovic, B. V., 405, 406, 408
Zoccolillo, M., 338
Zoellner, C. D., 340
Zohar, S., 482
Zoico, E., 324
Zoja, C., 413
Zoncu, S., 387
Zong, Q., 206
Zong, W. X., 69
Zordan, M. A., 219
Zou, K., 388

Author Index

Zou, S., 206
Zou, Y., 96, 165, 166, 260
Zouboulis, C. C., 145
Zoumakis, M., 285
Zuccala, G., 360
Zuccollo, A., 16
Zuk, P., 129
Zulian, A., 219
Zünd, G., 285
Zunich, S., 32
Zuo, X., 301
Zuo, Y., 412
Zuo, Z., 357
Zureik, M., 169
Zvan, B., 349, 352
Zwaan, B. J., 28
Zweiner, J., 286
Zwerina, J., 283
Zwetsloot, K. A., 64, 84, 149
Zwicker, K., 48
Zwiener, J., 13, 15, 16

Subject Index

A

- AD, *see* Alzheimer's disease
- Adipocyte
  - aging effects, 125–128
  - function, 125–126
  - precursor cells, 128–130
  - turnover, 126–128
- Adipose tissue, *see also* Adipocyte; Obesity
  - aging effects
    - body composition, 119–120
    - brown adipose tissue, 123
    - ectopic fat accumulation, 122–123
    - redistribution, 122
  - composition, 120
  - dietary restriction effects, 125
  - functions, 120–122
  - prospects for study, 130
  - sirtuin regulation, 252–253
- β-Adrenergic receptor, sex differences in primate desensitization, 394, 397
- Adult stem cell, features, 143–144
- Advanced glycation end-products (AGEs), inflammatory processes and aging, 283–284
- AGEs, *see* Advanced glycation end-products
- AICAR, dietary restriction mimetic, 16
- AKT
  - conservation across species, 217
  - target of rapamycin signaling, 205
- Alveolar ventilation, *see* Pulmonary function
- Alzheimer's disease (AD)
  - cerebral microvascular disease, 356–357
  - inflammation, 280–282
  - sirtuin protection, 262
- AMP-activated protein kinase (AMPK)
  - dietary restriction pathway
    - AICAR activation,16
    - *Caenorhabditis elegans*, 9–10
    - mammals, 14
  - mediation of target of rapamycin-induced longevity effects, 208
  - sirtuin interplay, 251–252
- AMPK, *see* AMP-activated protein kinase
- Angiotensin II
  - cerebral microvascular disease therapeutic targeting, 360
  - sirtuins and receptor modulation, 259
  - vascular changes in aging, 413–414
- Antimycin A, Complex III inhibition, 50–51
- Aortic stiffness, sex differences in primates, 395–398
- ApoE, *see* Apolipoprotein E
- Apolipoprotein E (ApoE), alleles and longevity, 285, 340
- Apoptosis repressor with caspases recruitment domain protein (ARC)
  - mitochondrial-mediated signaling in sarcopenia, 75
  - muscle apoptosis suppression, 72
- Apoptosis, *see* Muscle apoptosis
- ARC, *see* Apoptosis repressor with caspases recruitment domain protein
- Atherosclerosis, *see* Cardiovascular disease
- Autophagy
  - aging and longevity, 307–309
  - dietary restriction genes in *Caenorhabditis elegans*, 12
  - physiological functions, 306
  - protein aggregate handling, 307
  - soluble protein removal, 306–307
  - types, 305–306

B

- Basal metabolic rate, aging effects, 468
- Bax
  - mitochondrial-mediated signaling in sarcopenia, 75–76
  - modulation in aging heart, 98–99
- BBB, *see* Blood–brain barrier
- BCL-2
  - mitochondrial-mediated signaling in sarcopenia, 73, 76
  - modulation in aging heart, 98–99
  - muscle calcium stress apoptosis signaling, 83
  - muscle intrinsic apoptotic pathway, 70–71
- Biosphere 2, calorie restriction findings, 455
- Blood–brain barrier (BBB)
  - aging effects, 407
  - pathology, 406
- Body size
  - life span relationship, 26–27
  - sex differences in longevity, 491
- Body weight
  - aging changes, 321–322
  - frailty, 322–323
  - terminal weight loss
    - food intake decrease, 325–326
    - non-dietary factors, 326–327
    - prospects for study, 327–328
    - unintentional weight loss and longevity, 324–325
- Brain, *see* Alzheimer's disease; Blood–brain barrier; Cerebral blood flow; Cerebral microvascular disease; Myelination, brain
- Brown adipose tissue, loss in aging, 123
- *N-tert*-Butyl-α-phenylnitrone (PBN), life span effects in mice, 181, 186

C

- β-Carotene, life span effects in mice, 181, 185
- *Caenorhabditis elegans*
  - aging model, 220–221
  - dietary restriction
    - approaches for dietary restriction, 3–5
    - pathways
      - autophagy genes, 12
      - criteria for gene dependence, 5, 8–9
      - energy sensors, 9–10
      - mitochondrial genes, 12
      - table of genes, 6–7
      - transcriptional regulators, 10–11
  - longevity pathway conservation with yeast, 226–227
  - target of rapamycin and link between nutrients and life span, 207–209
- CALERIE phase 1 study, calorie restriction findings
  - Pennington Center, 456
  - Tufts University, 456–457
  - Washington University, 455–456
- Caloric restriction, *see* Dietary restriction
- Calorie Restriction Society, 457
- Cancer
  - inflammation, 282–283
  - sirtuin oncogenic versus tumor suppression activity, 255–258
  - somatotropic axis role, 34–35
- Carbon dioxide, arterial partial pressure in aging, 439
- Carboxyfullerene superoxide dismutase, life span effects in mice, 181, 186
- Cardiomyocyte apoptosis, *see* Muscle apoptosis
- Cardiomyopathy, sex differences in primates, 392–393, 396–397
- Cardiovascular disease (CVD)
  - inflammation in atherosclerosis, 280–282
  - leukocyte telomere length in atherosclerosis, 169–170
  - mortality, 165
  - sex differences
    - prevalence, 386
    - primate studies, 391–392
  - sirtuins and cardiovascular system modulation in aging, 258–260
- Caspases
  - mitochondrial-mediated signaling in sarcopenia, 73–75
  - muscle calcium stress apoptosis signaling, 83
  - muscle extrinsic apoptotic pathway, 79–80
  - muscle intrinsic apoptotic pathway
    - dependent signaling, 71
    - independent signaling, 71–72
- Catalase
  - conservation across species, 218
  - knockout effects on mouse longevity, 191
  - mitochondrial, 54
  - overexpression effects on mouse longevity, 188–189
  - somatotropic axis modulation, 28–29
  - transgenic mouse life span, 56
- CBF, *see* Cerebral blood flow
- CBP-1, dietary restriction pathway in *Caenorhabditis elegans*, 11
- Cellular senescence, obesity, 125
- Cerebral blood flow (CBF)
  - aging effects, 407
  - cognition studies, 357
  - determinants, 407
  - neurovascular coupling, 349–350, 407–408
- Cerebral microvascular disease
  - brain structural changes in chronic hypoperfusion, 351–352
  - clinical implications
    - Alzheimer's disease, 356–357
    - cognitive dysfunction, 356–357
    - falls, 353–356
    - gait disorders, 353–356
  - covert stroke incidence, 348
  - endothelial dysfunction in aging, 349, 352
  - hemodynamic changes in aging, 349
  - magnetic resonance imaging and white matter hyperintensity, 348–349
  - neurovascular coupling, 349–350, 407–408
  - prospects for study, 361–362
  - therapeutic interventions
    - flavonoids, 359–360
    - hypoxia-inducible transcription factor-1, 358–359
    - renin-angiotensin system inhibitors, 360
  - vascular remodeling in aging, 405–406
- Chaperones, *see* Heat shock proteins
- Circadian rhythm
  - central control of temperature, 473
  - sirtuin regulation, 253
- CLK-1, dietary restriction pathway in *Caenorhabditis elegans*, 11
- Coenzyme Q10, life span effects in mice, 180, 184
- Cognition
  - cerebral microvascular disease effects, 356–357
  - somatotropic axis role, 30–32
- Collagen
  - pulmonary changes in aging, 427–429
  - somatotropic axis in cross-linking, 35
- Creatine, life span effects in mice, 181, 185
- CVD, *see* Cardiovascular disease
- Cytochrome *c*, mitochondrial-mediated signaling in sarcopenia, 73, 75

D

- DBC1, SIRT1 modulation, 250, 255
- Death receptors, *see* Tumor necrosis factor-α
- Dehydroepiandrosterone (DHEA), thermogenesis, 469
- Dementia, cerebral microvascular disease, 356
- Desferrioxamine, hypoxia-inducible transcription factor-1 activation, 358–359
- DHEA, *see* Dehydroepiandrosterone
- Diabetes, *see also* Insulin
  - age-related hyperglycemia modeling, 381–382
  - complications, 374
  - diagnosis, 373
  - preventive interventions, 374
  - primate studies, 392
- Diet-induced thermogenesis, *see* Thermogenesis
- Dietary restriction (DR)
  - adipose tissue effects, 125
  - *Caenorhabditis elegans*
    - approaches for dietary restriction, 3–5
    - pathways
      - autophagy genes, 12
      - criteria for gene dependence, 5, 8–9
      - energy sensors, 9–10
      - mitochondrial genes, 12
      - table of genes, 6–7
      - transcriptional regulators, 10–11
  - conservation of effects across species
    - insulin/insulin-like growth factor-I system, 228–229
    - overview, 222, 228
    - sirtuins, 229–230, 247–248
    - target of rapamycin, 230–231
  - differences by regimens

    - mimetics, 16
    - non-dietary restriction parameters, 16
    - nutrient types, 14–15
    - temporal effects, 15
    - tissue specificity, 15–16
  - *Drosophila* pathways, 12–13
  - growth hormone receptor knockout studies, 28
  - human observations
    - Biosphere 2, 455
    - CALERIE phase 1 study
      - Pennington Center, 456
      - Tufts University, 456–457
      - Washington University, 455–456
    - Calorie Restriction Society members, 457
    - food restriction during world wars, 452
    - Okinawa Island, 452, 455
    - overview, 452–454
  - mammalian pathways, 13–14, 248–249
  - muscle apoptosis reduction in aging
    - cardiac muscle, 99–100
    - skeletal muscle, 96
  - oxidative stress defenses, 30
  - prospects for study, 17
  - rhesus monkey studies
    - comparison of studies, 449, 452
    - health span versus life span, 448
    - National Institutes of Health studies, 449–450
    - overview, 392
    - University of Wisconsin studies, 450–451
  - yeast pathways, 12
- Diphenyleneiodonium (DPI), superoxide production inhibition, 49–50
- DNA polymerase-γ, transgenic mouse life span, 55–56
- DPI, *see* Diphenyleneiodonium
- DR, *see* Dietary restriction
- *Drosophila melanogaster*
  - aging model, 221
  - dietary restriction pathways, 12–13
  - target of rapamycin and link between nutrients and life span, 205–206

E

- EDHF, *see* Endothelium-derived hyperpolarizing factor
- Elastin, pulmonary changes in aging, 427–429
- Electron transfer flavoprotein–ubiquinone (ETTF-Q) oxidoreductase, mitochondrial free radical production, 51–52
- Embryonic stem (ES) cell, features, 141–142
- Endothelial cell, aging effects, 410
- Endothelial progenitor cell (EPC), leukocyte telomere length in atherosclerosis, 169–170
- Endothelium-derived hyperpolarizing factor (EDHF), vasodilation, 410
- End replication problem (ERP)
  - overview, 165
  - oxidative stress, 167
- EPC, *see* Endothelial progenitor cell
- ERP, *see* End replication problem
- ES cell, *see* Embryonic stem cell
- ETTF-Q oxidoreductase, *see* Electron transfer flavoprotein–ubiquinone oxidoreductase
- Evaporative heat loss, aging effects, 472
- Extrinsic hazards hypothesis, sex differences in longevity, 485–487

F

- FAAH, *see* Fatty acid amide hydrolase
- Falls, cerebral microvascular disease in etiology, 353–356
- Fat, *see* Adipose tissue; Obesity
- Fatty acid amide hydrolase (FAAH), knockout mouse and aging effects, 100
- Finger tapping speed (FTS), brain myelin integrity correlation, 339
- FLIP, muscle apoptosis suppression, 72
- FoxA, dietary restriction pathway in *Caenorhabditis elegans*, 10–12
- FoxO
  - conservation across species, 217
  - dietary restriction pathway
    - *Caenorhabditis elegans*, 10
    - *Drosophila*, 13
    - mammals, 14
  - sirtuin modulation, 259
- Frailty
  - definition, 322–323
  - etiology in elderly, 323
- Free radicals, *see* Mitochondrial free radicals; Oxidative stress
- FTS, *see* Finger tapping speed

G

- Gait, cerebral microvascular disease in disorders, 353–356
- GCN4, mediation of target of rapamycin-induced longevity effects, 207
- Gender differences, *see* Sex differences
- GH, *see* Growth hormone
- α-Glycerolphosphate dehydrogenase, mitochondrial free radical production, 51
- Glucagon, thermogenesis, 469
- Glutathione, life span effects in mice, 180, 185
- Glutathione peroxidase
  - knockout effects on mouse longevity, 191
  - mitochondrial, 53
  - overexpression effects on mouse longevity, 189
  - somatotropic axis modulation, 28–29
- Growth hormone (GH)
  - age-related pathology, 34–35
  - cognitive effects, 30–32
  - direct metabolic effects, 26
  - immune function, 32–33
  - inhibition and aging delay in mice, 26–27
  - insulin sensitivity effects, 27
  - oxidative stress induction and defenses, 28–29
  - receptor knockout and caloric restriction effects, 28
  - thermogenesis, 469
- GRP78, muscle calcium stress apoptosis signaling, 83

H

- Heart failure, cardiomyocyte apoptosis, 97
- Heat shock proteins (HSPs)
  - chaperone-mediated autophagy, 305–307, 309
  - cytosolic chaperones, 300–301
  - organelle chaperones in stress response, 301–302
- Heat shock transcription factor-1 (HSF-1)
  - dietary restriction pathway in *Caenorhabditis elegans*, 11
  - dysfunction in aging, 300–301
- Hematopoietic stem cell (HSC)
  - aging effects, 149–152
  - telomere dynamics
    - aging ramifications, 170–171
    - atherosclerosis studies, 169–170
    - longevity considerations, 170
    - overview, 167–168
    - race differences, 169
    - sex differences, 168–169
- Hepatic steatosis, aging, 122–123

Heterogametic sex hypothesis, sex differences in longevity, 487–488
HIF-1, *see* Hypoxia-induced transcription factor-1
Histone deacetylase, *see* Sirtuins
HSC, *see* Hematopoietic stem cell
HSF-1, *see* Heat shock transcription factor-1
HSPs, *see* Heat shock proteins
Hypercapnia, chemoreceptors, 425
Hypoxia-induced transcription factor-1 (HIF-1)
  cerebral microvascular disease therapeutic targeting, 358–359
  dietary restriction pathway in *Caenorhabditis elegans*, 11
  mediation of target of rapamycin-induced longevity effects, 208–209
  sirtuin regulation, 255

I

Id2
  mitochondrial-mediated signaling in sarcopenia, 76
  satellite cell apoptosis role, 85
IGF-I, *see* Insulin-like growth factor-I
IL-15, *see* Interleukin-15
Induced pluripotent stem cell, features, 142
Inflammation
  Alzheimer's disease, 280–282
  atherosclerosis, 280–282
  cancer, 282–283
  diet, metabolism, and exercise, 284–285
  dietary restriction response, 260
  ecological perspective on environmental influences, 286–287
  genetics, 285–286
  glucose elevations in inflammatory processes, 283–284
  obesity, 123–125
  osteopenia, 283
  overview of responses, 275–277
  sirtuin modulation, 260–261
  systemic manifestations and aging, 278–280
  terminal weight loss role, 327
  tissue changes in aging, 280–281
  vasculature, 412–413
Insulin, *see also* Diabetes
  autophagy inhibition, 308
  beta cell secretion
    function and aging studies
      humans, 378–380
      rodents, 376–378
    regulation, 375–376
  conservation of effects across species, 222–223, 228–229
  dietary restriction pathway
    Caenorhabditis elegans, 9
    mammals, 14, 27, 381
  glucose elevations in inflammatory processes, 283–284
  inhibition and aging delay in mice, 26, 28
  receptor conservation across species, 217
  sensitivity
    growth hormone effects, 27
    long-living animals, 27
  signaling and longevity, 380–381
  thermogenesis, 469
Insulin-like growth factor-I (IGF-I)
  age-related pathology, 34–35
  cognitive effects, 30–32
  conservation of effects across species, 224, 228–229
  immune function, 32–33
  inhibition and aging delay in mice, 26–28
  receptor conservation across species, 217
Interleukin-15 (IL-15), muscle extrinsic apoptotic pathway, 80
Intestinal epithelial stem cell (ISC), aging effects, 152–154
ISC, *see* Intestinal epithelial stem cell

J

JAK-STAT pathway, growth hormone/cytokine crosstalk, 32

K

α-Ketoglutarate dehydrogenase, mitochondrial free radical production, 52
Klotho, vasculature protection, 412

L

LAMP-2A, aging effects, 309
Left ventricular function, sex differences in primates, 394
Life expectancy at birth, 164
α-Lipoic acid, life span effects in mice, 180, 184–185
Lung, *see* Pulmonary function

M

Macroautophagy, *see* Autophagy
Magnetic resonance imaging (MRI), cerebral microvascular disease, 348–349, 351, 354
Melatonin, life span effects in mice, 181, 185
Methionine sulfoxide reductase, knockout effects on mouse longevity, 192–193
Mitochondrial DNA, mutation in aging, 55
Mitochondrial free radicals
  antioxidant systems, 52–54
  free radical theories of aging, 54–56, 177–178, 193–196
  measurement *in vivo*, 52
  myonuclear apoptosis, 70–71
  sources
    Complex I
      inhibitors, 49–50
      reactive oxygen species production, 48–50
    Complex II, 51
    Complex III
      inhibitors, 50–51
      reactive oxygen species production, 50
    electron transfer flavoprotein–ubiquinone oxidoreductase, 51–52
    α-glycerolphosphate dehydrogenase, 51
    α-ketoglutarate dehydrogenase, 52
    overview, 47–48
    pyruvate dehydrogenase, 52
MRI, *see* Magnetic resonance imaging
Msn2/4, mediation of target of rapamycin-induced longevity effects, 207
mTOR, *see* Target of rapamycin
Muscle apoptosis
  cross-talk between pathways
    intrinsic and calcium stress pathways, 84
    intrinsic and extrinsic pathways, 81–82
  extrinsic apoptotic pathway
    cellular conditions for activation, 77
    tumor necrosis factor-α and death receptors, 77–81
  heart
    aging effects on mitochondria-associated signaling, 92
    Bcl-2 and Bax modulation in aging heart, 98–99
    cardiac function and aging, 97–98
    cardiomyocyte apoptosis
      aging effects, 96–97

    heart failure role, 97
    longevity interventions, 99–100
    p66Shc role, 99
  intrinsic apoptotic pathway
    BCL-2, 70–71
    caspases
      dependent signaling, 71
      independent signaling, 71–72
    cellular conditions for activation, 66–67
    mitochondrial dysfunction
      bioenergetics, 67–68
      oxidative stress, 69–70
      permeability, 68–69
      sarcopenia, 67
    mitochondrial-mediated signaling in sarcopenia, 72–76
    suppressors, 72
  modulation in aging
    aerobic exercise, 95–96
    caloric restriction, 96
    death receptor signaling response, 90–91
    extrinsic pathway response, 91
    mitochondria-associated signaling response
      cardiac muscle, 93
      skeletal muscle, 86–89
    muscle loading, 85, 94
    unloading and disuse, 94–95
  myonuclear apoptosis
    overview, 64–65
    signaling, 65–66
  overview in aging, 64
  prospects for study, 100–101
  respiratory muscle function, 433–434
  sarcoplasmic/endoplasmic reticulum–calcium stress signaling
    cellular conditions for activation, 82–83
    overview, 83–84
  satellite cell apoptosis
    aging effects, 147–149
    loading adaptation effects, 94
    overview, 84
    role
      Id2, 85
      MyoD, 84–85
Myelination, brain
  aging changes, 336–338
  developmental changes, 334–337
  myelin model, 338–341
  species comparison, 333–334
MyoD, satellite cell apoptosis role, 84–85
Myxothiazol, Complex III inhibition, 51

N

Neural stem cell (NSC), aging effects, 154–156
Neurovascular coupling, *see* Cerebral microvascular disease
NF-κB, *see* Nuclear factor-κB
NFE2, dietary restriction pathway
  Caenorhabditis elegans, 11
  mammals, 14
Nitric oxide synthase (NOS)
  endothelial function, 408–409
  sirtuin modulation, 259
NOS, *see* Nitric oxide synthase
Nrf2, dietary restriction pathway in mammals, 14
NSC, *see* Neural stem cell
Nuclear factor-κB (NF-κB), sirtuin modulation, 260–261

O

Obesity
  aging changes in body weight, 321–322
  cellular senescence, 125
  comorbidity, 123
  epidemiology, 123
  inflammation, 123–125
Ogg1, knockout effects on mouse longevity, 193–194
Optic neuritis, sirtuin protection, 262
Osteopenia, inflammation, 283
Oxidative stress
  end replication problem, 167
  free radical theories of aging, 54–56, 177–178, 193–196
  sex differences in longevity, 489–491
  somatotropic axis role, 28–30
  vasculature, 410–412
Oxygen, arterial partial pressure in aging, 438–439

P

p53, mitochondrial-mediated signaling in sarcopenia, 76
p66Shc, cardiomyocyte apoptosis role, 99
Parental care hypothesis, sex differences in longevity, 488
PBN, *see* N-tert-Butyl-α-phenylnitrone
PDH, *see* Pyruvate dehydrogenase
Peripheral blood flow, aging effects, 471–472
Peroxiredoxin, knockout effects on mouse longevity, 191
Peroxisome proliferator-activated receptors (PPARs)
  PPAR-γ and vasculature protection, 412–413
  sirtuin regulation, 251
Peroxynitrite, formation, 411
PHA-4, mediation of target of rapamycin-induced longevity effects, 208
Phosphoinositide 3-kinase (PI3K)
  conservation across species, 217
  dietary restriction pathway in *Caenorhabditis elegans*, 9
Phospholipid glutathione peroxidase, knockout effects on mouse longevity, 191–192
Physical activity, baseline physical activity in aging, 468
PI3K, *see* Phosphoinositide 3-kinase
Posture, thermogenesis, 468
PPARs, *see* Peroxisome proliferator-activated receptors
Primate models, *see* Rhesus monkey
Proteasome, *see* Ubiquitin/proteasome system
Protein homeostasis
  autophagy
    aging and longevity, 307–309
    physiological functions, 306
    protein aggregate handling, 307
    soluble protein removal, 306–307
    types, 305–306
  heat shock proteins in quality control
    cytosolic chaperones, 300–301
    organelle chaperones in stress response, 301–302
  overview, 298–299
  prospects for study, 309–310
  proteome maintenance, 297–298
  ubiquitin/proteasome system
    catalytic machinery, 302–304
    ubiquitinization code, 304
Pulmonary function
  aging changes
    alveolar ventilation and perfusion, 436–438
    arterial partial pressures
      carbon dioxide, 439
      oxygen, 438–439
    dynamic lung volume and ventilatory flow, 436–437
    mechanical considerations
      airway resistance, dynamic airway collapse, and closing volume, 432–433
      collagen composition, 427–429

- Pulmonary function (*Continued*)
        - elastic recoil of lung tissue and chest wall, 429–430
        - elastin composition, 427–429
        - muscle function, 433–434
        - static work of breathing, 431–432
    - morphological considerations, 426–427
    - static lung volume and capacity, 434–435
  - experimental design for aging studies
    - cross-sectional versus longitudinal designs, 423–424
    - sampling considerations, 424–425
  - overview of regulation, 421–423
  - sensory feedback
    - central integration, 426
    - chemoreceptors, 425
    - secondary receptors, 426
  - terminology, 422
- Pyruvate dehydrogenase (PDH), mitochondrial free radical production, 52

Q

- Quercetin, hypoxia-inducible transcription factor-1 activation, 359

R

- Rapamycin, longevity effects, 209
- Reactive oxygen species, *see* Mitochondrial free radicals; Oxidative stress
- Renin–angiotensin system, *see* Angiotensin II
- Reproductive senescence, primate studies, 391
- Resveratrol
  - Alzheimer's disease studies, 262
  - sirtuin modulation, 250–251
- Rhesus monkey
  - advantages of model system
    - gene expression, 390
    - life span, 390
    - physiology and metabolism, 390
    - reproductive physiology and endocrinology, 390–391
  - dietary restriction studies
    - comparison of studies, 449, 452
    - health span versus life span, 448
    - National Institutes of Health studies, 449–450
    - University of Wisconsin studies, 450–451
  - sex difference studies
    - cardiovascular aging sex differences
      - β-adrenergic receptor desensitization, 394, 397
      - aortic stiffness, 395–398
      - cardiomyopathy, 392–393, 396–397
      - left ventricular function, 394
      - prospects for study, 398
    - cardiovascular disease, 391–392
    - diabetes, 392
    - dietary restriction, 392
    - neurobiology, 391
    - reproductive senescence, 391
- RNA interference, *Caenorhabditis elegans* genes in dietary restriction and longevity, 8
- Royal jelly, life span effects in mice, 180, 184

S

- S6K
  - conservation across species, 217
  - mediation of target of rapamycin-induced longevity effects, 205, 208–209
- *Saccharomyces cerevisiae*
  - chronological aging, 220
  - dietary restriction pathways, 12
  - longevity pathway conservation with worms, 226–227
  - replicative aging, 216, 220
  - target of rapamycin and link between nutrients and life span, 206–207
- Sarcopenia, *see* Muscle apoptosis
- Sarcoplasmic reticulum calcium-ATPase (SERCA), muscle apoptosis signaling, 82
- Satellite cell
  - aging effects, 147–149
  - apoptosis, *see* Muscle apoptosis
- SERCA, *see* Sarcoplasmic reticulum calcium-ATPase
- Sex differences
  - birth ratio, 483
  - cardiovascular disease prevalence, 386
  - leukocyte telomere dynamics, 168–169
  - longevity in humans
    - age-specific mortality, 482–484
    - demographics across countries, 480–482
    - mortality–morbidity paradox, 484
    - theories for sex differences
      - body size difference, 491
      - extrinsic hazards hypothesis, 485–487
      - heterogametic sex hypothesis, 487–488
      - oxidative stress hypothesis, 489–491
      - parental care hypothesis, 488
  - mechanisms of differences in aging
    - hormones, 388
    - overview, 387
    - sex-linked genes, 387–388
  - models for aging studies
    - limitations
      - human models, 389
      - rodent models, 389
    - primate model advantages
      - gene expression, 390
      - life span, 390
      - physiology and metabolism, 390
      - reproductive physiology and endocrinology, 390–391
  - primate studies
    - cardiovascular aging sex differences
      - cardiomyopathy, 392–393, 396–397
      - β-adrenergic receptor desensitization, 394, 397
      - left ventricular function, 394
      - aortic stiffness, 395–398
      - prospects for study, 398
    - cardiovascular disease, 391–392
    - diabetes, 392
    - dietary restriction, 392
    - neurobiology, 391
    - reproductive senescence, 391
  - rodent studies of longevity, 488–489
  - species-specific differences in aging, 388–389
- SIR2, *see* Sirtuins
- Sirtuins
  - cardiovascular system modulation, 258–260
  - discovery, 244
  - endogenous modulators
    - chemical inhibitors and activators, 250–251
    - gene expression, 248
    - NAD$^+$, NADH, and nicotinamide, 248–250
    - protein–protein interactions, 250
  - energy metabolism regulation
    - SIRT1, 251–253
    - SIRT3, 253–254
    - SIRT4, 254
    - SIRT5, 255
    - SIRT6, 255

  - enzymology, 245–246
  - functional overview, 243–244
  - inflammation and immunity modulation, 260–261
  - neuroprotection, 261–262
  - oncogenic versus tumor suppression activity, 255–258
  - prospects for study, 262–263
  - SIR2
    - conservation across species, 217, 224–225
    - dietary restriction pathways
      - *Caenorhabditis elegans*, 9
      - comparison between species, 229–230, 246–247
      - *Drosophila*, 13
      - yeast, 12
  - SIRT1
    - dietary restriction pathway in mammals, 13–14, 99, 247–248
    - prosurvival activity, 255
    - tumor suppression activity, 255–258
- SOD, *see* Superoxide dismutase
- SRT1720, sirtuin modulation, 251
- Statins, anti-inflammatory mechanisms, 285
- Stem cell
  - adult stem cells, 143–144
  - aging effects
    - hematopoietic stem cell, 149–152
    - intestinal epithelial stem cell, 152–154
    - muscle stem cell, 147–149
    - neural stem cell, 154–156
    - overview, 145–147
  - embryonic stem cell, 141–142
  - induced pluripotent stem cell, 142
  - longevity significance, 156
  - niches, 144–145
- Stigmatellin, Complex III inhibition, 51
- Strawberry extract, life span effects in mice, 181, 185
- Stroke, *see* Cerebral microvascular disease
- Superoxide dismutase (SOD), *see also* Carboxyfullerene superoxide dismutase
  - conservation across species, 218
  - knockout effects on mouse longevity
    - SOD1, 190–191, 195
    - SOD2, 191, 193–194
  - mitochondrial, 53–54
  - overexpression effects on mouse longevity
    - SOD1, 186–187
    - SOD2, 187–188
  - somatotropic axis modulation, 28–29
  - vasculature defenses, 411–412
- Superoxide, *see* Mitochondrial free radicals; Oxidative stress

T

- Target of rapamycin (TOR)
  - complex, 204–205
  - conservation of effects across species, 218, 226–228, 230–231
  - dietary restriction pathway
    - *Caenorhabditis elegans*, 10, 12
    - *Drosophila*, 13
    - mammals, 14
    - yeast, 12
  - links between nutrients and life span
    - *Caenorhabditis elegans*, 207–209
    - *Drosophila*, 205–206
    - mouse, 209
    - yeast, 206–207
  - nutrition–growth coupling, 203–204
  - signaling between nutrients, growth factors and stress, 204–205
- T cell, growth hormone effects, 33
- Telomere
  - comparative biology between mouse and humans, 166
  - end replication problem, 165, 167
  - length, replication and evolution, 165–167
  - leukocyte dynamics
    - aging ramifications, 170–171
    - atherosclerosis studies, 169–170
    - longevity considerations, 170
    - overview, 167–168
    - race differences, 169
    - sex differences, 168–169
  - shortness in humans, 166–167
- Terminal weight loss, *see* Body weight
- Testosterone, thermogenesis, 469
- Tetrahydrocurcumin, life span effects in mice, 181, 185–186
- Thermogenesis
  - central control
    - circadian rhythm in temperature, 473
    - thermoregulatory control, 472–473
  - facultative thermogenesis and aging effects
    - classical neuronal shivering thermogenesis, 469
    - nonclassical nonshivering thermogenesis, 469
    - physical activity, 469
    - shivering thermogenesis, 469
  - heat gain and retention
    - behavioral measures, 469–470
    - body capacitive and insulative properties, 470
    - peripheral vasoconstriction and autonomic heat retention, 470
  - heat loss and reduction of heat gain
    - evaporative heat loss, 472
    - peripheral blood flow, 471–472
  - obligatory thermogenesis and aging effects
    - basal metabolic rate, 468
    - baseline physical activity and posture, 468
    - diet-induced thermogenesis, 468
  - types, 466–467
- Thermoreception
  - aging effects, 466
  - anatomy and physiology
    - central nervous system, 465–466
    - deep body thermoreception, 465
    - skin, 464–465
  - overview, 464
- Thioredoxin
  - conservation across species, 219
  - knockout effects on mouse longevity, 192
  - overexpression effects on mouse longevity, 56, 189
- Thioredoxin reductase, knockout effects on mouse longevity, 192
- Thymulin, growth hormone modulation, 33
- TNF-α, *see* Tumor necrosis factor-α
- TOR, *see* Target of rapamycin
- Tumor necrosis factor-α (TNF-α), muscle extrinsic apoptotic pathway death receptors, 77–81

U

- Ubiquitin/proteasome system (UPS)
  - aging effects, 302–304
  - catalytic machinery, 302–304
  - ubiquitinization code, 304
- Unfolded protein response (UPR), dysfunction in aging, 301–302
- UPR, *see* Unfolded protein response
- UPS, *see* Ubiquitin/proteasome system

V

Vascular remodeling, aging, 405–406
Vascular stiffness, *see* Aortic stiffness
Visceral fat, function, 121
Vitamin C, life span effects in mice, 179, 182–183
Vitamin E, life span effects in mice, 178–179, 182

W

Wallerian degeneration, sirtuin protection, 261–262
Weight, *see* Body weight; Obesity
White matter hyperintensity, *see* Cerebral microvascular disease

X

XIAP, *see* X-linked inhibitor of apoptosis
X-linked inhibitor of apoptosis (XIAP)
  mitochondrial-mediated signaling in sarcopenia, 75
  muscle apoptosis suppression, 72

Y

Yeast, *see Saccharomyces cerevisiae*