Espen E. Spangenburg *Editor*

Integrative Biology of Women's Health

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Preface

The word integrative is defined as "bringing together parts to make up a whole." Hence, this book was designed to bring together viewpoints of scientists across a variety of disciplines, all relevant to women's health. The invited authors have all published significant material on matters relevant to women's health. Thus, it is expected that each author will provide the reader novel insight that might encourage new avenues of thought or research that would benefit women.

A common problem often faced when designing an experimental approach to test a hypothesis is that scientists will simplify the biological complexity that likely underlies the problem to more easily identify the mechanism. Often dragged into this methodology is the assumption that the mechanism will respond in a similar fashion across both sexes. However, numerous seminal investigations conducted over past few decades have clearly demonstrated that biological mechanisms often behave differently across the sexes. In other words, men and women are different. Although the statement is obvious, when you examine the statement solely from the cellular or the tissue perspective the mechanisms that explain these differences remain poorly defined. Unfortunately, this has often led us to assume that clinical approaches used for treating chronic disease will work with equal fidelity in both men and women. The evidence is mounting that these assumptions are often not true and are likely the result of mechanistic differences that exist between sexes.

A recent wave of scientific momentum has begun to develop where investigations are focusing on regulatory mechanisms specifically in women. The resulting data are often unique and in some cases challenge the previously defined dogma. The purpose of this book is to capture this momentum in the form of a collection of review-based chapters across a variety of biological areas. The book seeks to address the basic biology of women's health across a number of tissues including cardiac muscle, bone, skeletal muscle, and brain while discussing critical questions from a physiological and metabolic perspective. In addition, the book was designed to have translational appeal in that chapters contained within the book discuss scientific results that were obtained utilizing a wide array of approaches including cell culture of animals or humans.

In order to develop appropriate treatment interventions for women, this critical knowledge gap in women's health research must be addressed. The former Deputy Director of NIH, Dr. Ruth Kirschstein, once stated, "Researchers must continue to make more intensive efforts to address the health needs of the whole woman." Overall, it is the hope of the authors of each chapter that the reader will be enlightened to the complex biological issues that are often presented in a unique fashion in women.

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Chapter 1
Influence of Ovarian Hormones on Skeletal Muscle Contractility

Dawn A. Lowe and Sarah M. Greising

Abstract There is a loss of skeletal muscle strength around the time of menopause in women, probably due to the decline of ovarian hormone production. The maintenance of muscle strength and contractility with age and with loss of ovarian hormones are critical issues because the risk for disability and dependent living increases with muscle weakness. There is substantial evidence that estradiol is beneficial to muscle strength. Thus, better understanding of the mechanisms by which estradiol affects contractility and how the loss of this hormone is detrimental to skeletal muscle function is critical. This chapter focuses on ovarian hormones, specifically how the lack of estradiol affects skeletal muscle contractility in both postmenopausal women and rodent models.

Keywords Aging • Estradiol • Estrogen • Hormone replacement therapy • Muscle force • Myosin • Postmenopausal • Power • Ovariectomy • Strength

Introduction

The primary function of skeletal muscle is to contract, generate force, and enable movement. A broad array of factors can influence the ability of muscle to contract including ovarian hormones in females. The major ovarian hormones are estrogens and progesterone; inhibin and relaxin are considered to be minor ovarian hormones.

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Since the identification of estrogen receptors (ER) in skeletal muscle [21, 55, 56], subsequent studies have been conducted to determine the function of estrogens and ER in this tissue. Estradiol (E_2) is the estrogen most investigated because it is the predominant estrogen in females with functioning ovaries in terms of both serum levels and biological activity. Thus, it is likely that E_2 is the most relevant estrogen affecting skeletal muscle in females during reproductive years. The other naturally occurring estrogens in females are estrone (E_1) and estriol (E_3). E_1 predominates in aged females following menopause and E_3 is the dominant estrogen during pregnancy. How E_1 and E_3 affect skeletal muscle during these time frames of the female life have not been investigated. Therefore, this chapter focuses on how E_2, and the lack of E_2, affect skeletal muscle contractility.

Menopause and hormone therapy are the foremost conditions in women in which the absence of and treatment with E_2, respectively, have been studied for effects on skeletal muscle contractility. Rodent models are commonly used to probe the mechanisms by which E_2 affects skeletal muscle. In those models, ovariectomy and ovarian senescence are conditions that have been exploited to investigate the loss of E_2 in young and aged mammals, respectively. Some of the advantages of utilizing rodent models include genetic homogeneity, standardized nutrition and housing conditions, highly reproducible and sensitive methods of measuring muscle contractility, relatively short life-span making longitudinal study designs feasible, and more easily controlled hormonal treatments relative to women. The effects of ovarian hormone-altering conditions on muscle contractility in women and rodents are beginning to be identified and, in general, support the supposition that ovarian hormones, particularly E_2, are beneficial to skeletal muscle function. The evidence to support these statements is summarized below.

Estradiol and Muscle Strength

E_2 and muscle strength in women. The aspect of skeletal muscle contractility that is investigated the most is strength, typically measured as force or torque. An early report of declining muscle strength was by Asmussen who showed that back, arm, and leg muscle strength start to gradually decline around the 6th decade of life in men and women [3]. This result is similar to others [2, 18, 31, 35, 41]; however, some reports show that women have an accelerated decline in strength around the time of menopause [27, 35, 41]. It was postulated that the greater decline in strength in postmenopausal women was due to the loss of estrogen production by the ovaries.

One way of determining whether or not the additional decline in strength in postmenopausal women can be attributed to loss of estrogen is to compare strength between women who are and are not on estrogen-based hormone therapy (HT). A highly cited study in which this was done was published by Phillips and coworkers in 1993 [35]. They measured adductor pollicis muscle strength in 25

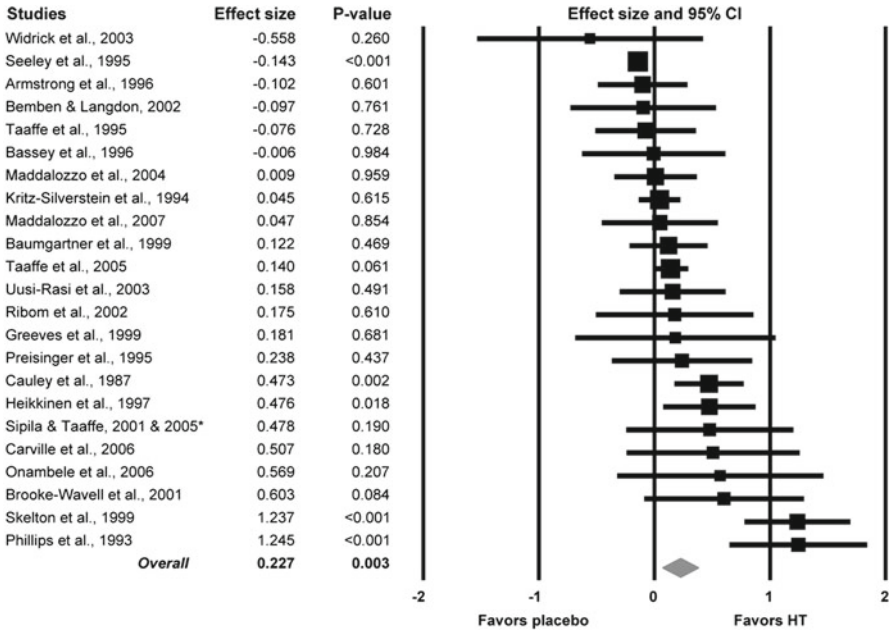

Fig. 1.1 Forest plot of effect sizes from 23 publications between 1987 and 2007 that reported muscle strength in postmenopausal women who were and were not on an estrogen-based hormone therapy (HT). An effect size for each study is represented by a *square*, with the size of the *square* equating to the weight of the study in the overall meta-analysis. *Horizontal lines* through the *squares* represent 95 % confidence intervals. The *diamond* at the *bottom* illustrates the significant overall, beneficial effect of HT on muscle strength in postmenopausal women. Refer to Greising et al. for citations abbreviated in the graph [12]. Figure is reprinted with permission from Oxford University Press, copyright 2009

postmenopausal women aged 42–72 years who were taking an estrogen-based HT and compared them to 67 postmenopausal women not on HT. The main finding was that HT prevented the loss of strength. As such, these data have been the most robust evidence of a positive effect of estrogen on muscle strength in postmenopausal women. Conversely, other studies of postmenopausal women have shown little or no impact of HT on strength. In fact, narrative reviews that summarize findings from those studies conclude that there are inconsistent results regarding estrogenic effects on muscle strength in older women and that further research is needed [27, 44].

A systematic review and meta-analysis of the research literature was conducted to directly address inconsistent reports regarding muscle strength in postmenopausal women and HT [12]. Twenty-three studies were identified in which strength was compared between postmenopausal women on an estrogen-based HT and those not on HT, that is, women who remained estrogen deficient. This yielded strength data for a total of 2,668 postmenopausal women on estrogen-based HT and 7,288

postmenopausal women not on any estrogen treatment. When data from the subjects were statistically combined and analyzed, the overall result was that strength was significantly greater in women on HT. The impact was small with an effect size of 0.23, equating to women taking estrogenic hormones being approximately 5 % stronger (Fig. 1.1). However, when only randomized, controlled trials were considered [1, 15, 38, 45, 47], the combined effect size of estrogen treatment on strength was much higher at 0.46, though this was not quite statistically significant due to the small number of studies. Overall, the results of the meta-analysis pooling data from 23 studies show that postmenopausal women taking an estrogen-based HT have greater muscle strength compared to women not taking HT.

Positive correlations between circulating E_2 levels in women and muscle strength would also be expected and have been reported. Pollanen and co-workers showed that serum concentration of E_2 was positively correlated with quadriceps femoris muscle force among pre- and postmenopausal women [37]. Interestingly, while strength was significantly correlated to serum E_2, it was not significantly correlated to the concentration of E_2 in the quadriceps muscle. Further understanding of the mechanisms underlying estrogenic effects on muscle contractility will also need to include the importance of local, non-ovarian E_2 production [37].

E_2 and muscle strength in rodent models. Ovariectomy in rodent models is an approach that has been utilized to investigate the influence of ovarian hormones on muscle contractility, particularly strength. The ovarian hormone responsible for any such influence can then be determined by treating ovariectomized rodents with a specific hormone, such as 17β-E_2. An advantage of strength measurements in rodents is that maximal force or torque is assessed, whereas most studies on humans depend on effort and performance of the subject and their ability to recruit motor units, which is unlikely to be maximal.

One of the first reports to compare muscle strength in a rodent ovariectomy model was by Suzuki and colleagues [48]. They ovariectomized very young, 3-week-old Wistar rats and then followed with placebo or E_2 treatments for 10 weeks. An age-matched control group was also studied with those rats undergoing a sham operation and placebo treatment. Soleus and extensor digitorum longus (EDL) muscles from ovariectomized rats that were treated with E_2 had the lowest maximal isometric tetanic forces. These data do not support the contention that estrogens are beneficial to muscle strength, rather the results suggest that estrogens impact skeletal muscle during development and growth. Similarly, young growing Sprague Dawley rats were used in a study by McCormick and colleagues [26]. In that study, 7-week-old rats were sham operated or ovariectomized and then rats that were ovariectomized received a placebo or an E_2 treatment for 4 weeks. No differences in soleus muscle maximal isometric tetanic force among sham, ovariectomized, and ovariectomized plus E_2 treatment groups were detected. These results again do not lend support to any beneficial estrogenic effects on muscle strength using a rodent model in which substantial growth and development were occurring during the hormone manipulation.

Fisher and colleagues conducted one of the first studies of ovarian hormone effects on strength using a mature, fully grown rodent model [9]. Sprague Dawley

rats, 6.5 months of age, were randomized to either sham surgery or ovariectomy surgery; some rats were further randomized to hindlimb unloading groups. One month later, results from the control (i.e., normal loaded) rats were mixed in regard to strength. Forces generated by soleus and plantaris muscles did not differ between rats that were sham operated and ovariectomized, but EDL muscle force was greater in the ovariectomized rats, and peroneus longus muscle force was greater in the sham, ovary-intact rats. Overall, the results of this study do not support or refute effects of ovarian hormones on muscle strength.

Collectively, studies utilizing the rat ovariectomy model do not support beneficial effects of estrogen on muscle strength. This is directly shown by results of a meta-analysis as the combined data from the above three studies utilizing rat models yield a nonsignificant effect size of estrogen on strength [12].

Relatively more studies have been conducted using mouse models to investigate estrogenic effects on muscle strength. Warren and colleagues designed a study with the aim of determining the effects of E_2 on the functions of the musculoskeletal system [52]. Young ICR mice, 6 weeks of age, were ovariectomized and randomized to receive E_2 or placebo treatment for 3 weeks. Maximal isometric torque of the ankle dorsiflexor muscles was 18 % greater in mice treated with E_2 than placebo and maximal isometric tetanic force of EDL muscles was 14 % greater in the E_2-treated than the E_2-deficient mice. These results support a beneficial estrogenic effect on muscle strength. Conversely, Wohlers and co-workers reported that 8-week-old C57BL/6 mice that were ovariectomized and studied for contractility 8 weeks later did not differ in tibialis anterior muscle strength compared to age-matched, sham-operated mice [57]. A study by Schneider and colleagues also investigated the influence of ovarian hormones on muscle contractility in young, 6-week-old, C57BL/6 mice [42]. Mice that were ovariectomized were randomized to receive placebo, E_2, progesterone, or a combination of E_2 and progesterone for 16 days. Maximal isometric torque of the hindlimb plantarflexors was ~27 % greater in control, ovary-intact mice compared to the ovariectomized mice that were treated only with placebo, indicating positive estrogenic effects. Hubal and colleagues reported on plantarflexor muscle strength in ICR mice before, and then 4 and 8 weeks following, sham and ovariectomy surgeries [16]. Mice in that study were 13 weeks of age at the time of surgery. Peak isometric torque of the plantarflexors did not significantly change over time in either group and there was no difference between groups at any time point. Peak torques in the E_2-deficient mice tended to be lower than those in sham mice and it was noted that the study may have been underpowered to detect this small difference. While not unanimous, these four studies provide support for estrogenic effects on strength of the major muscles and muscle groups of the mouse lower hindlimb, that is, the ankle dorsi- and plantarflexors in young mice.

Studies by Moran and colleagues further probed the question of effects of ovarian hormones on skeletal muscle strength by analyzing contractility of small muscles isolated from legs of C57BL/6 mice [29, 30]. To avoid any potential confounding effects of manipulating ovarian hormones during growth and development, ovariectomy and hormone interventions were not initiated until mice were 4–6 months of age. This is an important consideration because estrous cycles in C57BL/6 mice

become consistent at about 4 months of age and by ~12 months of age the consistency of the four-day cycles starts to decline [32]. The two studies by Moran et al. showed that soleus and EDL muscles from mice that were ovariectomized for 4 or 8 weeks had ~15 % lower maximal isometric force compared to those from age-matched, ovary-intact mice [29, 30]. This was attributed specifically to estrogen because E_2 treatment prevented or reversed force reductions caused by ovariectomy. Furthermore, plasma E_2 levels were positively correlated with maximal isometric tetanic force of soleus muscle [30]. Those studies showed that estrogenic effects on muscle strength are evident in mature mice with as little as 4 weeks of hormone manipulation. When similar outcome measures were evaluated in mature mice after only 2 weeks of hormone manipulation, strength effects on soleus but not EDL muscles were detected [13]. Specifically, soleus muscles from C57BL/6 mice that were ovariectomized and remained E_2 deficient for 2 weeks generated ~20 % less maximal isometric tetanic force than soleus muscle from mice that were sham operated or ovariectomized and simultaneously treated with E_2. Therefore, it appears that in a mature mouse model, 2 weeks of estrogen manipulation is sufficient to impact the strength of mouse soleus muscle, but a longer duration is required to influence contractility of EDL muscle. What underlies subtle differences of muscle responsiveness to E_2 between muscles remains to be determined.

The collective results of the estrogenic effects on muscle strength in mouse models of ovariectomy and E_2 treatment were also synthesized by meta-analysis [12]. As opposed to the combined data on rat studies, it was shown that E_2 has a large effect on muscle strength in mice with an effect size of 0.88.

If the overall goal of using the rodent ovariectomy model is to mimic the biology of female aging, then a limitation is that typically animals are relatively young and as such do not reflect concomitant influences of aging that occur in postmenopausal women. Thus, a critical issue is to determine to what extent E_2 treatment in aged, ovarian-failed rodents improves muscle strength. To address this, 20-month-old female mice confirmed to be ovarian senescent were treated with E_2 or placebo for 8 weeks [14]. Strength, as measured by soleus muscle maximal isometric twitch and tetanic forces, was not different between E_2- and placebo-treated mice. The amount of strength loss during a series of fatiguing contractions was blunted by E_2 treatment, suggesting some beneficial estrogenic influence on submaximal strength during repetitive contractions in muscle from aged mice. Additional studies are needed to further investigate timing and dosage of estrogenic hormone treatment in aged female rodents and the resulting effects on skeletal muscle contractility. Furthermore, if such additional studies confirm that estrogenic effects on muscle differ with age, it will be critical to determine why that is so. It is interesting that aged rodent models are available and are widely utilized to study the impact of aging on muscle contractility. Unfortunately, the vast majority of those studies have been done on male rats and mice, so very little is known about the effects of aging intermingled with ovarian failure in female rodent models.

E_2 and intrinsic muscle strength. Loss of muscle mass is a major contributor to declining strength associated with aging and menopause in women [20, 28]. However, it appears unlikely that estrogenic effects on muscle mass can completely

explain decrements in muscle strength that occur with loss of E_2 due to menopause in women or ovariectomy in rodent models. This is shown by analyses of muscle strength that is normalized to muscle size and is thus indicative of the functional quality of skeletal muscle (as opposed to the quantity of muscle). In studies on women, imaging techniques such as computer tomography can be used to measure cross-sectional area of muscles that are tested for strength. In studies that are performed on muscles of rodents, mass, cross-sectional area, or contractile protein content are measured specifically in the muscle tested for torque or force. By determining force production relative to the size of the muscle generating that force, the intrinsic functional capacity of skeletal muscle can be assessed.

Three studies that investigated the strength of the thumb adductor muscle in postmenopausal women also measured cross-sectional area of that muscle and reported data as specific force (i.e., force generation normalized by size) [33, 35, 47]. Two of the studies showed that women on HT had significantly greater specific force than women not on HT [35, 47]. Studies by Sipila and Taaffe and colleagues also measured quadriceps muscle cross-sectional area as well as strength in postmenopausal women who were and were not on HT [45, 49]. When these five studies were statistically combined by meta-analysis, results show that estrogen has a moderate effect on muscle strength normalized to size (effect size of 0.45), though it was not quite statistically significant ($P=0.07$) [12]. Overall, these data indicate that estrogen influences the intrinsic ability of skeletal muscle to generate force because any estrogenic influence on muscle size is accounted for.

A large effect size of 0.66 was determined by meta-analysis for E_2 benefits to rodent muscle strength when accounting for muscle size, which equated to rodents with estradiol having 7 % greater normalized strength [12]. A confounding effect of ovariectomy in rodent models is that skeletal muscles accumulate fluid as a result of losing ovarian hormones [25, 30, 46]. Thus, normalizing strength by muscle mass or cross-sectional area is not appropriate and normalizing to myofibrillar protein content is recommended [50]. Maximal isometric force generated by mouse soleus and EDL muscles normalized to contractile protein content of those muscles were less in ovariectomized mice compared to those in control or E_2-replaced mice [13, 29, 30]. These data indicate that the quality of skeletal muscle in terms of intrinsic force generating capacity is significantly impacted by altered levels of E_2 in the circulation.

Single and small bundles of permeabilized muscle fibers have also been studied for estrogenic effects on contractile function. Fibers in this type of preparation do not have intact sarcolemma and contraction is initiated by exogenous delivery of calcium, instead of calcium release from the sarcoplasmic reticulum. As such, excitation (i.e., events at the neuromuscular junction and conduction of action potentials along the plasmalemma) and excitation–contraction coupling (i.e., events in the transverse tubular system and at the dihydropyridine–ryanodine receptor interface) are completely bypassed when contraction is initiated. Therefore, estrogenic effects on fiber strength can be directly attributed to function of the thick- and thin-filament contractile proteins in this type of experiment. Cross-sectional areas of the fibers that are tested are measured to account for differences in fiber size. One study using

this strategy has been completed on fibers of vastus lateralis muscle biopsies from postmenopausal women on and not on HT [54]. Results from that study do not support the hypothesis that estrogen affects contractile protein function because maximal isometric force, as well as force normalized to fiber cross-sectional area, was not different between muscle fibers from women on HT and those not on HT.

Wattanapermpool and Reiser conducted a similar study on contractile characteristics of muscle fibers from ovariectomized and sham-operated Sprague Dawley rats [53]. They report that after 10 or 14 weeks of ovarian hormone deprivation, fibers from ovariectomized rats that were permeabilized and tested for calcium-activated force were weakened. Specifically, soleus muscle fibers produced 20 % less force per fiber cross-sectional area than did fibers from sham-operated rats. Authors speculated that ovarian hormone deprivation affected strength by either decreasing the number of cross-bridge attachments during contraction or decreasing the force per cross-bridge during contraction. A cross-bridge hypothesis had been put forth by Phillips and co-workers in regard to HT beneficial effects on muscle strength in postmenopausal women [35].

The hypothesis that ovarian hormones, specifically E_2, influence muscle strength by affecting function of contractile proteins was directly tested by Moran and colleagues. This was done by three types of analyses. First, calcium-activated force by bundles of permeabilized muscle fibers was 25 % less in those that came from mice that had been ovariectomized for 8 weeks compared to those that came from sham-operated mice with normal circulating levels of E_2 [29]. Those data indicate that ovarian hormones affect contractile protein function. Second, active stiffness of intact soleus and EDL muscles was measured as an indicator of actin and myosin strong-binding during contraction [43]. Active stiffness was ~12 % lower in muscle from ovariectomized compared to sham-operated mice [29] and the decrement was reversed with E_2 treatment [30]. The most direct evidence that contractile protein function is affected by E_2 was shown by studies using electron paramagnetic resonance spectroscopy paired with site-directed spin labeling on the catalytic domain of myosin in muscle fibers. Those studies directly showed that the fraction of myosin heads strongly bound to actin during contraction (i.e., producing force at the molecular level) was 15–20 % lower in muscle from ovariectomized mice, and that the decrement was reversed or prevented by E_2 treatment [29, 30]. Because the ovariectomy-induced decrements in strength and myosin strong-binding were similar in magnitude, it was proposed that myosin dysfunction is the major factor causing strength loss when E_2 declines.

Estradiol and Parameters of Muscle Contractility Other than Strength

E_2 and muscle contractility in women. Skeletal muscle power is often reported as a functional and integrated aspect of skeletal muscle contractility and is reflective of both muscle strength and speed of contraction. A study of monozygotic twin pairs

who were discordant for HT usage showed that vertical jump height, a surrogate of muscle power, was 21 % greater in co-twins taking HT compared with those not [40]. Two other studies examined the power of the leg extensors in postmenopausal women and reported no differences between those on and not on HT [4, 23]. Women in those studies had a mean age of ~51 years and were tested about 1 year after the onset of menopause. In contrast, Carville and colleagues measured leg extensor power in an older group of postmenopausal women, mean age of ~69 years, with those who were on an estrogenic HT for an average of 13 years [5]. These older postmenopausal women taking HT generated 20 % greater leg extensor power than women without HT and had power outputs similar to those of younger healthy women, ~28 years of age. Because power was affected by HT usage but maximal force was not, the authors hypothesized that HT may have positive effects on skeletal muscle contractile speed. Collectively, these results imply that chronic estrogen treatment may be needed to impact speed of muscle contractions.

A small number of studies have examined twitch kinetics of muscles in postmenopausal women. This analysis gives some insight into intrinsic contractile speed of muscle. One example involved postmenopausal women averaging 64 years of age and 16 years past onset of menopause, in which stimulation of ulnar nerve was used to determine twitch kinetics of the adductor pollicis muscle [33]. In both dominant and non-dominant hands twitch force, time-to-peak twitch force, and twitch half-relaxation time were independent of HT usage. Finni and co-workers examined contractility of plantarflexor muscles in the monozygotic twin pairs who were discordant for HT usage [8]. While there was no difference between twins in voluntary plantarflexor tetanic torque, twins on HT had 32 % higher peak twitch torque that was elicited by tibial nerve stimulation. There was no difference in electromyography activity of the medial gastrocnemius and soleus muscles measured during stimulation of the tibial nerve related to HT usage indicating that estrogenic effects were intrinsic to the muscle. While involuntary peak twitch force was higher in co-twins on HT, there were no differences in time-to-peak twitch force or twitch half-relaxation time. Taken together, these limited results are inconclusive in determining how involuntary, electrically stimulated twitch kinetics are affected by estrogen.

Often investigations of muscle strength measure force or torque during isometric contraction, but some studies on postmenopausal women have employed shortening (concentric) or lengthening (eccentric) contractions. While a few of these studies found beneficial effects of HT on strength measured during isokinetic types of muscle contraction, not all studies did. For example, Greeves and colleagues examined quadriceps strength of postmenopausal women longitudinally over 39 weeks [11]. Women on HT maintained isometric strength of the quadriceps, while those not on HT lost 11 % over the duration of the study. Similarly, those on HT maintained dynamic strength measured at the lowest of the three angular velocities tested while women not on HT lost ~10 %. Quadriceps muscle strength at the two higher velocities tested did not change during the study in either group of women. Similarly, Dieli-Conwright and colleagues showed that eight 59-year-old postmenopausal women taking HT did not differ in peak concentric and eccentric torque of the knee extensors from six age-matched women not taking HT [6]. The issue of muscle contraction type was addressed in the meta-analysis by Greising and co-workers

[12]. Among the 23 studies that compared the strength of postmenopausal women on and not on HT, strength measurements were reported on 8 and 20 isokinetic and isometric types of muscle contractions, respectively. No differences in effect sizes between these groups of studies were detected indicating that muscle contraction at a given velocity (isokinetic) and static muscle contraction (isometric) are not preferentially influenced by estrogens.

E_2 and muscle contractility in rodent models. Akin to studies on muscle contractile indicators such as twitch kinetics, velocity, and power in estrogen-deprived and -replaced women, few studies have been done in rodent models. Time-to-peak twitch force and twitch one-half-relaxation time in soleus and EDL muscles were faster in very young, ovariectomized rats compared to sham rats or ovariectomized rats treated with E_2 [9, 26]. Conversely, in aged ovarian-senescent mice, E_2 treatment did not affect soleus muscle time-to-peak twitch force, twitch one-half-relaxation time, or tetanic rates of contraction or relaxation [14]. Whether or not the species studied or the age of the rodent can explain the discrepancy in the responsiveness of twitch kinetics to E_2 remains to be determined.

Eight weeks of E_2 deficiency via ovariectomy of adult mice resulted in 9 % faster maximal shortening velocity (V_{max}) of soleus muscles [29] while two weeks of ovariectomy-induced E_2 deficiency was not long enough to affect V_{max} of soleus muscle, but maximal power was 25 % greater in ovariectomized mice treated with E_2 than those treated by placebo [13]. V_{max} of EDL muscles was not affected [29]. No differences in power were detected in terms of absolute or normalized peak power of soleus or EDL muscles. Using permeabilized soleus muscle fibers from rats, Wattanapermpool and Reiser showed that calcium sensitivity was not affected by 10 or 14 weeks of ovarian hormone deprivation [53]. Although not statistically significant, the trend was for soleus fibers from ovariectomized rats in that study to have faster V_{max}.

Collectively, these six studies that report indices of muscle contractile speed show either no effect of ovarian hormones or that the lack of ovarian hormones resulted in faster muscle contractility. No study indicated that ovariectomy was associated with the slowing of muscle contractility.

Influence of E_2 on contractile protein expression. Changes in muscle contractility, especially those related to speed of contraction, are heavily influenced by changes in the expression of contractile proteins, such as myosin heavy-chain (MHC) isoforms. Whether or not estrogens can alter contractile protein expression is not clear. Widrick and colleagues examined the relationships between HT and vastus lateralis muscle fiber characteristics related to MHC in postmenopausal women [54]. Using both electrophoresis and histochemistry they found no differences in MHC isoform distribution (i.e., distribution of slow and fast fiber types in the vastus lateralis muscle) or cross-sectional area of fibers composed of type I or IIa MHC between women on and not on HT. These data on human muscle did not indicate that estrogens influence MHC isoform expression.

Relatively more studies have been conducted on contractile protein expression in muscles of estrogen-manipulated rodents, particularly MHC composition and fiber type distribution based on myosin ATPase reactivity or MHC isoforms. Two studies compared muscles of female rats that were ovariectomized at 3 weeks of age to

those that were ovariectomized and simultaneously treated with E_2 [19, 48]. No differences in fiber type distributions were detected in soleus, EDL, or caudofemoralis muscles based on histological analyses of myosin ATPase reactivity. Thus, it appears that in very young, developing female rats estrogens do not affect MHC expression in muscle fibers.

Results of estrogenic effects on MHC isoform expression and fiber types from muscles of slightly older, yet still growing, young female rats (7–10 weeks of age) are quite mixed. Kadi and co-workers reported that rats which were ovariectomized for 5 weeks had muscles with MHC isoform expressions shifted toward the slower isoforms [17]. That is, gel electrophoresis showed that soleus muscle of ovariectomized rats had relatively more type I and less type IIa MHC than sham rats and EDL muscles from ovariectomized rats had relatively more IIa and less IIb MHC than sham rats. The changes in MHC isoform composition were on the order of ~10 %. In a similar study on rats, type I, IIa, and IIx MHC composition of soleus muscle was not affected by 4 weeks of ovariectomy or ovariectomy plus E_2 treatment [26]. In those same rats, plantaris muscle MHC isoform composition was slightly altered [36]. MHC type IIx was ~5 % less in plantaris muscle from ovariectomized compared to sham rats and MHC type IIb was ~10 % lower in ovariectomized rats that were treated with E_2 compared to sham rats. Overall, these data indicate a slight *shift toward slower MHC isoforms* with the loss of estrogens.

In contrast, other studies using rats of similar ages showed *shifts toward faster MHC isoforms* with the loss of estrogens. Velders and co-workers studied soleus and gastrocnemius muscles 2 weeks after ovariectomy with and without E_2 treatment [51]. They reported that MHC type I expression was lower in soleus muscle of ovariectomized rats compared to sham-operated rats or those ovariectomized and treated with E_2. Liu and colleagues examined the genioglossus muscle of young rats 4 weeks following ovariectomy using both histological myosin ATPase staining and electrophoretic analyses of MHC composition [22]. No difference in fiber type distribution (types IIa and IIb/IIx) was detected between groups based on histochemistry, but genioglossus muscles from ovariectomized rats contained a lesser percentage of MHC IIa and greater percentages of MHC IIx and IIb compared to sham or pure control rats. Wattanapermpool and Reiser used electrophoresis and silver staining to measure MHC isoforms and isoforms of thin-filament proteins in segments of single fibers from rat soleus muscles [53]. Though the data are not shown, the authors state that no changes in isoform expression were detected in soleus muscles from ovariectomized rats compared to sham rats. Because these same fibers from ovariectomized rats were shown to have reduced force generation, these data indicate that decrements in strength that result from the loss of estrogens are not directly due to changes in the expression of contractile protein isoforms.

There is one report of type I MHC expression in soleus muscle of fully grown, adult Wistar rats, that is, rats 5 months of age when ovariectomy surgeries were done [10]. This study is also unique because muscle analyses were done 9 months following the ovariectomy or sham operation. The authors state that immunohistochemistry qualitatively revealed an increase in fibers expressing MHC type I in ovariectomized compared to sham rats. However, quantitation was not performed because many fibers appeared to be hybrid, expressing variable amounts of type I

MHC. Immunoblot analyses showed that MHC type I protein expression was ~7 % greater in soleus muscles from ovariectomized compared to sham rats, indicating a slight shift toward the slow contractile protein isoforms.

Further analyses of MHC isoform expression in response to estrogen manipulation and at specific ages in female rats are needed to clarify conflicting results in the literature. Particular consideration should be given to soleus muscle and the age at which analyses are made because shifts in fiber distributions are significant in this muscle during the first year of life [24]. Also to note, others have reported that rat soleus muscle is composed of nearly 100 % MHC type I, so shifts toward slower isoforms could be difficult to establish in the soleus muscle of rats [34].

Results of estrogenic influences on contractile protein isoforms in mice are also not conclusive. The masseter muscle of 10-month-old mice that were ovariectomized for 6 weeks did not have different fiber type composition based on immunohistochemistry and immunoblotting than sham-operated mice [7]. Similarly, soleus muscle fiber type distributions among types I, IIa, IIx, and IIb were not different between sham mice and mice that were ovariectomized and treated with placebo or E_2 [30]. Mice in that study were fully grown adults at the time of ovariectomy, nearly 4 months of age, and muscle analyses were conducted 4 and 8 weeks later. In contrast, 10-week-old mice that were ovariectomized and studied 12 weeks later showed several signs of contractile protein shifts toward faster isoforms [39]. Specifically, gene expression of slow isoforms of myosin light chain and troponin was analyzed by quantitative polymerase chain reaction and shown to be lower in quadriceps muscle from ovariectomized mice compared to sham mice. Supporting evidence for ovariectomy-induced shifts away from slow, type I and toward fast, type II isoforms included changes in MyoD, myogenin, peroxisome proliferator-activated receptor-γ, and FoxO1 gene expressions. It remains to be determined whether or not these altered gene expressions correlate to changes in contractile protein isoforms and corresponding contractility characteristics.

Of the 13 published studies summarized in this section (one human, nine rat, three mice), 7 studies reported no effect of the loss of estrogen on fiber type or contractile protein isoform expression, 3 studies reported shifts toward slower or type I isoforms, and 3 studies reported shifts toward faster or type II isoforms. Moreover, when significant shifts in contractile protein isoforms were detected, the magnitude of the shifts were typically small, on the order of 10 %. Given the mixed results, it seems unlikely that an estrogen-mediated change in the expression of contractile proteins, such as MHC isoforms, is a mechanism underlying alterations in muscle contractility. However, much more work is needed to substantiate this.

Summary

The maintenance of muscle strength and contractility with aging and with loss of estrogen are critical issues because the risk for disability and dependent living increases with muscle weakness. Women live longer than men and do so in

postmenopause for nearly one-third of their lives. Because of these facts, it is becoming increasingly important to understand the effects of E_2 and the loss of this hormone on skeletal muscle function. There is substantial evidence that E_2 is beneficial to muscle strength. This evidence comes from the collective results of studies on postmenopausal women comparing those who did take HT with those who did not. There is also good evidence from several studies utilizing mouse models that the loss of ovarian hormones via ovariectomy is detrimental to muscle strength, particularly muscle's intrinsic ability to generate force, and that E_2 treatment reverses or prevents the losses. Estrogenic effects on contractility parameters other than strength are not clear. The direction of any such effect in rodent studies is that the lack of ovarian hormones is related to faster muscle contractility. Whether or not contractile protein isoform expression is affected by E_2 and contributes to changes in muscle contractile speed is even less clear.

The influence of E_2 on skeletal muscle extends beyond its effects on strength and contractility as skeletal muscle has additional functions, such as its contributions to metabolism and heat generation. Finally, estrogenic effects on skeletal muscle should not be completely evaluated in isolation. That is, integral to skeletal muscle are adjacent tissues that are also estrogen sensitive, such as bone and fat. To completely understand estrogen's role in women's health multisystem evaluations are necessary.

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Chapter 2
Novel Findings in Bone Biology: Impact on Bone Health for Women

Susan A. Bloomfield and Corinne E. Metzger

Abstract The maintenance of bone integrity throughout life is critical for minimizing the risk of debilitating fractures, which most frequently occur in those with low bone mass (osteopenia) and frank osteoporosis. Bone dynamically adapts to mechanical stresses placed on it, as with increased exercise, but this adaptation may be modified by changes in circulating estrogen, altered oxidative status, and nutritional factors. This review addresses novel findings of the last decade as they affect bone health in women. Specific topics discussed include the negative impact of low energy availability due to prolonged caloric restriction, the surprising role of estrogen receptor-alpha in bone mechanotransduction, and how oxidative stress may be an important mechanism contributing to bone loss with aging and estrogen insufficiency.

Keywords Skeleton • Estrogen receptor • Dietary energy • Caloric restriction • Oxidative stress

Introduction

The skeleton's key functions are to provide levers for locomotion, protect vital organs, and serve as a calcium reservoir. These are rarely appreciated functions until, of course, one incurs a bone fracture and appreciates anew the mobility afforded by the intact skeleton. "Bone health" is an oft-used phrase reflecting the mechanical integrity of bone and its ability to resist fracture in all but extreme

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loading situations. The most commonly used clinical measure of bone health is bone mineral density assessed by dual-energy X-ray absorptiometry (DEXA), with which most postmenopausal women are very familiar. This technique measures only the mineral content of bone, which is also composed of organic matrix (collagen and multiple non-collagenous proteins). Since BMD levels are reasonably predictive of fracture rates, this simple noninvasive test provides the handiest tool to track bone health in the general population, with a minimal radiation dose.

There are many varieties of metabolic bone disease (tumor metastasis to bone, disorders of osteoclast biology, osteomalacia with, e.g., renal failure). The best known and most prevalent is osteoporosis, which is simply a lower bone mass than expected for one's age and affects an estimated ten million Americans over 50 years of age [1]. Although the majority of these are female, aging men (and those on certain medications causing bone loss, such as corticosteroids) are also at risk. Intermediate bone loss that does not reach the criterion mark for osteoporosis is termed osteopenia, analogous to glucose intolerance that may precede a diagnosis of Type 2 diabetes. Osteopenia is very common in middle-aged women and in select populations of young women, particularly those who restrict dietary energy intake for prolonged periods. The accelerated bone loss observed in the osteoporotic patient dramatically diminishes bone strength and contributes towards 1.5 million so-called fragility fractures and health care costs of $18 billion/year in the USA [1]. The survival rate 5 years after a hip or a vertebral fracture is ~80 % of that in men and women with no fractures [2], with higher mortality rates in men and in the more aged [1].

Bone cells behave quite similarly in men and women, but what is unique to women is, of course, the hormonal milieu and the dramatic changes in estrogen status at mid-life (or, on occasion, in younger women with amenorrhea or surgical menopause). Bone loss occurs fairly rapidly with the onset of estrogen deficiency due to a multitude of cellular mechanisms [3]. Briefly, bone resorption activity by osteoclasts, normally inhibited by circulating estrogen, accelerates dramatically with estrogen withdrawal. In addition, both differentiation and activity of mature bone-forming osteoblasts are impaired in the absence of estrogen. There is a dearth of evidence about the impact of estrogen deficiency on osteocytes, those cells embedded within bone matrix that are thought to serve as the essential mechano-sensors.

This short review discusses some novel findings in the bone biology field over the last 10 years that have direct relevance for bone health in women. The first section of this review considers how dietary energy intake, the critical "other bone nutrient," can impact bone mass and fracture risk in both young athletes and in the frail elderly. Low circulating estrogen levels can also impact negatively the expression of estrogen receptor-alpha (ER-α) in bone cells and on mechanotransduction, which may provide the mechanism for the lower responsiveness of bone to exercise in postmenopausal women; this topic is covered in the second section. The final topic briefly presents the growing evidence for an alternate mechanism for age-related and estrogen-deficiency bone loss: oxidative stress.

Impact of Reduced Dietary Energy Intake on Bone Integrity

Most individuals, when queried, will name calcium and vitamin D as the two most important nutrients supporting optimal bone health. Millions of older Americans focus on getting adequate intake of these two nutrients, which do indeed help to minimize (but cannot abolish) age-related bone loss. However, relatively few are aware of the impact that reduced energy (calorie) intake has on bone mass. In a recent NHANES survey, nearly 60 % of American women (and 40 % of men) are pursuing some weight loss regimen [4]; given the rising prevalence of obesity, the fraction of Americans who will pursue serious weight loss regimens can only increase. Others at risk for bone loss coincident with restricted energy intake include those with involuntary caloric restriction (frail elderly or those without regular access to adequate caloric intake, e.g., the food-insecure poor); active military personnel in the field; and athletes in training who voluntarily restrict food intake. On average, each 10 % decrement in body weight is associated with a 1–2 % decline in BMD [5]. The consequences of a 2 % decline in bone mass, particularly with reference to fracture risk, vary significantly across these different populations.

Both epidemiological evidence and controlled intervention trials provide evidence for significant bone loss and elevated fracture risk in individuals losing weight. A history of >10 % weight loss in community-dwelling older women [6] and men [7] increases the risk of hip fractures. Even more convincing are results from controlled randomized intervention trials, most frequently performed in obese postmenopausal women, in whom losses in bone mass consistently occur with reductions in body weight [5]. Notably, the bone lost may not be regained after the end of a weight loss period. Serum markers of bone resorption remained elevated [8] and BMD values remained lower after the end of an exercise-based weight loss intervention in obese individuals, even in the face of significant weight regain [9]. A similar lack of bone recovery was found in premenopausal women who had followed a very-low-calorie diet for 3 months; a full 33 months later, decreases in lumbar spine BMD were not restored even in those subjects who regained all bone lost. (However, in this case, trochanter BMD (at proximal femur) did recover with weight regain [10].) These data suggest that a burst of remodeling activated during a period of negative energy balance (at least in vertebral bone) does not switch off as soon as energy balance is resumed, resulting in continued loss of bone mineral even after the individual resumes usual caloric intake.

Many obese individuals enter a period of caloric restriction with higher than average BMD values [11]. Hence losing 1–3 % of this elevated bone mass may result in a minimal impact on fracture resistance as compared to the same proportional bone loss in a near-normal weight population. Military recruits in basic training and those in intense combat operations often incur rapid weight loss [12]; in this case, the negative energy balance is more likely due to the enormous increase in energy expenditure with hours of vigorous activity each day. A classic illustration of this is provided by the data of Friedl et al. [13], who monitored caloric intake and energy expenditure during an 8-week field training experience by Army Rangers,

during which average energy intake was 1,000–1,200 kcal/day below average energy expenditure and body weight loss averaged 12 kg.

In the extreme instance, individuals with eating disorders or frank anorexia nervosa incur significant bone loss; fracture rates are sevenfold higher than expected for these (usually) young individuals [14]. A wealth of literature now documents the impact of the prolonged and severe negative energy balance on bone health in these populations, which may be complicated by shifts in neuroendocrine function. Less severe restriction of energy intake occurs frequently in those on the other end of the age spectrum: the frail elderly. Reduced eating may be voluntary (lack of appetite) or involuntary (food insecurity, inability to prepare food) in this population, but the result can be accelerated bone loss beyond that observed in well-nourished individuals of the same age [15]. Fracture risk in this group is further exacerbated by loss of muscle mass with inadequate energy intake, which reduces both the mechanical loading delivered to bone as well as the energy absorption effect of the overlying soft tissue should a fall occur.

Another unique population at risk of bone loss are those athletes who overrestrict energy intake over prolonged periods (often years) to maintain extremely lean physiques and lower body weights. Early findings of Drinkwater et al. [16] confirming spinal BMD values in amenorrheic varsity rowers equivalent to those typical in 51-year-old women galvanized the research community into explaining how bone mass could *decline* with vigorous physical activity. A plethora of studies in the subsequent decades confirmed these findings in athletes in weight-classed sports (like rowing), in sports (e.g., distance running) where low body weights provide a competitive advantage, and in the aesthetic sports that demand an extremely lean appearance (dance, gymnastics, figure skating). Recently published data using high-resolution CT scans in adolescent amenorrheic athletes provide evidence of impaired cancellous bone micro-architecture (reduced trabecular number, increased trabecular spacing), which can independently elevate fracture risk [17].

Whether bone loss due to reduced energy intake is more specific to cortical or cancellous bone seems to vary some with the bone site examined. Human studies generally rely on DEXA measures of bone mass, which focus on the clinically relevant sites of lumbar spine and proximal femur. These are "mixed" bone sites, with a dense cortical shell surrounding a core of cancellous bone, which forms a latticework of connected rods and struts that strengthen the structure. Several intervention trials have documented a twofold higher rate of bone loss at anatomic sites with more cancellous bone (e.g., distal radius and proximal femur trochanter) over that observed in total body scans [9] or in cancellous compartments within the distal tibia, as separated out by peripheral quantitative computed tomography (pQCT) [18]. Bone "quality," if not bone quantity, may be protected in older adults practicing caloric restriction but getting adequate protein and other nutrients, in contrast to the amenorrheic adolescent athletes mentioned earlier. Even in the face of significant reductions in BMD, middle-aged adults consuming a healthy diet with ~35 % fewer calories exhibit no changes in indicators of trabecular bone micro-architecture as measured by high-resolution MRI [19]. A number of animal studies utilizing peripheral quantitative or micro-computed tomography document that cancellous

bone is preferentially affected in adult female rats [20, 21]; however, the primary deficits in young male mice subjected to similar reductions in energy intake are limited to cortical bone [22]. It remains unclear whether these disparate results reflect species and/or gender differences.

The functional concern related to loss of bone mass with chronic energy restriction is two-pronged. The first is a long-term health issue: should this bone loss occur in a younger individual, it may result in "premature" osteoporosis, some years before that individual might have otherwise reached osteoporotic BMD levels. Hence the risk of fragility (low-trauma) fractures can be significant at a relatively young age. The second functional consequence is of more immediate concern to younger individuals, particularly military personnel and athletes: an increased risk of stress fracture [23, 24]. The military in particular has a vested interest in reducing the incidence of stress fractures in recruits, since the required 6 weeks' healing time can seriously disrupt basic training schedules.

What are the systemic or the cellular mechanisms for these losses of bone mass with reduced energy availability? Early research on low BMD values in amenorrheic women athletes presumed that reduced circulating serum estrogen associated with cessation of normal menstrual cycles was the culprit, creating a "premature menopause" endocrine profile conducive to accelerated bone loss. However, the limited success of oral contraceptives in restoring lost bone mass in amenorrheic athletes or in anorexia nervosa patients [25] suggests that estrogen-independent mechanisms are also important contributors to this bone loss.

Chronic negative energy balance impacts two primary endocrine axes: the metabolic hormones that are sensitive to energy intake and the reproductive hormone axis, each of which can impact bone cell activity leading to loss of bone mass. An elegant illustration of this derives from tightly controlled studies of nonathletic women subjected to combined treadmill running and graded energy restriction, resulting in 10–30 kcal/kg LBM/day reductions in energy availability [26]. (In this context, "energy availability" was computed as total energy intake minus exercise energy expenditure.) After only 5 days of this regimen, reductions in circulating IGF-I and leptin, evident even at the milder levels of reduced energy availability, were highly predictive of reductions in serum biomarkers of bone formation activity. Conversely, elevations in bone resorption biomarkers (but only at the most extreme reduced energy availability) tracked closely with reductions in serum estrogen.

Although prolonged hypoestrogenemia almost certainly contributes to bone loss in women with prolonged amenorrhea, metabolic hormones related to energy status such as IGF-I and leptin may play an important role in mediating bone loss, particularly in those individuals experiencing milder (subclinical) changes in estrogen status, such as luteal phase defects [27]. More recent data indicate that estrogen-deficient exercising women who are energy-replete exhibit few perturbations of bone resorption or formation, reinforcing the key role of energy status in modulating bone cell activity and hence bone mass [25]. These data provide the mechanistic endocrine link between the reduced BMD frequently observed in amenorrheic athletes and their reduced energy intake, which together describe the phenomenon labeled the female athlete triad (for a comprehensive review [28]).

Research in relevant mammalian models provides valuable insight into tissue-level mechanisms for bone loss with restricted energy intake. One often unrecognized contributor is the reduced absorption of dietary calcium with estrogen deficiency. Ovariectomized (OVX) rats exhibit a fourfold lower net calcium absorption when subjected to a 40 % restriction of energy intake; this effect can be reversed with estrogen replacement [29]. The impact of energy insufficiency on reduced bone formation and/or increased resorption activity can derive from reduced activity of mature osteoblasts or increased vigor of mature osteoclasts, respectively. Alternatively, chronic restriction of dietary energy can alter the differentiation of stem cell populations, decreasing the availability of mature osteoblasts [30], and expanding adipocyte populations in bone marrow [30, 31]. This impact on stem cell populations is coincident with reductions in circulating IGF-I and leptin, reinforcing the notion that these metabolic hormones that reflect energy balance are important mediators of bone loss with energy restriction.

An important related question is the following: Does concurrent exercise mitigate bone loss with restricted energy intake? Most physicians recommend increased caloric expenditure (most frequently, a walking program) in addition to reduced caloric intake for patients pursuing weight loss. Although it makes intuitive sense that the metabolic shift that occurs with regular exercise would enhance the weight loss with energy restriction alone, the evidence that this results in significantly greater weight loss is surprisingly weak, even in well-controlled randomized interventions [32]. Even if exercise during a caloric restriction regimen does not improve weight loss outcomes, it is reasonable to hypothesize that the mechanical loading effect of an exercise regimen might mitigate the negative impact of reduced energy intake on bone mass. Circumstantial evidence is provided by multiple cross-sectional studies comparing amenorrheic sedentary and athletic populations, in which the athletes have higher BMD values than their nonathletic peers [33]. More solid experimental evidence for this derives from well-controlled clinical trials comparing BMD losses in obese populations seeking weight loss by caloric restriction alone vs. exercise-induced weight loss. For example, middle-aged men and women achieving weight loss by increased exercise expenditure only experienced no loss of BMD, whereas those restricting energy intake by up to 20 % experienced significant (2 %) declines in spine and hip BMD [34]. Increases in serum sclerostin (an osteocyte-specific protein that inhibits bone formation) and negative changes in hip geometry observed in those losing weight through calorie restriction alone are prevented with exercise training concurrent with the restricted energy intake [35]. At least one published study in dieting humans demonstrated that exercise during a period of reduced energy intake increased serum IGF-I and reduced markers of inflammation while mitigating bone loss [36].

It is far easier to strictly control energy intake and expenditure in animal studies, particularly for long-duration experiments. Recent work testing the impact of reductions in energy availability achieved by reducing dietary energy intake alone vs. combining smaller reductions in energy intake with increased energy expenditure in adult female rats provides several interesting findings. If energy availability is reduced by 25 %, significant bone loss does occur in cancellous-rich bone

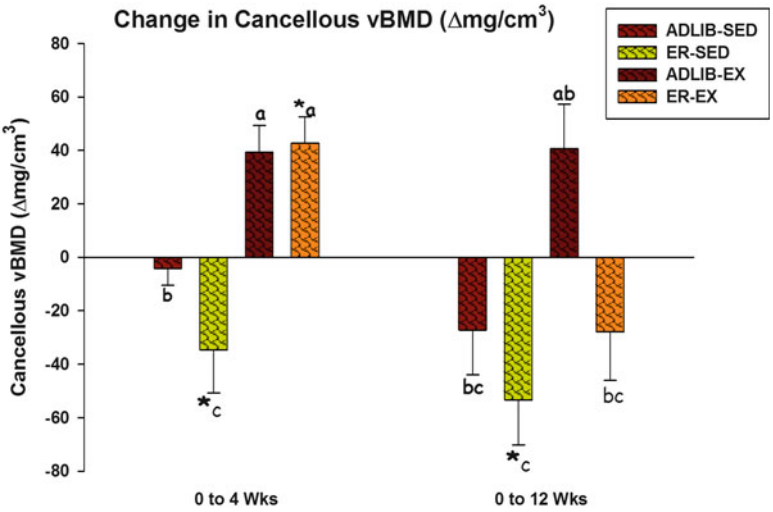

Fig. 2.1 Short-term protection observed at 4 weeks against loss of cancellous bone mass in rats achieving reduced energy availability with exercise and dietary energy restriction (ER) disappears after 12 weeks. Decline in volumetric bone mineral density (vBMD) at 12 weeks in ER-exercised animals is still half that observed in ER-sedentary (SED) rats. Delta values with the same letter superscript are not significantly different; $*p<0.05$ vs. baseline value on day 0. Data from Swift et al. [21]

compartments in sedentary animals after 4 weeks but, interestingly, not in rats subjected to treadmill running (see Fig. 2.1) [21]. However, the implied protective benefit of exercise disappears after 12 weeks of reduced energy availability, by which time even exercising animals incur a 9 % deficit in volumetric BMD (vs. a 17 % deficit in sedentary rats). This pattern of bone loss may be related to alterations in serum IGF-I, declines in which are mitigated in exercising animals [37]. If indeed the anabolic effect of exercise attenuates negative changes in metabolic endocrine factors during energy restriction, this may provide some protection over short-term dieting periods. It appears, however, that should energy availability be reduced on a chronic basis, the impact of energy insufficiency overwhelms metabolic or mechanical loading advantages concurrent exercise might provide.

Estrogen Receptor-Alpha and Mechanotransduction in Bone

Recently, a fascinating new line of research has examined the effect of reduced circulating estrogens may have on bone's ability to adapt to mechanical strains and exercise. It has long been known that bone structure adapts to the typical loading patterns to which it is subjected. Current recommendations focus on weight-bearing exercise and resistance training as effective means of increasing bone mass across most of the life-span. However, bone is less responsive to even optimal exercise

modes with increasing age [38]. Interestingly, the sensitivity of bone's response to loading is also reduced in individuals with estrogen deficiency, including postmenopausal women, amenorrheic athletes, and men with progressive estrogen deficiency [39]. This observation led to the hypothesis that low serum estrogen or a closely related factor is responsible for this diminished sensitivity of bone to mechanical loading; key candidates were the intracellular estrogen receptors, of which there are two: ERα or estrogen receptor-β (ERβ). ERα became a likely candidate for this role as it is expressed in osteoblasts, osteoclasts, and osteocytes [39]. Also, it has been previously shown in humans as well as animals that the estrogen receptor expression varies with levels of circulating estrogen [40, 41]. Therefore, a decline in circulating estrogen would lead to fewer estrogen receptors expressed in bone cells. If these receptors have any influence on key signaling pathways important to the regulation of bone formation activity in osteoblasts, an individual with low estrogen status would be predicted to have an attenuated bone response to exercise.

The first clue regarding ERα's response in mechanical loading of bone was provided by in vitro experiments in which osteoblast-like cells were exposed to mechanical strain and treated with the estrogen receptor modulators ICI-182,780 and tamoxifen [42]. These two compounds, both estrogen receptor antagonists, eliminated or reduced osteoblast proliferation to the mechanical strain, suggesting that bone's adaptive response to mechanical strain is mediated through a mechanism which involves ERα. To confirm these findings in vivo, Lee et al. [43] studied the response to mechanical loading of bone in ERα-null mice. This commonly used model provides axial loading of the ulna using a servomotor device to cyclically deform the bone in an anesthetized animal; the key advantage of this model is to provide very precise control over the loading signal delivered (strain magnitude and strain distribution), but it does not involve muscle contraction or the integrated physiological response to voluntary exercise. While wild-type mice exhibited an 8 % increase in cortical area at the midshaft ulna, the response in ERα-null mice was nearly fourfold lower (2.4 % gain in bone area) (see Fig. 2.2). Primary osteoblast cultures from the ERα knockout mice exhibited no proliferative response when exposed to strain in vitro, while cells derived from wild-type littermates increased in number by 58 %. These data demonstrate some critical role for ERα activity in the adaptive response of bone to strain-related signals. The loss of bone and reduction in ability to respond to exercise in estrogen deficiency could be explained through the estrogen receptor's role in adaptation to loading.

The role of ERβ is not as well established as that of ERα, but it, too, might have a role in the loading response of bone. The fact that there are two estrogen receptors present in bone has made determining the role of each individual receptor a greater challenge. ERβ is also present in osteoblasts, but early research was inconclusive as to its impact on the loading response. With in vivo loading of ERα knockout mice and ERβ knockout mice, similar changes in bone are seen when the estrogen receptors are individually silenced. ERβ knockout mice exhibit only half the increase in cortical area due to periosteal expansion as do ERβ-intact (wild-type) mice [44]. This impact of the missing ERβ on the cortical bone gain is about half of that

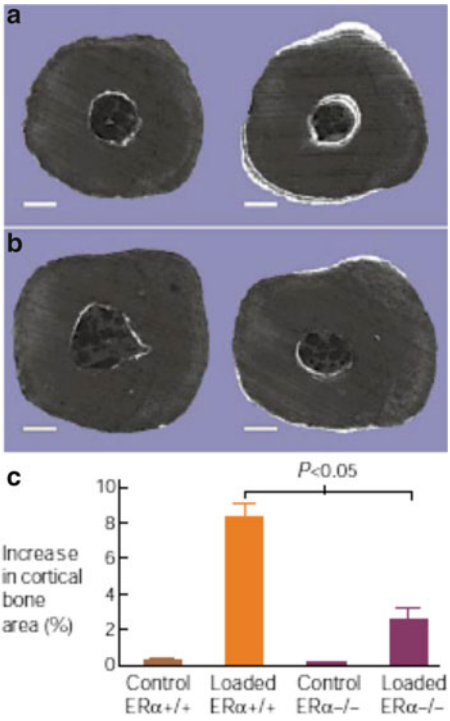

Fig. 2.2 Mechanotransduction is impaired in the absence of estrogen receptor-alpha (ERα). Transverse sections of unloaded control (*left*) and loaded (*right*) ulnae from (**a**) ERα wild-type and (**b**) ERα knockout mice. *White* fluorochrome label on bone surfaces represents newly formed bone. (**c**) Quantification of increased cortical bone area in the loaded versus control wild-type and knockout mice. From Lee et al. [43]

observed in ERα-null mice (nearly a fourfold decrease) [43], but it appears that the absence of either ERα or ERβ results in attenuation of the osteogenic response to loading.

To complicate this story, more recent research demonstrates that there may be differential roles for ERα and ERβ in males and females. With ERα or ERβ deleted, the different effects of either reduced loading on tibial bone (via sciatic neurectomy) or increased loading were investigated in male and female mice [45]. In both genders ERα-null, but not ERβ-null, mice exhibited an attenuated loss of cancellous bone after prolonged disuse, as compared to their wild-type counterparts. Those female mice lacking ERα had a diminished osteogenic response to loading on cortical bone but, interestingly, no effect was observed for cancellous bone surfaces. By contrast, ERα knockout males had a *greater* osteogenic response (vs. wild-type littermates) to increased loading on both cortical and cancellous bone surfaces. Deletion of ERβ, on the other hand, resulted in an increased osteogenic response on cortical bone surfaces in both male and female mice, suggesting a tonic inhibition of periosteal osteoblast response to loading in the intact animal [45]. The precise role of these two estrogen receptors in mechanotransduction may be more complex than initially thought, given these gender-specific responses.

Another intriguing question is if mechanical loading or exercise is capable of independently affecting estrogen receptor expression in bone cells. Osteoblast and

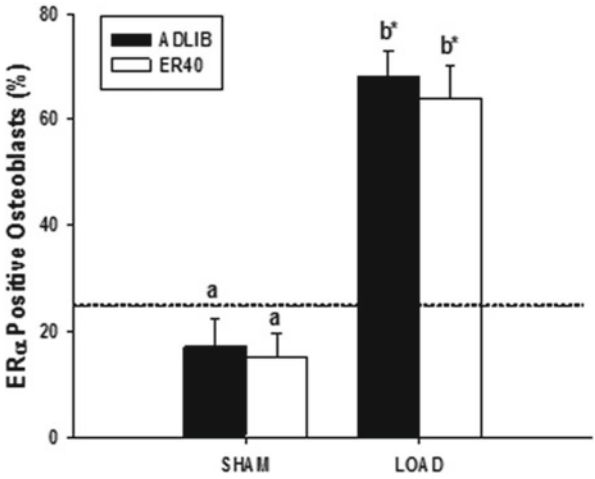

Fig. 2.3 Short-term mechanical loading (LOAD) upregulates ER-α expression in (**a**) osteoblasts in the distal femur metaphysis, even in animals subjected to 12 weeks of 40 % reduction in energy intake (ER-40). Dashed line denotes baseline control (BC) animals' values. Those groups not sharing the same letter are significantly different from each other ($p<0.05$). *$p<0.05$ versus baseline control mean value. Data from Swift et al. [46]

osteocyte expression levels of ER-α protein are 6- and 26-fold greater, respectively, in female rats after 1 week (3 bouts) of loading achieved by active muscle contraction (see Fig. 2.3) [46]. Interestingly, 12 weeks of energy restriction preceding the week of imposed loading, which usually diminishes circulating estrogen, does not diminish these increases in ER-α expression, but does blunt the bone formation response to mechanical loading. Hence, in this case, the anabolic response of osteoblasts to exercise does not appear to be caused by a down-regulation in ER-α protein in osteoblasts or osteocytes.

While more research needs to be conducted to fully understand the roles of each of the estrogen receptors, there may be interesting implications of this work. For example, could a pharmacological agent that manipulates estrogen receptors improve mechanosensitivity and diminish the negative effects of estrogen deficiency on bone? In conjunction with an appropriate exercise program, could manipulation of estrogen receptors increase the effectiveness of exercise and improve bone mass? Novel treatments for postmenopausal osteoporosis and age-related bone loss may capitalize on the role of estrogen receptors in bone's adaption to mechanical loading.

Oxidative Stress: New Mechanism for Aging-Related Bone Loss?

Oxidative stress has been a leading theory of aging for decades [47], but only very recently has it been advanced as a mechanism for age-related bone loss [48]. The primary mechanism leading to oxidative stress is the formation of reactive oxygen species (ROS) by electrons escaping from the electron transport chain during aerobic metabolism which then join with oxygen to form superoxide ($O_2^{\bullet}$), hydrogen

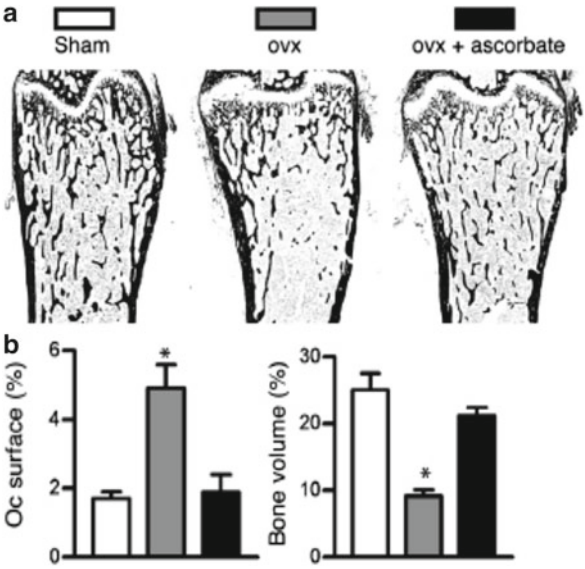

Fig. 2.4 Antioxidant treatment can reverse the impact of estrogen deficiency on cancellous bone mass and osteoclast surfaces. (**a**) Photomicrographs of distal femurs of mice subjected to sham ovariectomy (*white bar*), ovariectomy (*gray bar*), and ovariectomy with ascorbate treatment (*black bar*). Mineralized bone is in *black*. (**b**) Bone volume normalized to tissue volume (%BV/TV) and (**b**) Percent osteoclast surface (Oc.surface %) and cancellous bone volume normalized to tissue volume (%BV/TV) in the three groups. Adapted from Lean et al. [52]

peroxide (H_2O_2), and the hydroxyl radical ($OH^{\bullet}$) [48]. ROS cause damage to proteins, lipids, and DNA, initiating cell death and also trigger important signaling pathways, such as the $p66^{shc}$ pathway initiating cell apoptosis.

For several decades, it has been known that ROS in bone tissue are involved in the formation and activation of osteoclasts, thereby increasing bone resorption [49]. This led to an interesting hypothesis that oxidative stress could enhance, or even provide an alternate mechanism for, bone loss associated with menopause and aging. In a large-scale study of postmenopausal women, that subset with a diagnosis of osteoporosis had a higher oxidative status and lower antioxidant status as assessed from serum markers. There was a significant negative correlation between a key indicator of oxidative stress in this population and bone mineral density in the lumbar and femoral neck region [50].

Recent research has shown that estrogen may play a key role in protecting bone tissue against the damage caused by ROS. Estrogens have been identified as antioxidants in many tissues, effectively functioning as radical scavengers and suppressing peroxidation reactions [51]. Lean et al. [52] hypothesized that this antioxidant function of estrogen might be an important mechanism for its well-known salutary effect on bone health. Glutathione and thioredoxin, two major oxidative defense enzymes, are much reduced in rodent bone marrow after ovariectomy. A single dose of 17β-estradiol rapidly normalizes these antioxidant enzyme levels. More convincingly, administration of exogenous antioxidants, *N*-acetyl cysteine (NAC) and ascorbate, prevents bone loss in OVX mice (see Fig. 2.4) [52]. To further validate the effect of oxidative stress on bone, administration of L-buthionine-(S,R)-sulfoximine, an inhibitor of glutathione, resulted in similar loss of bone as observed in OVX animals. These data show, first of all, that ROS can lead to bone loss and, secondly, estrogens help to prevent bone loss by increasing intracellular oxidative defense

mechanisms. The loss of estrogens at menopause could lead to a substantial decrease in the ability of the bone cells to defend against ROS and thereby contribute to increased osteoclastic activity and bone loss. Hence, the loss of estrogens in women at menopause may amplify the oxidative stress accounting for aging-related bone loss.

Besides antioxidant enzymes (glutathione, thioredoxin, and superoxide dismutase), there are other pathways that lead to protection against ROS. The FoxO genes, so named for their unique structure with a special winged DNA helix known as a Forkhead box, are upregulated by ROS. Once FoxOs are activated and translocated into the nucleus of the cell, they lead to the transcription of free radical scavenging enzymes (MnSOD, catalase) and DNA repair genes (such as Gadd45). The FoxOs are also able to induce apoptosis in cells damaged by ROS [48, 53]. Mice null for FoxOs exhibit increased oxidative stress and a loss of both cortical and cancellous bone due to deficient bone formation [54]. If FoxO1 is conditionally deleted in osteoblasts, osteoblast proliferation and bone formation are significantly impaired. Administration of the antioxidant NAC normalizes osteoblast number, bone formation rate, and bone volume in mice lacking FoxO1 [55] and rates of osteoblast apoptosis in mice with osteoblast-specific deletions of FoxO1, O3, and O4 [56]. These studies further highlight the effects of oxidative stress on bone loss and the importance of defense mechanisms against an increase in ROS. Also of importance, exogenous antioxidant compounds are able to counteract the deleterious effects of the deletion of oxidative defense genes.

As Lean et al. hypothesized, the antioxidant abilities of estrogens may be critical to bone health due to its capacity to defend against oxidative stress [52]. Further research has shown that loss of estrogens and androgens accelerate the effects of aging by decreasing defenses against oxidative stress [48]. The loss of estrogen results in increased lipid peroxidation and hydrogen peroxide, and a decrease in oxidative defense enzymes (SOD, glutathione peroxidase, and glutathione *S* transferase) in femoral bone tissue of OVX mice, resulting in increased oxidative stress [57]. Almeida et al. compared normally aging mice (up to 31 months of age) of both genders with mice that underwent gonadectomy at 5 months of age [58]. Similar changes in ROS, glutathione reductase, and phosphorylation of p53 and $p66^{shc}$ were observed within 6 weeks of gonadectomy as observed over 31 months of aging. The administration of NAC protected against all measures of oxidative stress damage and was as effective at preserving spinal BMD as administration of either estrogen or testosterone. This exogenous antioxidant also mitigated the increase in osteocyte and osteoblast apoptosis caused by gonadectomy and maintained cancellous bone mass [58]. Later studies by the same laboratory confirm that antioxidants are as effective at maintaining bone integrity as sex steroid replacement [48, 54]. Taken together, these studies demonstrate the importance of oxidative defense mechanisms in bone tissue and demonstrate the powerful antioxidant effect of sex steroids.

Other potentially important mechanisms for estrogen's antioxidant function relate to osteoblast and osteocyte apoptosis and to immune cell function. (Osteocytes are those cells embedded inside bone that are essential to signaling targeted remodeling of damaged bone, as well as sensing the mechanical loads placed on bone.)

Pretreatment of MLO-Y4 osteocyte cells with either 17β-estradiol or selective estrogen receptor modulators (SERMs) prevents osteocyte apoptosis [59]. This suggests that estrogen deficiency may lead to a loss of osteocytes due to an inability to protect against ROS-induced damage. Oxidative stress also impacts osteoblast apoptosis. Aging or loss of androgens or estrogens leads to activation of nuclear factor-κB (NF-κB), and phosphorylation of $p66^{shc}$, which increases the production of ROS and stimulates apoptosis. Administration of 17β-estradiol effectively inhibits ROS-stimulated activation of NF-κB and $p66^{shc}$ by acting on PKCβ, thereby attenuating osteoblast apoptosis [54]. OVX mice also exhibit an increase in ROS in bone marrow, which enhances the activity of bone marrow dendritic cells that activate T cells, leading to a signaling cascade resulting in bone loss. Bone loss is effectively prevented by supplying either an antioxidant or an inhibitor of the CD80/CD28 pathway, which is involved in T cell activation [60]. Taken together, these studies demonstrate that the loss of estrogen leads to a decreased ability to defend against ROS acting on key bone and immune cells important for maintaining bone integrity.

The evidence that oxidative stress leads to bone loss and the effect antioxidants exert on reversing this damage could lead to novel concepts for treating bone disorders like osteoporosis. Could a diet high in antioxidants help prevent the increase in ROS seen with aging? Could administration of antioxidants prevent the sharp decline in oxidative defense seen with postmenopausal osteoporosis and, therefore, preserve bone mass or attenuate its decline? In a cross-sectional study of postmenopausal women, Rao et al. [61] demonstrated a strong inverse correlation between serum levels of the antioxidant lycopene (found in tomatoes, red bell peppers, watermelon, and other red fruits and vegetables) with N-telopeptides (NTx), a well-accepted serum marker of bone resorption. There is also strong epidemiological evidence for the beneficial impact of flavonoids, polyphenolic compounds found in many plant foods that have anti-inflammatory and antioxidant properties, on bone health [62]. However, intervention trials provide less definitive conclusions to date.

Conclusion

The preceding provide a fine example of how integrative physiology informs the field of bone biology, since nutritional status, endocrine regulation, and oxidative stress are all important modulators of bone cell differentiation and activity and, ultimately, bone structural integrity and fracture risk. It has become clear that prolonged negative energy balance is an important contributor to bone loss in a variety of populations. Given the large number of Americans repeatedly attempting to lose weight, useful interventions to minimize this loss of bone should be pursued, especially as it remains uncertain if bone loss associated with dietary energy restriction is reversible. Estrogen deficiency clearly impacts estrogen receptor function, which may explain why exercise appears to have a diminished osteogenic effect in postmenopausal women. The interaction of estrogen withdrawal at menopause and

oxidative stress, with its myriad effects on bone cell function, may lead to interesting new therapeutic or dietary interventions for improving antioxidant defenses in aging individuals. Defining the interplay of these factors in animal models and in human intervention trials provides a stimulating challenge to integrative physiologists interested in optimizing bone health for all women.

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Chapter 3
Estrogen Effects on Skeletal Muscle

Marybeth Brown

In the past several decades the importance of the sex hormone estrogen for the overall health and well-being of skeletal muscle has become apparent, particularly for women, but also in men. In the early 1990s an article appeared indicating that skeletal muscle may be an estrogen (E2) target and that E2 impacted muscle strength. This seminal article was published by Phillips et al. [1] and indicated that women who were on hormone replacement therapy (HRT) through the menopause maintained the strength (force/muscle mass) of their adductor pollicis muscle compared with women who were not on HRT. Findings suggested that E2 provided a protective effect on muscle strength loss with aging that was maintained until women were approximately 70 years of age. Since that time hundreds of studies have been conducted and our understanding of E2 effects on skeletal muscle has grown substantially. The purpose of this chapter is to summarize recent findings on known E2 effects on skeletal muscle but also indicate some of the questions that remain.

Women are living to an average age of 80 years but for most women E2 levels begin to decline at around age 50. Thus, approximately one-third of a woman's lifetime is spent with very little circulating E2. Even for younger women, there are a number of circumstances that result in low E2. For example, more than 600,000 hysterectomies are performed each year, resulting in tens of thousands of young women with drastically reduced E2. Many younger women with breast cancer are put on drugs or chemotherapy which brings circulating E2 levels to near zero. Women who sustain head injury, spinal injury, and other forms of trauma also have E2 values that are, in many instances, unmeasureable [2–4]. Thus, a substantial number of women are E2 deficient, which may impact their quality of life through diminished muscle (and bone) quantity and quality.

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Estrogen Effects on Muscle Mass

Cross-sectional studies indicate that muscle mass declines with aging beginning in the third decade [5]. Approximately 10–15 % of mass is lost between the ages of 20 and 50 but once menopause commences, muscle mass declines at an accelerated rate, approximately 1 % per year [6–8]. These studies indicate that the accelerated rate of muscle mass loss with menopause coincides with the reduction in circulating E2. Thus, by the time a woman is postmenopausal she has likely lost another 5 % of muscle that is over and above the loss in muscle that has occurred prior to the menopause. It warrants repeating that the average life-span for women is approximately 80 years while the average age for menopause is 53 years, which leaves a sizeable portion of the life-span where women are E2 deficient.

Initially, only cross-sectional studies indicated an association between muscle mass and E2 but now most population studies also support an E2 effect on muscle mass [9]. For example, Taaffe et al. conducted a double-blind study of perimenopausal women between the ages of 50 and 57 years, some of who received 1 year of HRT [10]. Those in the HRT group had significantly increased cross-sectional area of the quadriceps and hamstring muscles compared to the women who were not suggesting a preservation of lean mass during menopause if E2 levels remained within normal limits. Chen et al. [11] analyzed a large number of women from the Women's Health Initiative (WHI) study and found after 3 years that those taking HRT lost 0.04 kg of lean tissue mass compared to women on placebo who lost 0.44 kg of lean mass, determined by DXA. Thus, there is reasonable evidence that E2 alone and E2 plus progesterone attenuate muscle mass loss during the menopause.

Probably the most remarkable study of E2 effects in women who had transitioned to postmenopause was conducted by Ronkainen et al. [12]. In this instance the investigators identified 13 monozygotic twin pairs who had gone through the menopause (54–62 years of age when studied). One of the twins was on HRT for the duration of menopause and the other was not and the average duration of hormone use was 6.9 years. Twins receiving HRT had an average of 11 % more muscle area of the thigh, providing strong evidence that the presence of normal values of sex hormones during menopause attenuates muscle mass loss. One caveat, however, is that the independent effects of E2 alone rather than E2 combined with progesterone could not be readily discerned in this study.

Most of the studies of E2 preservation effects on muscle mass were conducted on women who were transitioning through menopause. There are several studies, however, of younger women that support the androgenic effects of E2 on muscle. Gonadotropin-releasing hormone (GnRH) agonist, if given to women, produces profound hypogonadism and causes a nearly immediate loss in E2 production which subsequently has a remarkably rapid negative effect on lean muscle mass [13]. In summary, the evidence supporting E2 as an anabolic agent is mounting. Although most studies of HRT have not evaluated the independent effects of E2 and progesterone, there are still enough data to indicate that E2 alone is important for the maintenance of muscle in women.

One question that remains unanswered is whether E2 continues to have anabolic effects through the entire lifetime or if E2 loses its effectiveness shortly after menopause. Also unclear is whether women who take E2 through menopause, and thus lose less muscle, maintain this advantage if E2 use is continued or discontinued. Data from Phillips et al. suggest that the advantages of E2 use are lost by age 70 but the number of subjects in their study is too few for conclusions to be drawn [1]. Kenny et al. studied the prevalence of sarcopenia (age-related decline in muscle mass) in older women who were long-term users of hormone replacement [14]. Their results indicated that the incidence of sarcopenia was as high in women taking HRT (23 %) as women not taking HRT (22 %). Complicating interpretation of their results however is the fact that women on HRT took a variety of compounds and had dosing regimens that varied substantially. More research is needed to better understand the long-term effects of hormone use on muscle and how long the benefits of HRT are maintained.

E2 effects are mediated through two estrogen receptors (ER) termed alpha and beta. Interestingly, skeletal muscle fibers in men appear to have as much ER as women, suggesting that muscle is a target tissue in men as well as in women. A few studies are beginning to indicate the independent effects of E2 on muscle mass in men, independent of testosterone, but data are too few at the moment for definitive conclusions [15]. There are data that indicate that DHT is converted to both T2 and E2 within male skeletal muscle suggesting a role for each during protein synthesis. Data from our lab on male mice indicate that the absence of E2 results in a 10–12 % loss in muscle mass with no effect on strength/mass. In this instance we studied groups of wild-type (WT) mice and those without the ERα, ERβ, and the enzyme aromatase, which converts T2 to E2. Mice without a specific ER had occasional differences in muscle mass, particularly those missing the ERα, but data were too inconsistent for conclusions to be drawn. Mice lacking aromatase, those with E2 values that were undetectable, had significantly less mass than the WT controls. Force per unit of muscle mass or force/muscle protein were comparable to values for those of WT controls suggesting that E2 regulates, in part, muscle mass in the male (Fig. 3.1).

When the WHI study was terminated prematurely, millions of women were taken off of HRT and E2 alone due to the potential negative side effects of sex hormone therapy. Since that time it has been realized that the WHI data presented a very incomplete picture of hormonal effects on peri- and postmenopausal women but clinical practitioners are still reluctant to prescribe E2. There are other estrogenic compounds that potentially can stimulate skeletal muscle, possibly through ER, compounds such as plantlike estrogens (phytoestrogens). Soy-based products have been evaluated to a modest extent and it appears that soy isoflavones are weakly estrogenic (anabolic), particularly when combined with exercise. Future research should yield additional insights into other phytoestrogens, synthetic steroids, and specific selective estrogen receptor modulators, compounds that may have the desirable effects on skeletal muscle (and bone) without any deleterious side effects.

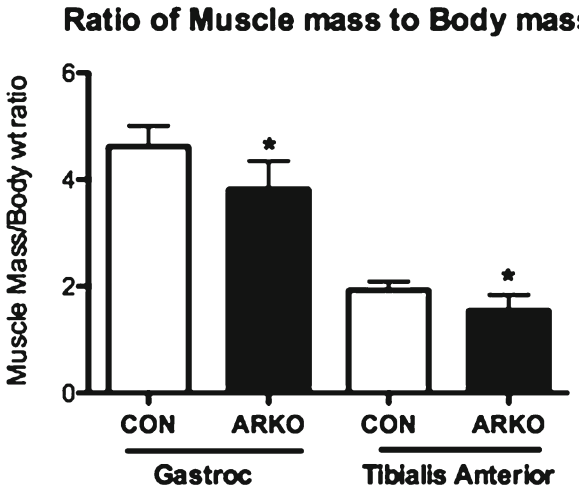

Fig. 3.1 Groups of male mice ranging in age from 4 to 6 months were studied. Control mice (CON, $n=11$) were hormonally intact while those in the aromatase knockout (ARKO, $n=9$) group were deficient in E2. The muscle masses of two representative muscles, gastrocnemius and tibialis anterior, are presented. The mass deficiency for the gastrocnemius (17 %) and tibialis anterior (20 %) is significant ($p<0.01$). Force per muscle mass was comparable between groups

Estrogen Effects on Muscle Strength

The inevitable outcome for women who have less muscle mass is less muscle strength. Unfortunately, the typical association of mass and strength is lost in women who are E2 deficient. There is ample evidence, from both the human and animal literature, indicating that E2 impacts muscle quality, specifically force/unit area, such as muscle mass or cross-sectional area [12, 16–19]. Thus, with E2 deficiency, a greater amount of force than skeletal muscle is lost, which may explain the decline in functional capacity in many postmenopausal women. A recent meta-analysis of the human and animal literature indicated that muscle force/unit of muscle (specific force) is approximately 10 % less in the ovariectomized (OVX) rodent, although results from a variety of studies range from no change to almost 20 % less specific force [18]. The cause(s) associated with the loss in force/unit area is unclear. Moran et al. determined, using electron paramagnetic resonance spectroscopy, that the fraction of strong-binding myosin was ~15 % less in EDL single fibers from OVX mice [16]. The loss of strong binding was consistent with the decline in EDL specific force (19 %). These findings suggested to the investigators that the loss of ovarian hormones causes a loss in the total number of actin and myosin molecules, a reduction in the fraction of myosin that is strongly bound to actin during the contraction cycle or, possibly, that the force generated per molecule of myosin is reduced [16]. These results could potentially explain the lower specific force but findings have never been replicated.

To put the loss of specific force in context, van Geel et al. reported in 329 healthy postmenopausal women between the ages of 55 and 85 years (cross-sectional study) that muscle mass loss was 5 % when those in the 55–65-year-old group were compared to the 75–85-year-old women [20]. Within the same comparison groups the decline in maximal knee extension strength (32 %) and grip strength (23 %) far exceeded the loss in lean mass, suggesting a dramatic decrease in muscle efficiency with age, possibly compositional change in lean mass. The declines in strength (and lean mass) were associated with bioavailable serum E2 levels. Although all of the women were aging, it was not possible to separate age-related muscle decline from the changes associated with loss of ovarian hormones. Nonetheless, findings strongly support the importance of active E2 (and testosterone).

Muscle force loss was also measured in the Finnish twin study. To reiterate, 13 postmenopausal monozygotic twin pairs were identified, one of whom had taken HRT through the menopause (average duration of use was 6.9 years) while the other twin had not [12]. The twin taking HRT had more muscle mass and significantly more mobility than the twin not taking HRT. Lower body muscle power measured as vertical jump height was 16 % higher in the HRT users. Average walking speed was 7 % greater in women taking hormones. This study is important for two reasons: first, the use of monozygotic twin pairs eliminates biologic subject variability and second, the investigators demonstrated the functional consequences of lost ovarian hormones. Older women are far more likely than men to require nursing home placement due to loss of functional mobility. Findings from the twin study suggest that the loss of E2 plays a role in the decline in physical function.

E2 and Exercise Effects

Because the loss of ovarian hormones results in a decline in muscle mass and strength, it begs the questions of whether strengthening exercise can offset or mitigate these losses. Surprisingly, little is known about the effects of exercise alone in the perimenopausal women or if there is an interaction of E2 and exercise. Several studies have reported that the postmenopausal woman does not gain strength to the same magnitude as men or premenopausal women, suggesting a role for E2 to augment muscle force increases with exercise. Our lab strength-trained older men and women (average age 82.3 years) at 70–80 % of 1-RM, 3x/week, for 3 months and found the overall increase in strength for the men (67 %) to be significantly higher than the strength increase for older women (45 %) even though both groups worked at the same relative intensity (Fig. 3.2). However, Petrella et al. studied young (20–29 years) and older men and women (60–75 years, mean age 63.7 years) before and after 4 months of rigorous strength training for the knee extensors [21]. Biopsies were taken from the vastus lateralis muscle before and after training and myofiber cross-sectional area determined. Hypertrophy occurred in all groups (young and older men, young and older women) but the greatest increase occurred in the young men (~32 %). Younger women had an ~32 % increase in strength whereas older women showed a 25 %

Fig. 3.2 Strength gains following 3 months of traditional training at 60–80 % of 1-repetition maximum. On average, participants trained rigorously 2 days/week and stretched or worked with therabands during a third session. The training stimulus was the same for both men and women

improvement. Curiously, the number of myonuclei per muscle fiber increased only in the young men. The number of NCAM-positive cells (indicative of satellite cell incorporation) was higher only in the young men. These findings suggest that there is little difference in training adaptation for young vs. older women. Whether these two studies are comparable given a nearly 20-year difference in age for older subjects remains to be seen. One important point to emerge from both of these studies is that older postmenopausal women are adaptable to strength-training and capable of making gains in muscle mass and force, whether the magnitude of change is the same or lower than that of younger women. Gains in muscle mass and strength are important for the maintenance of independence in old age.

Several additional studies provide results that are challenging to interpret and it is still unclear whether E2 augments gains in muscle mass and strength with training in the woman who is hormone deficient. Technically, exercise and E2 effects should be additive if there is an independent hormone augmentation of muscle during exercise. In one study of postmenopausal women Sipila et al. (2001) randomized their 50–57-year-old subjects into exercise, exercise-plus-HRT, HRT-only, and untrained control (placebo HRT) groups. Women in the exercise groups strength-trained 2–3 days/week for 12 months [22]. Explosive power significantly increased in the HRT–exercise and HRT groups. Knee extension torque and muscle fiber cross-sectional area also increased in the HRT–exercise and HRT-alone groups. While the differences between these two groups were not significant, the magnitude of increase in muscle torque and fiber area in the combined HRT–exercise group was greater than that observed in the HRT-only subjects suggesting an additive beneficial effect of hormone replacement. Subject variability was substantial which likely masked potential differences given subject numbers in each group. Subsequent analyses of the same women [23] revealed that quadriceps and posterior compartment (e.g., hamstrings) muscle loss was attenuated in women taking HRT compared to controls. In a separate study Brown et al. (1997) strength-trained 42 women, half of whom were on HRT. Women were between the ages of 60 and 72 and all of them

exercised 3 days/week. The first three months were spent in stretching, balancing, and light calisthenics in preparation for the subsequent 6 months of strength training at ~75 % of 1-RM. At the end of 9 months strength was significantly increased in leg press, hip extension, and isokinetic knee extension/flexion. However, the gains made by the HRT-plus-exercise group were no greater than the gains made by women who were not on HRT, which raises the question of whether women were too far beyond the menopause to benefit from hormonal intervention [24]. Regardless, studies of strength training in postmenopausal women indicate that they are capable of making significant gains in muscle strength. Whether the increases in muscle mass and strength were augmented by HRT seems unclear but few studies have addressed this issue and more are needed.

One rodent study of exercise and E2 effects on skeletal muscle warrants mention. Grieseing et al. (2011) determined if E2 effects on skeletal muscle were independent of physical activity. To that end they randomized mice to groups that had increased physical activity (wheel running), diminished activity (HLU for 2 weeks), and no muscle activity (nerve transection). In all instances, E2 effects on muscle force were independent of activity level [25]. Soleus muscles in OVX mice from the HLU and nerve transected groups had 31 % less muscle force/muscle protein content than mice subjected to the same conditions but supplemented with E2. Findings strongly support the concept that E2 significantly influences muscle power independent of physical activity levels.

E2 Effects on Muscle May Be Fast Acting or Exerted Through ER

The apparent anabolic effects of E2 appear to be mediated through the ER. In rat myotubes [26] Wiik et al. studied first whether ER were present and secondly whether ER expression increased with E2 and electrical stimulation (exercise). Investigators first demonstrated that both ERα and ERβ are present in myotubes. Next E2 was used to determine if mRNA levels of ERα and ERβ would increase in response to hormone stimulation. In their cells only ERβ increased in response to E2 while ERα remained unchanged. In a separate set of studies Galluzzo et al. studied the effects of E2 on ERα and ERβ using rat myoblast L6 cells [27]. In their cells, Akt activation was the consequence of ERα stimulation, not ERβ. Akt stimulation has been linked to muscle hypertrophy and muscle development. Both receptors ERα and ERβ were involved in E2-mediated activation of p38. The p38/MAPK pathway has been identified as critical for muscle cell differentiation and the fusion of myoblasts into myotubes, a key step in muscle regeneration. Whether these studies are complementary is unclear. A third ER has been postulated which may modulate ER activity. This putative third receptor is called Gper and is expressed in both the soleus and EDL muscles. Gper is responsive to E2 and appears to be associated with antioxidant gene expression [28]. More study is needed to better understand the potential role of Gper in muscle function.

To further elaborate the role of ER, our laboratory studied female knockout (KO) mice without ERα or ERβ. Muscle mass and contractile tensions were determined for four separate muscles with different anatomic and fiber type profiles. Muscle mass was essentially unaffected in ERα and ERβ KO females. Peak tetanic tension/anatomical cross-sectional area was significantly reduced in the ERα KO gastrocnemius and tibialis anterior but not in the plantaris or soleus muscles. The absence of ERβ had no impact on muscle force. Total myosin content was unaffected by KO status [19].

These studies suggest that the ERs are important determinants of pathway activation in response to E2. Receptor function has been studied extensively but not in skeletal muscle, so the role of ER in muscle has not been precisely determined. What has become apparent recently in other cell types is that ERβ can act as a negative or a positive regulator of ER activity and that both receptors can be involved simultaneously as α/β heterodimer which may explain some of the results that seem contradictory [29–31]. More study of nuclear ER involvement is needed.

17-β estradiol can also stimulate non-transcriptional responses by non-genomic signaling. Activation of the PI3K/Akt and MAPK pathways, for example, can occur with or without the classic stimulation of the estrogen response element in the nucleus. The non-genomic effects of E2 are hypothesized to activate receptors outside the nucleus such as those on the cell membrane, in the cytoplasm, and in the mitochondria. Non-genomic signaling has been found to increase the expression of a variety of signaling molecules including ERK1/2, JNK1/2, p38, CREB, and c-fos. Cellular processes associated with non-genomic signaling include the regulation of apoptosis, mitochondrial function, and blood flow to muscle [26, 32].

E2 Effects on Gene Expression

Several studies have addressed the question of what happens to muscle when E2 is absent or when an E2-deficient organism is given E2 treatment. Overall, results indicate that E2 has direct effects on gene expression in a variety of ways but most effects still need to be elaborated in more detail.

Cell culture experiments provide strong evidence of E2 effects on gene expression. Wiik et al. [26] cultured rat myoblasts (curiously, from one male rat) and once cells reached confluency, they were transfected with estrogen response element (ERE)—luciferase. Cells were subsequently differentiated to myotubes and either stimulated to contract (exercise) or exposed to ICI 182,780, an ER antagonist, or the MAPK inhibitor PD-98059. Electrical stimulation activated the ERE-LUC reporter construct. To determine if activation was ER dependent, ICI 182,780 was used. Activation of the ERE was unaffected by ICI 182,780 but activation was abolished when the MAPK inhibitor PD 98059 was added to the cell culture. E2 also stimulated ERE-LUC activity but activation involved the ER. Thus, results indicate that exercise and E2 stimulate ERE activation but one involves the ER and the other does not.

Kahlert and colleagues [31] cultured L6 and C2C12 myoblasts with 17-β estradiol or estrone and determined if there is evidence of E2 effects on skeletal muscle growth. Gene transactivation, as evidenced by activation of ERE-LUC, did occur in response to 17-β estradiol in a dose-dependent fashion. Myoblast proliferation, however, as measured by BrdU incorporation, was induced by estrone but not 17-β estradiol. Further, investigators determined whether estrone or 17-β estradiol can induce expression of the immediate early genes egr-1 and c-fos. Treatment of myoblasts with 17-β estradiol for 30 min resulted in a 1.7-fold increase in c-fos and a 2.3-fold increase in egr-1 expression. Treatment of myoblasts with estrone for 30 min led to a 3.9- and 4.6-fold increase, respectively, in c-fox and egr-1. Treatment of cells with ICI 182,780 abolished responses indicating that E2 effects were mediated by the ER. More study is needed to understand why both forms of E2 induced the transcription factors egr-1 and c-fos but did not have similar effects on myoblast proliferation.

To further establish a role for E2 in skeletal muscle cell growth (myogenesis), Galuzzo et al. [27] studied the response of L6 cells to E2. Specifically, they examined ER-mediated nuclear signal transduction pathways. Briefly, they determined that E2 increased myogenin and myosin heavy chain (MHC) levels. Further, when they added the ER antagonist ICI 182,780 to the culture medium, myogenin and MHC expression failed to occur, indicating that the E2 effects on muscle cell growth were mediated by the ER. E2 also induced phosphorylation of p38 which is required for the expression of myogenin and MHC, and is crucial for transcriptional control of skeletal muscle differentiation. Interestingly, when these investigators added the extranuclear ER blocker 2-bromopalmitate, myogenin and MHC expression failed to occur. Thus, data indicate that E2-dependent rapid signaling from the membrane and nuclear action are responsible for the induction of L6 differentiation. These results need further elaboration.

Human skeletal muscle cells (myoblasts) were cultured by Dieli-Conwright et al. [29] and treated with E2. RT-PCR was used to determine the expression levels of mRNAs for steroid receptor coactivator (SRC), a positive regulator of ER activity, and silencing mediator for retinoid and thyroid hormone receptors (SMRT), a negative regulator of ER activity. E2 treatment for 24 h resulted in increased mRNA expression of SRC and decreased expression of SMRT. Additionally, E2 resulted in increased mRNA expression for MyoD, a potent stimulator of myoblast differentiation. Results suggest a role of E2 in the maintenance of skeletal muscle mass and function and have implications for aging women.

These important studies strongly indicate a role of E2 as a hormone impacting skeletal muscle cell growth and may explain why women who are postmenopausal lose strength and muscle mass at an accelerated rate. Given the number of younger women who are premenopausal undergoing oophorectomy each year coupled with the enormous number of women with breast cancer who are receiving drugs to reduce E2 levels, there may be an enormous number of women of all ages who are strength deficient.

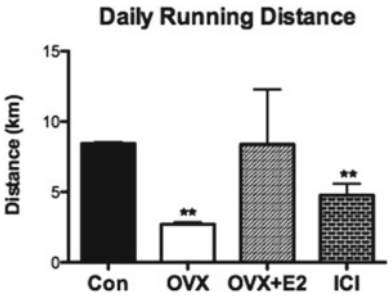

Fig. 3.3 Mature female mice were placed in cages with running wheels and studied for 7 weeks. Data for running distance were collected daily after steady-state running distances were observed, which typically occurred within 2 weeks. Four groups of mice are presented: intact (sham OVX) females, OVX females, OVX females supplemented with E2, and intact mice given 3 weeks of ICI 182,780 (10–12 mice/group). **Daily distances for OVX and ICI 182,780 mice are significantly below those of the intact and E2-supplemented groups ($p < 0.01$)

Indirect Effects of E2 on Skeletal Muscle

E2 exerts a powerful behavioral effect on spontaneous physical activity. Rodent studies are particularly dramatic in that if ovarian hormones are removed (OVX), spontaneous physical activity (wheel running) plummets almost immediately. Indeed, distances are 20–25 % of those prior to OVX. When the ovarian hormone E2 is provided back to the organism, running distances return to pre-OVX levels (Fig. 3.3). In our lab mature 4-month-old mice were placed in cages with running wheels and distances traveled were recorded daily for 6 weeks. Subsequently an OVX was performed and mice were returned to their cages with running wheels and followed another 3 weeks. With OVX running distances plummeted to values that were approximately 20 % of those recorded when ovaries were functioning normally. After the 3-week recording period following OVX, E2 was then provided to confirm that it was the ovarian hormone influencing spontaneous activity. Running distances returned to baseline levels, those recorded before OVX.

Data for women suggest the same finding, a reduction in physical activity with the loss of estrogen [12]. Coincidentally, menopausal women lose muscle mass but gain fat mass, particularly in the abdominal region. Unclear is whether the loss of spontaneous activity results in an increase in fat mass or the loss of hormone precipitates fat gain which subsequently impacts spontaneous activity. Women who continue to endure exercise through the menopause do not gain body fat compared to women who are sedentary suggesting that lifestyle is a more important determinant of body fat than E2 [33].

One of the strongest determinants of dependency in old age is lack of physical activity. Women who fail to exercise experience losses in factors that contribute to independence: muscle mass and strength, balance, bone mass, flexibility, and cardiovascular reserve. Women with a lifestyle that includes exercise have far less risk

for loss of independence because they have higher muscle mass and strength, better balance, better bones, higher cardiovascular endurance, and more reserve in all systems. One aspect about aging that has received little attention is whether the loss of E2 with the menopause, which contributes to inactivity, has long-term deleterious consequences. Long-term studies of women who take HRT into their 70s and 80s are needed.

Another indirect effect of E2 relates to food intake. Animal studies indicate that loss of E2 results in hyperphagia [34]. Data from out of the lab also indicate that loss of E2 affects metabolic rate suggesting that a food intake that maintained a stable body weight before loss of E2 will result in body mass gain afterwards. A common complaint of women who become postmenopausal is weight gain without a change in diet, which mirrors rodent studies. Body mass is a direct determinant of physical activity; the higher the body mass the more inactive the individual [35]. These findings and observations suggest that loss of E2 with age or losses in E2 that are artificially induced result in weight gain and loss of spontaneous activity, which in the long-term can independently contribute to losses in muscle mass and strength, losses that are over and above those that occur with low E2. Indirect effects of lost E2 may have more consequences than the direct effects of diminished levels of E2.

Protective Effects of E2 on Skeletal Muscle Injury

For the past decade the laboratory of Tiidus et al. has conducted a series of studies to examine the influence of E2 on the extent of muscle injury and the mechanisms behind the protection of E2-replete muscle. In one of their earliest studies Tiidus et al. ran male and female rats downhill on a treadmill for 90 min to induce muscle injury [36]. They determined that female rats sustained far less injury than males and speculated that E2 may have a protective effect on the muscle cell membrane. In a subsequent study male rats were again run downhill on a treadmill to induce muscle damage but one group was supplemented with E2 prior to the exercise bout. E2-supplemented male rats appeared to have less damage and greater number of satellite cells compared to male rats that were not supplemented. In the next study OVX female rats with and without E2 supplementation were run downhill for 90 min and sacrificed 72 h later. Soleus and white vastus lateralis muscles were immunostained for Pax7 (total # of satellite cells), MyoD (activated satellite cells), and BrdU (proliferating satellite cells). E2-supplemented rats had lower values of the injury marker β-glucuronidase and higher number of Pax 7-, MyoD-, and BrdU-stained cells [37]. Thus, results again indicated that E2 protects against muscle damage and that E2 plays an active role in muscle repair by activating satellite cells. In a subsequent study Enns et al. determined that the protective effects of E2 are mediated through the ER. To make this determination the ER blocker ICI 182,780 was given to rats to mitigate the effects of E2 supplementation. ICI 182,780 completely blocked the increases in activated and total satellite number and prevented satellite cell differentiation. Thus, findings suggest that ERs play an important role in the

activation and proliferation of satellite cells. In a further study Thomas et al. [38] determined further that the ERα was probably the route of E2-activated events. In this instance the ERα-specific agonist propyl pyrazole triol or PPT was administered 3 days before the exercise bout. PPT augmented myoblast activation and number to a similar extent as E2 indicating that E2 acts through the ER, particularly ERα, to stimulate myoblast proliferation.

Consistent with findings from rats, Dieli-Conwright et al. [39] determined that post-exercise muscle damage is attenuated in menopausal women who are on HRT. These investigators recruited 14 postmenopausal women 55–65 years of age, 8 of whom were on HRT. Each woman had a pre-exercise strength test and then underwent a rigorous bout of eccentric exercise (10 reps × 10 bouts) to induce damage to the quadriceps. Women on HRT had far less change in inflammatory cytokines following exercise compared to women with low E2 values. HRT significantly blunted increases in TNFα, and IL 6, 8, and 15. Further, women on HRT had lower serum values of CK, another indicator of muscle damage.

In summary, data from animal and human studies strongly support a protective effect of E2 against skeletal muscle damage. It has been hypothesized that E2 stabilizes the muscle cell membrane but there are few data to support this contention. There is evidence as well that E2 protects tissues other than skeletal muscle from damage such as neural and cardiac. Thus, E2 appears to have an anti-inflammatory role.

E2 Effects on Simulated Bed Rest

If, as hypothesized and demonstrated in some studies, E2 plays a role in muscle protein synthesis, then the recovery of atrophic muscle should be blunted by the absence of hormone. To that end, several studies have demonstrated that loss of E2 compromises the recovery of atrophic muscle. Mature female rats, half of whom were OVX, were hindlimb unweighted (HLU) for 4 weeks to induce atrophy. Subsequent to the unweighting, two groups of rats were cage recovered for 2 weeks. Those with E2 showed an increase in muscle mass and peak tetanic force toward baseline control values whereas rats lacking E2 failed to demonstrate recovery in muscle parameters [40]. Moreover, rats that were E2 deficient failed to increase total protein content or to activate the Akt/mTOR muscle protein synthesis pathway [41]. In a subsequent study McClung et al. [42] determined that the recovery of the atrophic soleus occurred in 7 days in rats that were HLU for 10 days. Rats that were OVX required 2 weeks for soleus to recover its mass but importantly, recovery ultimately did occur in this study. However, additional examination of muscle at the single fiber level revealed that the recovery of mass in OVX females was not reflected in fiber cross-sectional area, which remained essentially unchanged from HLU values. Noncontractile tissue was found to be responsible for the increase in soleus mass, particularly collagen and water. These results further support the importance of E2 for myofiber growth.

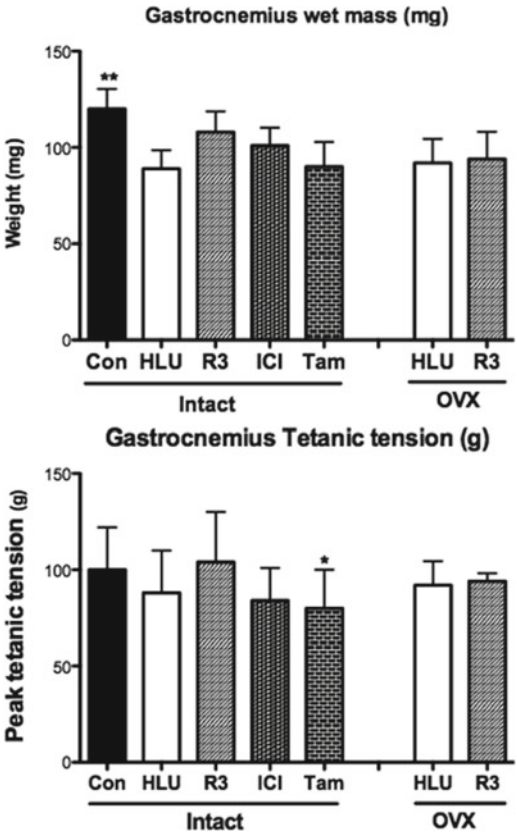

Fig. 3.4 Mature female mice were HLU for 4 weeks and studied or allowed 3 days of cage recovery. Control (sham OVX) mice were hormonally intact, and OVX were not. Recovery of muscle mass was diminished in OVX mice and in those mice receiving the estrogen receptor blocker ICI 182,780. Tamoxifen, an ERα agonist, failed to enhance recovery. **Controls had higher muscle wet mass than all other groups ($p < 0.01$). *Contractile tension was significantly less in the tamoxifen-supplemented mice ($p < 0.05$)

In a recent study from our laboratory, mice were HLU for 4 weeks and subsequently allowed to recover for 3 days or 1 week. As hypothesized, recovery of the atrophic soleus, plantaris, gastrocnemius, and tibialis anterior muscle mass was compromised at both time points in the OVX mice. Additionally, the force generating capacity of the predominately fast fibered plantaris, gastrocnemius, and tibialis anterior was diminished in OVX mice. The estrogen receptor antagonist ICI 182,780 completely blocked the recovery of muscle mass, contractile tension, and fiber cross-sectional area in the sham OVX group. Surprisingly, the addition of tamoxifen, an E2-related agonist that is preferential to ERα, failed to augment recovery in any of the muscles studied (Fig. 3.4).

Collectively these studies have implications for E2-deficient women who undergo bed rest for any reason. Over a million postmenopausal and hypogonadal women are hospitalized each year. Additionally, women who undergo significant physical trauma reportedly have sex hormone levels that are unmeasurable. These women have the potential additional compromise due to the ill effects of bed rest coupled with the likely negative effects of low E2 [2, 4]. There have been reports that women with low E2 values have less rehabilitation potential [43]. It has also

been noted that postmenopausal women do not respond to strength training to the same extent as premenopausal women or men [21]. These findings raise the question of whether short-term E2 supplementation should be considered to enhance rehabilitation potential and recovery from bed rest.

In a unique study Sugiura et al. [44] immobilized one hindlimb of male rats for 10 days. For 2 weeks prior to immobilization one group of animals was given E2 at a dose of 40 μg/kg of body weight or vehicle. This dose was maintained throughout the immobilization phase, so E2-supplemented rats received treatment for 24 total days. Subsequently the immobilized and non-immobilized soleus muscles were removed and frozen. Rats that received vehicle injections had more soleus atrophy than those that were treated with E2 suggesting attenuation in the rate of muscle atrophy in the presence of E2 in a male rodent. Additionally, E2-treated males had significantly less calpain expression (an indicator of muscle breakdown) compared to rats that had not been treated.

Alternatives to E2

When the original WHI study was terminated in 2002 (E2 plus progesterone) millions of women were taken off HRT. It has been realized that the original data collection was problematic for a variety of reasons including supplementing women that were 10–15 years post menopause, not pre-screening for heart disease, not delineating women with and without other pre-existing conditions, not including physical activity or body mass as variables, and treating all women with the same dose regardless of body size. A variety of secondary analyses have been performed in the interim and several additional studies have been performed that seem to indicate that women who are perimenopausal are actually protected from heart disease with E2 and that the risk of breast cancer is negligible [44]. Two negative side effects have persisted throughout the studies of women on HRT and they are deep vein thrombosis and stroke [45]. However, women on HRT are protected against colon cancer and osteoporosis (with attendant reductions in hip fracture as well). The evidence seems to indicate that many women in the perimenopausal phase of the menopause can benefit from HRT but as with any treatment, the benefits and risks must be weighed accordingly.

Because the pendulum has not swung back toward the midline in terms of E2 usage, and there are clearly identifiable situations in which hormonal supplementation would be ideal, some alternative to E2 needs to be identified. Not all women can take E2 (e.g., those with estrogen-fed breast cancers) and many physicians are not aware of the newer work that indicates that the original findings of the WHI are not applicable to women of all ages. There are, however, SERMS, estrogen receptor modulators, that have promise but are not well studied. There are as well phytoestrogens and other potential botanicals that may have the desired estrogenic effect on skeletal muscle without the secondary negative side effects.

Considerable work is needed to fully elaborate this new realm of treatment potential.

In summary, this chapter has identified the importance of E2 for the maintenance of muscle mass in aging women and, most importantly, for the preservation of muscle strength. As most women are living to the age of 80 years and beyond, it has become more imperative for women to maintain muscle mass and strength for the sake of independence. The average older woman spends her final 4 years in a nursing home, which is a dismal end with tremendous expense involved. It is entirely probable that an E2 or an E2-like substance could preserve muscle integrity for a decade or more and prevent some of the loss of independence associated with old age. Thus, additional research on the role of E2 is sorely needed.

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Chapter 4
The Contribution of Ovarian Hormones to the Cellular Regulation of Lipid Metabolism

Espen E. Spangenburg and Kathryn C. Jackson

Abstract Regardless of the reason any reductions in estrogen function leads to the development of obesity and insulin resistance. In this chapter, we explore the concept that low levels of circulating estrogen are associated with the accumulation of lipid in skeletal muscle. We explore multiple possible metabolic mechanisms to address the development of the altered lipid metabolism and place a broad emphasis on the importance to women's health.

Keywords Metabolism • Lipid • Women • Ovary • Muscle

Our Understanding of Metabolic Function in Various Tissues Is Advancing

With the advent of new technology, scientists have begun to unravel novel metabolic regulatory mechanisms that help to define metabolic control in various tissues. The advances have allowed us to gain a better understanding of how these mechanisms either prevent or contribute to the development of metabolic disease in humans. In addition, defining these mechanisms has made scientists cognizant of tissue-to-tissue cross talk and the important role circulating factors play in regulating metabolic function in peripheral tissue. Unfortunately, due to the difficulty in defining these mechanisms, a number of critical considerations are often overlooked such as sex or hormonal status.

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From a Metabolic Perspective the Differences Between Men and Women Are Underappreciated

For reasons that are often unclear, when cellular mechanisms are initially characterized it is often assumed that the mechanism operates or is regulated in the same manner across the sexes. However, a substantial amount of evidence provides a foundation for rationale of testing these mechanisms in both males and females before generating broad conclusions. For example, enzymatic activity of constituents in the β-oxidation pathway in skeletal muscle is often higher in untrained women compared to untrained men [1]. This finding corresponds with results suggesting that women oxidize a greater amount of fat in response to an acute exercise bout than men [2]. Similar results can be found in the liver, where peroxisome proliferator-activated receptor activity, a critical regulator of metabolic function, is significantly lower in female mice compared to male mice [3]. Finally, one of the most apparent metabolic differences that exist between men and women is the distinction in anatomical fat storage, with men often storing more lipid in the visceral region compared to women [4]. There are multiple more examples that could be provided to address the unique sex differences, with all of them contributing to defining the risk for developing metabolic based chronic health conditions.

The Ovary Is Critical for Defining the Metabolic Phenotype of Women

Most cellular mechanisms are initially defined in males because investigators often wish to avoid the hormonal influence of the menstrual cycle in humans or the estrous cycle in animal models. However, the cyclic release of hormones from the ovary in women plays a critical role in defining the cellular phenotype of a variety of insulin-sensitive peripheral tissues. Thus, to truly understand the importance of the mechanism one must consider it across both sexes. An enormous amount of evidence supports the importance of the ovary to overall physiological function of women. A majority of this evidence was collected using female animals in which the ovaries had been surgically removed (i.e., ovariectomy (OVX)). Using OVX animals is important for women's health research because the same surgical approach is often used as a clinical approach in women to treat and/or prevent a number of chronic clinical conditions. The OVX mouse model is often criticized by investigators because of the vast number of physiological and metabolic changes that develop in the animal making it often difficult to define mechanisms that are altered as a primary consequence of ovary removal. However, we would argue that this also points to the critical importance of ovary function to women and thus this model should not be discounted.

Loss of Ovarian Function Increases the Mortality of Women Independent of Age

As women age it is likely that they will experience a disruption in their ovarian-signaling axis at some point in their life-span. For example, reductions in ovary function are most commonly associated with the onset of age-induced menopause. However, it is often overlooked that numerous premenopausal women elect to undergo oophorectomy as an approach to treat or prevent chronic conditions such as breast cancer and chronic migraines [5, 6]. In addition, pharmacological or environmental disruption of the estrogen receptor can also result in premature ovarian failure [7]. Finally, for reasons that are often unknown a small percentage of young women develop primary ovarian insufficiency leading to abnormally low levels of circulating estrogens [8]. Regardless, a consistent outcome that arises with the loss of ovarian function is an increase in overall mortality irrespective of the age of the woman. The epidemiological data clearly point to the complexity of the biological impact of the ovary on women's health and the importance of understanding the consequences of disrupting ovarian function in women.

Ovariectomy Results in Reduced Physical Activity and Visceral Adiposity

In women, loss of ovarian function results in significant increases in fat mass in the visceral region; however this change does not always equate to increases in overall body weight [4]. Similar results are seen in surgical oophorectomy or OVX in animal models, with the most commonly studied being the mouse or the rat model. From a metabolic perspective, our lab has gone to great lengths to characterize the OVX mouse model [9, 10]. Specifically, we have shown that the OVX mouse exhibits significant increases in visceral adipose tissue mass (~150–250 %) (Fig. 4.1). This increase in fat mass is the result of significant increases in adipocyte size without a change in adipocyte number ([11]; data not shown). It has been suggested that the increase in adiposity is the result of hyperphagia; however this statement appears to only be true in the rat model [12]. Our data suggest that removal of the ovaries in mice results in a slight reduction in daily food consumption and a significant increase in feed efficiency (Table 4.1). The increase in feed efficiency is apparent when calculating for body weight or visceral fat mass; thus the data suggest that OVX mouse is more efficient at storing calories consumed when compared to the sham control mouse (Table 4.1) which is in agreement with the work from other labs [13]. The most pronounced behavioral change seen in the OVX mouse is lower levels of physical activity when compared to the sham mouse (Fig. 4.2). Interestingly, when we provided the OVX mouse access to a running wheel shortly after the surgery we found that even though the OVX mice ran significantly less than the sham mice the increased activity attenuated the increase in visceral fat mass [9, 10].

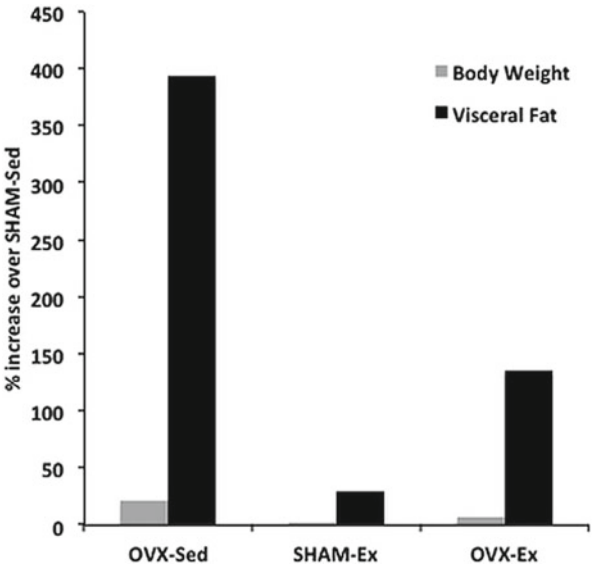

Fig. 4.1 Body weight and visceral adipose tissue mass are greater in sedentary OVX mice compared to age-matched sham mice. Increasing the cage activity of the OVX mice through access to a voluntary running wheel attenuated gains in visceral fat mass and resulted in minimal change in body weight. Data are expressed as percent change compared to age-matched sedentary sham group

Table 4.1 Food consumption in sham and OVX animals

	Food consumption (g/day)	Feed efficiency (BW/g*kcal)	Feed efficiency (VF/g*kcal)
Sham-Sed	5.21 ± 0.29	1.14 ± 0.007	5.34 ± 0.12
OVX-Sed	4.38 ± 0.06*	1.45 ± 0.007*	4.22 ± 0.06*
Sham-Exer	5.12 ± 0.06	1.14 ± 0.005	5.11 ± 0.11
OVX-Exer	4.37 ± 0.06*	1.45 ± 0.005*	4.14 ± 0.06*

*Different than sham $P < 0.05$

However, the OVX mice with access to the running wheel still gained significantly more fat mass compared to the sham-sedentary (Sed) group (Fig. 4.1) while retaining a higher feed efficiency (Table 4.1). Collectively, these data suggest that visceral adiposity in the OVX mouse is the result of an increased ability to store calories coupled with a reduction in physical activity levels. In addition, the model exhibits more similarities to humans experiencing loss of ovary function since clinically postmenopausal women do not consume excess calories, but are typically less active [14, 15]. An increase in visceral adiposity is significantly correlated with the development of metabolic disease and thus strongly associated with increased mortality. The anatomical location of white adipose tissue is a critical consideration, in that excess lipid storage in the visceral region results in more severe consequences on glucose disposal compared to the accumulation of lipid in subcutaneous depots. Based on the changes observed in visceral fat mass in women it is clear that ovarian hormones play a critical role in the regulation of lipid storage in females.

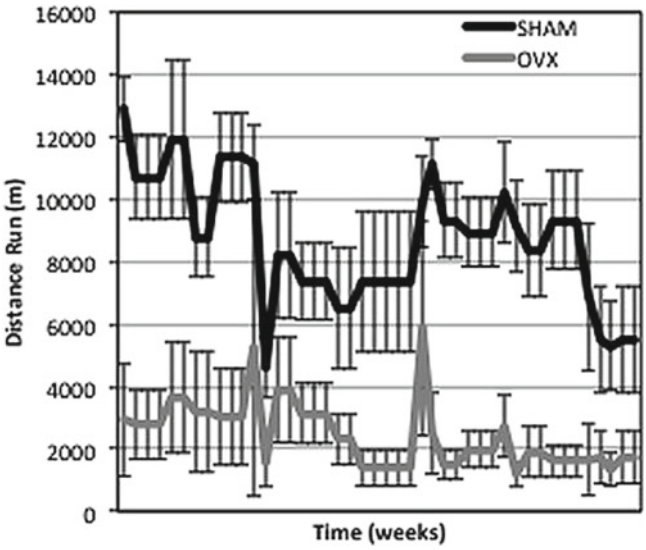

Fig. 4.2 Voluntary running distances of female OVX mice are lower than age-matched sham mice. Each point corresponds to distance run over 24 h

Development of Visceral Adiposity After Ovarian Ablation Leads to Lipolytic Dysfunction in Adipocytes and Fatty Acid Overflow

Under conditions of nutrient excess, free fatty acids (FFAs) are effectively removed from circulation and stored as triacylglycerol (TAG) in white adipose tissue. In contrast under conditions of energetic demand (i.e., exercise or starvation), FFAs are liberated from the stored TAG using a highly regulated enzymatic process termed "lipolysis." Lipolysis is a sequential sequence of enzymatic reactions that result in the complete catabolic breakdown of stored TAG leading to the release of FFAs into circulation. However, in certain disease states, mechanisms that regulate anabolic storage or catabolic breakdown of TAG are disrupted, and these alterations appear to significantly contribute to the onset of metabolic disease. In our hands, OVX animals exhibit significant elevations in circulating FFA, which appears to be a result of increases in basal lipolysis in the visceral adipose tissue [9, 10]. The in vivo results are confirmed by measures of enhanced lipolytic activation in visceral adipose tissue from OVX animals compared to sham using adipose tissue organ bath setups and isolated adipocyte measures [10, 16]. Coupled with dysregulation of basal lipolysis is a poor induction of the lipolytic response in OVX animals [10, 16, 17]. It is clear that derangements in lipolytic function are a characteristic of visceral adipose tissue in OVX mice, with the alterations resulting in a moderate increase in circulating FFA and a poor ability to mobilize FFA under conditions of metabolic stress.

We have previously reviewed the mechanisms that appear to contribute to altered regulation of lipolytic function in the OVX animals [11, 18]. Briefly, our data suggest

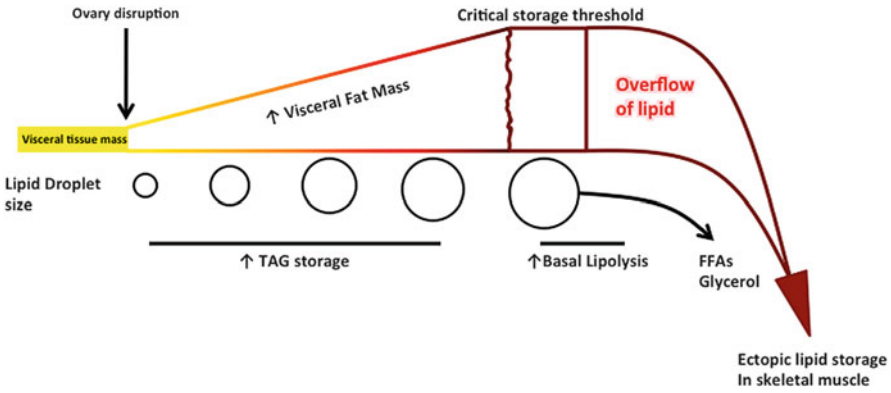

Fig. 4.3 Disruption of ovarian function leads to development of the "overflow hypothesis" in visceral adipocytes

that OVX animals exhibit a significant increase in visceral adipose tissue glycerol lipase (ATGL) protein content compared to the sham animals [10, 17]. ATGL is the rate-limiting lipase in the catabolic breakdown of stored TAG, which likely explains the increased FFA release from adipose tissue in the OVX animals. The alteration in lipase content was coupled with a decrease in perilipin (PLIN1) and an upregulation of adipose differentiation-related protein (PLIN2) content in the OVX mice compared to the sham mice. PLIN1 is a lipid droplet (LD) coating protein that regulates lipolytic rate by preventing unstimulated lipolysis and by facilitating activated lipolysis, while PLIN2, even though in the same protein family, does not adequately protect the LD from unstimulated lipolytic attack nor does it encourage stimulated lipolysis [19]. These alterations in LD coating protein content are likely critical in explaining alterations in adipocyte function under conditions of reduced ovarian function. With the loss of ovarian function in the female mice, there are dynamic changes in critical signaling proteins that result in dysregulation of lipolytic control contributing to significant increases in circulating lipid under conditions of nutrient excess.

The ability of the adipocyte to continually expand to accommodate more lipid storage is limited; thus under conditions of constant excess nutrient consumption the rate of TAG storage will decline [20]. The inability to continue to sequester more lipid in the white adipose tissue is termed the "overflow hypothesis" (Fig. 4.3) [21]. This hypothesis suggests that increased fatty acid levels in circulation lead to enhanced ectopic fat storage in other tissues such as skeletal muscle and/or the liver. An inability to effectively store lipid, due to alterations in the regulatory control of TAG dynamics, increases the risk of developing various forms of lipotoxicity in peripheral tissue. Lipotoxicity is defined as the excessive accumulation of intracellular lipid in peripheral tissue that contributes to the development of cellular dysfunction. Lipotoxicity is most commonly linked to the development of peripheral insulin resistance. Thus, under conditions of ovarian dysfunction there are unique cellular alterations that develop in white adipose tissue that would be a critical contributor to the development of increased risk for type 2 diabetes.

Skeletal Muscle Is a Primary Storage Depot for Excess Circulating Lipid Under Conditions of Reduced Ovarian Function

Skeletal muscle is known to act as a storage depot for lipid in the form of intramuscular triglycerides (IMTG). In 1999, Krssak et al. demonstrated a relationship between excessive accumulation of IMTG and the onset of insulin resistance in the muscle [22]. Investigations have attempted to pinpoint if the accumulation of lipid within the muscle cell plays a causative role in the development of insulin resistance; unfortunately the overall results remain equivocal. Although the exact mechanism has yet to be identified, current dogma suggests that elevation in stored lipid results in increased lipid-based molecules (i.e., ceramides) that are the likely contributors to the development of insulin resistance in skeletal muscle [23]. Using the OVX model, we have found across different skeletal muscle groups (i.e., plantaris, soleus, tibialis anterior, flexor digitorum brevis) an accumulation of a significant amount of intracellular IMTG compared to the sham group (Fig. 4.4). As in other models, this elevated amount

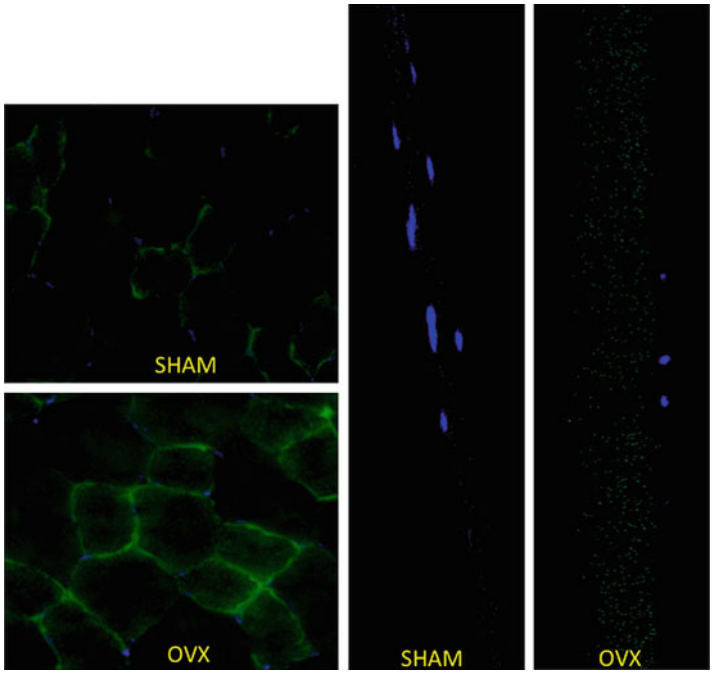

Fig. 4.4 Accumulation of intramuscular TAG in skeletal muscle fibers in OVX animals compared to age-matched sham animals. On the *left* are plantaris muscle cut in cross section and the *right* are single muscle fibers isolated from the flexor digitorum brevis. Lipid droplets are imaged using BODIPY (*green*) and nuclei (*blue*) as previously described [43]

of IMTG is associated with the development of glucose intolerance in the OVX mouse. The increase in IMTG in the OVX model is associated with increased protein content of the primary fatty acid transporters, CD36/FAT and FABPpm, in the skeletal muscle [24]. An increase in the protein content of these transporters would contribute to the elevated IMTG storage in the OVX model. As pointed out above, the OVX model results in a loss of regulatory control of lipolytic action in the visceral adipose tissue contributing to an increase in circulating FFAs. Based on the portal vein hypothesis, it would be expected that the triglyceride accumulation would develop in the liver potentially resulting in hepatic steatosis [25]; however our results did not entirely support this hypothesis [9]. A similar result has been found by another lab using the OVX mouse model [13]; however the development of hepatic steatosis is often found in the OVX rat model that may be a result of the transient hyperphagia that occurs in the rat [26, 27]. Collectively, these data suggest that the primary target of the increased circulating lipid in the OVX mouse model is the skeletal muscle and not the hepatic tissue. Thus, the development of overall peripheral insulin resistance in the OVX model is likely the result of a metabolic defect that is mediated in the skeletal muscle.

Uncovering the Metabolic Mechanisms That Contribute to Increased IMTG Content and Onset of Insulin Resistance in Skeletal Muscle Under Conditions of Ovarian Dysfunction Remain Elusive

An often-implicated target for accumulation of IMTG is the mitochondria. Arguments have been made that the development of dysfunction within the mitochondria contributes to a reduced ability to utilize lipid, thus encouraging fatty acids to enter esterification and consequently storage [28]. However, many have argued that for this to happen one must ignore the energetic state of the cell; thus it is hard to reconcile that an inability to oxidize lipid is the primary or only deficit that is responsible for the development of insulin resistance in skeletal muscle [29]. In our hands, we have found that mitochondria in single skeletal muscle fibers from OVX animals consume pyruvate and palmitate with equal efficacy as muscle fibers from the sham animals [24]. The only detectable deficiency in oxygen consumption by the muscle fibers from OVX animals was under basal conditions or in a state of maximal uncoupling. These results are difficult to extrapolate to a physiological state since neither state exists within tissue of the animal. For example, the uncoupled state represents an artificial condition used to maximally challenge or stress the mitochondria and it is rare to find mitochondria operating in this condition [30]. In addition, we have found little evidence of loss of mitochondrial density within skeletal muscle from OVX animals [24]. At this point, it is unclear if a mitochondrial pathology develops in the OVX animal that would contribute to the resulting metabolic dysfunction seen in the animal.

As a means to identify a possible mechanism, we undertook an unbiased metabolic profiling approach to identify potential metabolic limitations within the skeletal muscle from OVX mice. We found a significant reduction in the long-chain acylcarnitines and short-chain acylcarnitines [24]. These data suggest a reduced flux of long-chain fatty acids through β-oxidation or a reduced transport of long-chain fatty acids into the mitochondria. With respect to the former, we have found no differences in the protein content of VLCAD, LCAD, and MCAD in skeletal muscle of OVX mice, which would suggest that enzymatic capacity of β-oxidation is not the limiting factor. However it should be noted that other labs have found these same enzymes to be estrogen sensitive [2]. With respect to the latter suggestion it is possible that the results suggest an inability to transport long-chain fatty acids into the mitochondria. Indeed, Campbell et al. found a reduction in carnitine palmitoyltransferase I (CPT-1) activity in skeletal muscle from OVX rats compared to sham rats [31]. CPT-1 is the primary transporter of long-chain fatty acids into the mitochondria. These results suggest that the ovary influences lipid entry dynamics into the mitochondria through some undefined influence on CPT-1 or possibly some aspect of β-oxidation. Collectively, reductions in ovarian function result in increased lipid accumulation in skeletal muscle due to poor lipid entry into the mitochondria, which is a likely contributor to the development of peripheral insulin resistance.

Evidence Suggests That Estrogens Are Likely the Key Ovarian Hormone That Influences the Overall Metabolic Phenotype

Under conditions of reduced ovarian hormone function the subsequent increase in visceral fat mass in women increases their risk for developing metabolic and cardiovascular diseases. Although the ovary secretes a number of different hormones current evidence would suggest that it is a form of estrogen, likely 17β-estradiol, which predominantly mediates these metabolic effects. For example, the use of estrogen therapy (ET) in women can attenuate increases in visceral fat mass observed in women following the menopausal transition [32]. In a similar fashion, chronic 17β-estradiol treatment of animal models after ovariectomy attenuates the development of visceral adiposity [10], but does not always completely abrogate all of the physiological alterations from developing. All of these "rescue" approaches suggest that 17β-estradiol is the necessary factor missing after ovariectomy; it should be noted that the redelivery approaches are not without limitations. Specifically, the doses of 17β-estradiol used are often supra-physiological and the manner in which they are delivered results in a sustained exposure of the dose rather than a cyclic exposure that is seen during the estrous cycle. Thus, it is possible that these redelivery approaches have just overwhelmed the system resulting in a pharmacological effect rather than a physiological effect. In addition, a number of these metabolic changes seen in the OVX model also develop in a similar fashion across genetically

manipulated animal models. For example, genetic ablation of the estrogen receptor alpha or the aromatase enzyme results in adult-onset adiposity accompanied by the development of glucose intolerance [33, 34]. Interestingly, when comparing these genetic manipulated models a sex difference is often visualized with the male mice often exhibiting more severe metabolic dysfunction than the female mice [33], which might suggest that other female-specific hormones remain protective. Another critical point that should be considered in examining these data is that these mutant mice were born and developed in an environment of disrupted estrogen signaling; thus they never experience any form of estrogen exposure. Although this is a useful experimental approach it does not mimic clinical conditions found in humans; it is rare that a human would never experience some influence of estrogen exposure. In addition, due to the use of the global knockout approach the effects are not tissue specific; thus it is challenging to determine primary versus secondary effects, which is a problem that faces investigators using the OVX model. To overcome these limitations, investigators will need to develop tissue-specific knockout approaches to identify the true physiological role of the estrogen receptor in each tissue. Overall, the data suggest that the disruption of the estrogen signaling axis is likely the critical component to the development of these metabolic conditions; however it would be naïve not to consider the possibility that other ovarian derived hormones are important to peripheral tissue function as well.

Disruption of Ovarian Function in Women Is a Broader Health Implication and Deserves More Consideration Than Just Under Conditions of Age-Induced Menopause

With the knowledge that ovarian or estrogen signaling is likely key to defining the overall metabolic phenotype of women, it is possible to consider a bigger picture for women's health. Specifically, one would hypothesize that any intervention or environmental exposure that disrupts estrogen function would lead to visceral adiposity and/or metabolic disease. Recent evidence in both animal models and humans is beginning to confirm this hypothesis. For example, women being treated for estrogen-positive cancers with estrogen receptor antagonists are at increased risk for developing hepatic steatosis and alterations in glucose handling [35]. Female mice exposed to Bisphenol A, an environmental chemical found in plastics, results in inhibition of estrogen receptor function and increased risk for metabolic dysfunction [36]. These considerations are critical because not all research questions that are specific to women's health are focused on preventing alterations as a result of age-induced menopause. There are a number of reasons beyond age that contribute to loss of estrogen function in women. Overall the data clearly indicate that disruptions to the ovary signaling axis lead to the development of metabolic disease in women, thus delineating the mechanisms induced by ovarian derived hormones (likely estrogens) that define that the metabolic phenotype is critical in improving women's health.

Optimal Ovarian Function Is Necessary to Protect the Women from Lipid-Based Insults

The ovary provides women with an environmental exposure that induces or activates a metabolic phenotype in skeletal muscle that protects women from lipid-based insults (Fig. 4.5). Indeed, previous studies have shown for unknown reasons that females preserve muscle insulin sensitivity better than males when exposed to a high-fat diet [37]. Thus, we are proposing that the ovaries protect skeletal muscle from lipid-based insults (i.e., high-fat diet) through mechanisms that remain poorly defined, thereby preserving insulin sensitivity. Our data demonstrate that under conditions of reduced ovarian function there is an increase in visceral adiposity that mediates the development of adipocyte dysfunction leading to increases in circulating lipid and increased storage of the lipid in skeletal muscle. We have previously proposed a two-hit hypothesis to explain these results [11], in which we propose that the ovary provides a multifactorial means for regulating metabolic function in women. Specifically, the loss of ovarian function leads first to adipocyte dysfunction paralleled by a loss of a metabolic phenotype that would allow peripheral tissue to tolerate increases in lipid exposure (Fig. 4.5). Thus, future studies will be critical in identifying the underlying mechanisms by which the ovary communicates with adipose tissue and other peripheral tissues. The hope would be that these studies could identify the protective mechanisms that are lost in women under conditions of ovarian disruption.

The current impact of the results from the Women's Health Initiative (WHI) provides a more immediate concern of designing approaches that can be used to prevent the development of metabolic disease without the use of estrogen therapy.

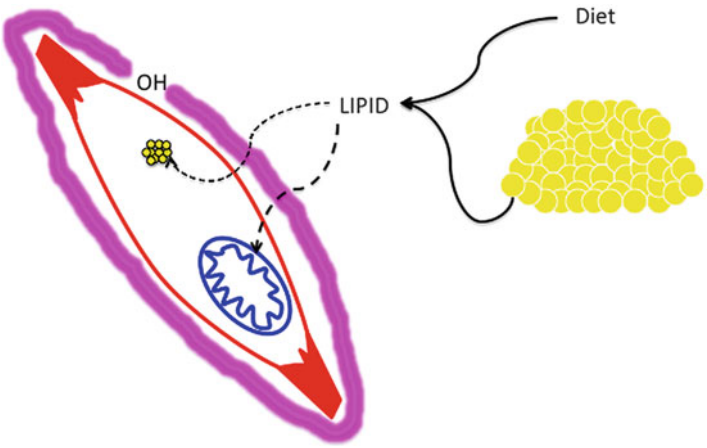

Fig. 4.5 Skeletal muscle in women is exposed to ovarian hormones that activate a gene program that induces a metabolic phenotype that is protective against lipid-mediated insults

In years past it was possible to preserve the cellular phenotype by prescribing ET; however evidence released from the WHI has discouraged this approach in practicing clinicians (http://www.nhlbi.nih.gov/whi/) due to increased risk of breast cancer and/or stroke. It should be noted that the initial results from the WHI were met with substantial vocal and written criticism that argued that we are discounting the use of ET in women too early [38]. Regardless, a clear impact of the WHI was a significant and measureable loss of prescribed ET by clinicians [39, 40]. Thus, what does a woman do when faced with a condition that results in reduced ovarian function? Interestingly, although often overlooked evidence indicates that exercise training prevents, attenuates, or even reverses most of these conditions suggesting that exercise should be considered a potential substitute for estrogen therapy [41, 42]. Moreover, the side effects of estrogen therapy as found by the WHI (i.e., increased risk of stroke, myocardial infarction, breast cancer) are not induced by exercise and in fact exercise training often prevents these conditions. Thus, we are suggesting that exercise training may act as an "estrogen-mimetic" acting to preserve the protective phenotype induced by the estrogen exposure. More studies will be necessary to confirm this hypothesis; however for women who are experiencing alterations in ovarian function implementing regular physical activity seems to be the most accessible alternative intervention. With respect to women's health, we are at crossroads where it is critical to gain a better understanding of the influence of the ovary on all peripheral tissues so that we can develop and implement usable interventions that complement the clinician's toolbox.

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Chapter 5
The Role of Estrogens in the Regulation of Peripheral Glucose Dynamics

Paige C. Geiger and Anisha A. Gupte

Abstract While estrogen is an important hormone regulating sexual function and reproduction, it also has numerous nonreproductive functions, including the regulation of glucose homeostasis. Clinical studies indicate that postmenopausal women are at greater risk for the development of type 2 diabetes, and new evidence in the literature supports a direct role for estrogen in regulating glucose metabolism. The primary estrogen receptors, ERα and ERβ, are now thought to play critical roles in the regulation of glucose in insulin-sensitive tissues. Increased adiposity occurs in humans and mice as a result of decreased ERα activation, and mice with global knockout of ERα exhibit impaired glucose tolerance and skeletal muscle insulin resistance. Based on this evidence, the beneficial effects of estrogens on glucose metabolism are thought to be mediated by ERα. ERα may regulate glucose by increasing insulin-signaling and insulin-stimulated glucose uptake, maintaining GLUT4 expression, and mitigating inflammation and oxidative stress implicated in the development of insulin resistance. ERα and ERβ are known to demonstrate a complex inter-regulatory relationship that varies with the target tissue. Both stimulated by estrogen, the ability of ERα or ERβ to regulate glucose may be dependent on the expression levels and activation of the two receptors in various metabolic tissues. ER isoform-specific agonists and antagonists may prove more beneficial in preventing insulin resistance in postmenopausal women than estrogen treatment alone and should be the focus of future research.

Keywords Estrogen receptors • ERα • ERβ • Insulin • Muscle • Adipose • Liver

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Estrogen deficiency is thought to play a critical role in the increased prevalence of metabolic dysfunction and the metabolic syndrome (dyslipidemia, hypertension, central obesity, and insulin resistance) in postmenopausal women (Executive Summary of the Third Report of The National Cholesterol Education Program (NCEP)) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III) [1]. Postmenopausal women experience a 60 % increase in risk of the metabolic syndrome, even after adjustments for age, body mass index, household income, and levels of physical inactivity [2]. In addition, women with metabolic syndrome are at increased risk for cardiovascular disease, the primary cause of death in women from westernized countries. While estrogen is an important hormone regulating sexual function and reproduction, it also has numerous nonreproductive functions, including the regulation of glucose homeostasis.

Glucose Homeostasis

Glucose is the body's primary source of fuel and is regulated by the hormone insulin. In response to postprandial glucose levels, β cells in the pancreas secrete insulin to stimulate glucose uptake into skeletal muscle and adipose tissue. At the same time, glycogen is formed in the liver through glycogenesis. Insulin functions by binding to its receptor at the cell surface membrane of skeletal muscle or adipose tissue, initiating a signaling cascade that ultimately results in translocation of the glucose transporter, GLUT4, to the cell membrane for glucose entry.

Without proper maintenance of glucose homeostasis, insulin resistance and type 2 diabetes can develop. Type 2 diabetes is characterized by high blood glucose levels in conjunction with insulin resistance and insulin deficiency. Insulin resistance, as previously mentioned, occurs when the body no longer responds to insulin signaling and results in decreased glucose uptake into skeletal muscle and adipose tissue, as well as a failure to suppress glucose production and release into the blood by the liver.

Clinical Evidence for the Role of Estrogen in Glucose Regulation

The risk for type 2 diabetes is greater in postmenopausal women, with postmenopausal women having higher fasting blood glucose and insulin levels compared to age- and body mass index-matched premenopausal women [3, 4]. In addition, postmenopausal women exhibit decreased insulin sensitivity and glucose tolerance compared to premenopausal women [3]. Compared with age-matched men, premenopausal women have increased insulin sensitivity and a lower prevalence of insulin resistance [5, 6]. These differences in insulin action between premenopausal women and age-matched men are at least partially due to higher levels of GLUT4 in women [7, 8].

A primary difficulty in determining the influence of estrogen on glucose regulation lies in differentiating the effects of menopause from normal aging in women or from differences in fat storage between men and premenopausal women. For example, menopause brings about changes in fat storage in women such that total body fat and abdominal fat increase [9–11]. Obese postmenopausal women with greater amounts of visceral adipose tissue are more likely to have decreased insulin sensitivity [12, 13]. Since abdominal fat is the number one risk factor for developing type 2 diabetes [14–16], this increase in abdominal fat could directly alter diabetes risk in postmenopausal women. Similarly, difference in fat storage between premenopausal women and men could account for differences in insulin sensitivity and insulin resistance between the sexes. While men tend to store fat in the abdominal area, premenopausal women tend to store fat in the gluteofemoral area [17–19]. Although other factors may be involved, women with predominately upper body obesity are more likely to have impaired glucose tolerance than women with predominately lower body obesity [20]. However, a study comparing non-obese pre- and postmenopausal women demonstrated that the increase in abdominal fat after menopause did not result in increased fasting insulin and glucose levels, or a lower glucose infusion rate during euglycemic-hyperinsulinemic clamp [10]. Increasingly, new evidence in the literature supports a direct role for estrogen in regulating glucose metabolism, independent of body fat distribution differences between sexes or with aging.

Cell culture studies have shown that estrogen treatment induces GLUT4 translocation to the membrane and increases insulin-stimulated glucose uptake [21], suggesting that estrogen has direct effects on glucose metabolism on muscle independent of adiposity. Estrogen and its receptors are important regulators of glucose metabolism and body weight not only in women but also in men. Previous studies have shown that men unable to synthesize estrogen display altered glucose metabolism and insulin resistance [22]. Men with mutations in the aromatase gene, and as a result cannot produce estrogen, or with mutations in ERα gene present with insulin resistance [23, 24]. It is believed that although plasma estrogen levels in men are lower compared to women, local concentrations at relevant sites of action such as the skeletal muscle may be of crucial importance in glucose homeostasis [25].

Clinical studies utilizing estrogen replacement in postmenopausal women indicate an important role for estrogen in glucose regulation. The Heart and Estrogen/Progestin Replacement Study (HERS) and the Women's Health Initiative Hormone Trial (WHI) were two large-scale, national studies with over 2,500 and 16,000 subjects, respectively, which assessed the overall potential benefits and risks of Hormone Replacement Therapy (HRT), including risk factors for type 2 diabetes. The HERS found that the incidence of insulin resistance in healthy, postmenopausal women on HRT for 1 year was 35 % less compared to postmenopausal women not on HRT [26]. The WHI demonstrates that fasting glucose and insulin levels also decreased in postmenopausal women after 1 year of HRT [27]. In addition, a double blind study by Andersson et al. [28] found that estrogen replacement for 3 months in postmenopausal women with type 2 diabetes improved their glucose homeostasis, as measured by euglycemic-hyperinsulinemic clamp, versus women with type 2 diabetes taking a placebo. A recent study by Gower et al. [29] randomized early

postmenopausal women to HRT or placebo for 2 years. This study assessed the independent effect of menopause on insulin sensitivity and found that HRT, even in the presence of increased abdominal fat, increased insulin sensitivity at the end of the 2-year study. Although the majority of HRT studies have used a combination of estrogen and progestin, estrogen alone is thought to confer the greatest effect on glucose regulation [3, 30–34].

While estrogen replacement benefits postmenopausal women in terms of glucose regulation, the overall risks may outweigh the benefits. The WHI sought to determine the overall benefits and risks of HRT in postmenopausal women to serve as a guideline for clinical practice. The trial ended early due to the increased risk of severe health complications including breast cancer, thrombosis, and coronary heart disease in the HRT-treated group [35]. Study investigators concluded that the overall risks of HRT exceeded the benefits. While estrogen has profound benefits on glucose metabolism, alternative approaches for the prevention of insulin resistance and type 2 diabetes based on targeted actions of estrogen are needed.

A major criticism to the HRT trials was that the study population was older than would normally be considered for initiating hormone replacement and the study did not tailor the hormones to the individual women. Cardiometabolic risk factors accumulate over years before clinical presentation. It was likely that menopause-related weight gain, and loss in estrogen protection in glucose metabolism and cardiovascular disease would be well advanced before HRT had been initiated in the trials. As a follow-up, the Kronos Early Prevention Study (KEEPS) has been initiated to study HRT in women aged 42–58 years who are within 36 months of their final menstrual period [36]. Early results from this trial confirm that early HRT to newly menopausal women protects from metabolic syndrome, including maintenance of insulin sensitivity and lowered cardiovascular risk factors [37].

Data from Animal Models Support Clinical Findings of Estrogen's Role in Glucose Metabolism

The use of rodent models has greatly contributed to the knowledge of cellular and molecular effects of estrogen on glucose metabolism. Ovariectomy (OVX) in rodents involves bi-lateral removal of the ovaries and models the postmenopausal state in humans. OVX in rodents results in total body weight gain [38–41], total body fat gain [40], and a high fat diet combined with OVX further increases weight gain in female rodents [38, 40, 41]. The decreased susceptibility to type 2 diabetes that females demonstrate over males also diminishes after rats undergo OVX [39] or become acyclic [42].

OVX has also been shown to impair insulin sensitivity and glucose metabolism in animal models [38, 43, 44]. Ten weeks of OVX in mice resulted in glucose intolerance [38]. Kumagai et al. [44] also found that 6 months of OVX in rats resulted in whole body insulin resistance and decreased glucose uptake into skeletal muscle. Estrogen replacement alone, or in combination with progesterone, ameliorated

the insulin resistance, but progesterone alone had no effect [44]. These studies demonstrate that OVX in rodents results in a phenotype similar to that seen in postmenopausal women and can serve as a model to study the cellular and molecular effects of estrogen on glucose metabolism.

Estrogen Receptors

ERα and ERβ are the primary estrogen receptors and are products of two distinct genes [45, 46]. Tissues such as the uterus, liver, kidney, and heart primarily express ERα, while the ovary, prostate, lung, gastrointestinal tract, bladder, and hematopoietic and central nervous systems express primarily ERβ [47, 48]. The ERs are members of the nuclear receptor superfamily [49] which includes the classical steroid hormones, orphan receptors, and adopted orphan receptors (reviewed in [50]). ERα and ERβ contain highly conserved DNA binding domains and as a result, both bind with similar affinity and specificity to estrogen response elements. Despite this similarity, ERα and ERβ have diverse physiological effects in multiple tissues depending on receptor level, and presence of various ligands, coactivators, and corepressors.

The ERs initiate cellular functions through both genomic and non-genomic mechanisms (Fig. 5.1). The ERs typically reside in the nucleus, although current literature indicates ERα is also present in the cytoplasm (reviewed in [51]). Following activation of the ligand-binding domain and receptor dimerization, the ERs bind to estrogen response elements on DNA to modulate gene transcription. The non-genomic mechanisms of ER action, thought to be important in the role of estrogen in nonreproductive tissues, occur following ligand binding to the ERs residing in the cytoplasm or at the membrane and can result in increased levels of nitric oxide and calcium, and activation of various signaling cascades and kinase activity [51–53].

The ERs also play a role in glucose metabolism, with ERα and ERβ knockout (KO) mice providing much of the initial understanding of ER regulation of glucose control. Increased adiposity occurs in humans and mice as a result of decreased ERα activation [23, 54], and mice with global knockout of ERα exhibit impaired glucose tolerance and skeletal muscle insulin resistance [54–56]. Based on this evidence, the beneficial effects of estrogens on glucose metabolism are thought to be mediated by ERα.

Mechanisms of ER-Mediated Glucose Metabolism

While activation of the estrogen receptors has the potential to positively modulate glucose metabolism, the exact mechanism of action is unknown. Some studies suggest a mechanism by which estrogen receptors modulate GLUT4, the critical glucose transporter in skeletal muscle. NF-κB is a transcription factor that is activated

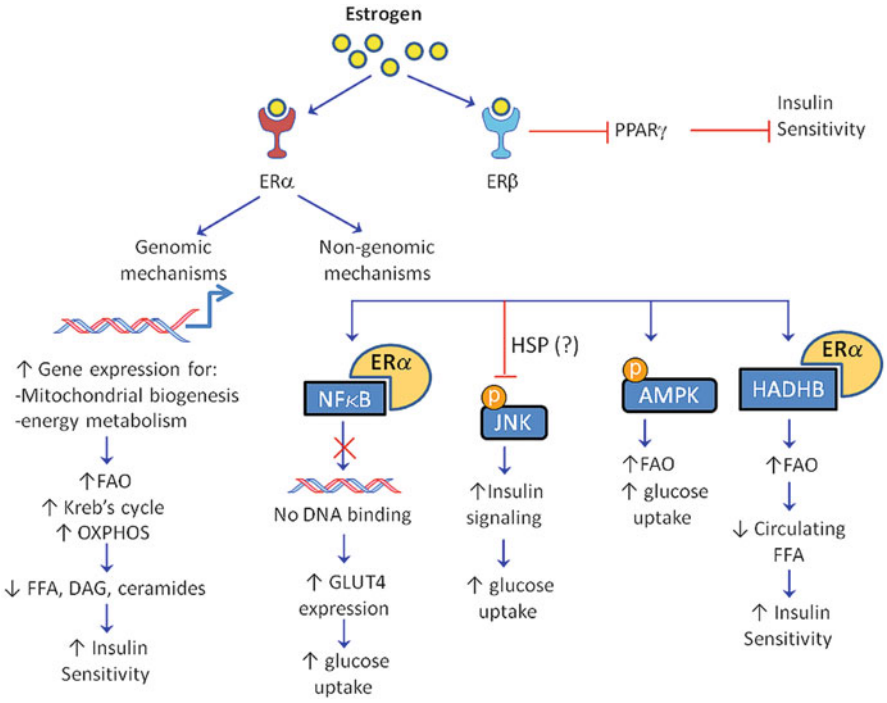

Fig. 5.1 Pathways indicating the integrative function of estrogen in regulating glucose uptake. Estrogen mediates glucose homeostasis via its two receptors ERα and ERβ. ERα, the predominant isoform in muscle, adipose, and liver tissues has genomic and non-genomic mechanisms regulating insulin action and glucose metabolism. *ER* estrogen receptor, *p* phosphorylated, *FAO* fatty acid oxidation, *OXPHOS* oxidative phosphorylation, *FFA* free fatty acids, *AMPK* AMP-dependent kinase, *NFkB* nuclear factor kappa B, *HSP* heat shock proteins

by stimuli such as cellular stress, cytokines, and inflammation. The promoter region of GLUT4 contains an NF-κB binding site [57], and NF-κB represses GLUT4 transcription [58]. In a basal state, NF-κB is bound by the inhibitor of kappa B α (IκBα) in the cytosol and remains inactive. Upon activation of the stress kinase proteins, IκBα is phosphorylated, which signals its degradation by the proteosome. The free NF-κB is then activated and translocates to the nucleus where it functions as a transcription factor. Tumor necrosis factor-α (TNF-α) is a cytokine that activates the NF-κB pathway and is highly expressed in obese humans [59, 60]. In addition, obese humans with T2DM have increased skeletal muscle NF-κB activation [61]. Previous studies have also shown that rats on an obesity-promoting high-fat diet have decreased skeletal muscle GLUT4 protein [62–64]. Overall, these studies suggest that obesity increases the amount of TNF-α and leads to NF-κB activation, which is followed by a decrease in GLUT4 protein levels.

New evidence suggests NF-κB, and Glut4 consequentially, is regulated by ERα. Rather than binding directly to DNA, ERα can also modulate gene expression by

binding transcription factors [65–67]. Previous studies have shown that activated ERα can directly bind to NF-κB and decrease NF-κB–DNA binding [65, 68–70]. Activated ERα could essentially serve a protective function in regards to GLUT4 expression. The opposite would also be true, that low levels of activated ERα may result in decreased GLUT4 levels. In fact, females with polycystic ovarian syndrome who have high androgen and low estrogen levels (and, therefore, low ERα activation) have 35 % less GLUT4 protein compared to control females [71]. In addition, ERα KO mice show a decrease in GLUT4 mRNA levels [72]. Therefore, increased NF-κB activation via a high-fat diet and obesity combined with low ERα activation could decrease GLUT4 transcription, leading to a subsequent decrease in glucose uptake and insulin resistance. This physiological condition (obesity and low E_2 levels) is present in most postmenopausal women, putting these women at a particular risk for insulin resistance.

Another postulated mechanism for estrogen-related protection of glucose homeostasis is the mitigation of inflammation and oxidative stress that are implicated in causing insulin resistance. ERα deficient mice have high levels of Plasminogen activator inhibitor-1 (PAI-1) and TNF-α, markers of inflammation, and reduced adiponectin, a suppressor of inflammation and inducer of insulin sensitivity [73]. Muscles of ERα knockout mice also exhibit greater proinflammatory lipid breakdown products diacylglycerol and ceramides, along with increased activation of stress kinases like JNK, all of which have been implicated in inducing insulin resistance in skeletal muscle. The mechanism by which ERα mitigates the proinflammatory state remains to be completely understood. One mechanism could include the ability of ERs to regulate fatty acid oxidation and mitochondrial function. Previous studies indicate that ERα can modulate mitochondrial function, including ATP production, mitochondrial membrane potential, and calcium concentrations [74, 75]. ERα directly interacts with mitochondrial fatty acid oxidation enzyme HADHB. 17-β Estradiol increases the activity of HADHB in wild-type cells but not in cells lacking ERα [76]. ERα is thought to have direct positive transcriptional effects on increasing mitochondrial biogenesis [77], and ERα binding sites are enriched in the promoters of genes involved in energy metabolism [78]. Recent studies also show the presence of ERs within the mitochondrial membrane [79–81]. Mitochondrial ERs can bind directly to estrogen receptor elements, suggesting that ERs may be directly involved in estrogen-induction of mtDNA transcription [82]. Therefore, with a coordinated action of nuclear ERs, mitochondrial ERs, and their coactivators, estrogen signaling may regulate mitochondrial function in tissues with high energy demand, like skeletal muscles. Increased mitochondrial oxidative phosphorylation and mitochondrial fatty acid oxidation improves complete fatty acid metabolism, preventing the buildup of proinflammatory intermediates like DAG and ceramides [83]. Reduction in DAG and ceramides improves glucose metabolism by reducing activation of stress kinases that are known to inhibit the insulin-signaling pathways.

Several additional mechanisms have been postulated by which estrogens can regulate glucose metabolism. Estrogens may improve insulin action by mitigating oxidative stress [84] and reducing mitochondrial ROS production [85].

Another mechanism by which ER can regulate glucose metabolism is the activation of PPARγ, a master regulator of insulin/glucose metabolism. ERβ inhibits ligand-mediated PPARγ-transcriptional activity to reduce adipogenesis, while high-fat diet-fed ERβ knockout mice are more insulin sensitive despite having more adiposity [86]. Packaging of fatty acids in adipose depots such that they become incapable of inhibiting insulin signaling in muscle tissue is an important mechanism of PPARγ ligands in maintaining glucose homeostasis. Estrogen may also affect glucose homeostasis by regulating muscle mass, which are the primary glucose disposal centers in the body. In L6 cells, estrogen treatment increases GLUT4 translocation to the membrane, along with expression of myogenesis markers, including myogenin and MHC [21]. Estrogen-dependent myogenesis was prevented by translation inhibitor cycloheximide and transcription inhibitor actinomycin, suggesting that nuclear mechanism of ER action was involved in muscle differentiation. The varied effects of estrogen on glucose metabolism are only now being uncovered and are not yet fully understood.

Estrogen Regulation of Glucose in Insulin-Sensitive Tissues

Glucose regulation in the body mostly occurs in the skeletal muscle, adipose tissue, and liver (Fig. 5.2). ERs are also present in the brain and have profound effects on food intake [87]. ERs are thought to play a critical role in the regulation of glucose metabolism by estrogen in each of these metabolic tissues.

Estrogen Regulation of Glucose Metabolism in Skeletal Muscle

Approximately 75 % of glucose regulation in the body occurs in skeletal muscle [88] and estrogen receptors are highly expressed in skeletal muscle. In mice that do not express ERα, a decrease in whole body glucose tolerance and a decrease in glucose uptake into the skeletal muscle is observed [54, 56, 89]. Even on a normal chow diet, a lack of ERα resulted in impaired glucose tolerance and reduced insulin sensitivity in skeletal muscle and liver [89]. Whether the whole body changes can be attributed to impaired insulin action in skeletal muscle or the liver is not clear [56].

Previous findings from our laboratory demonstrate an increase in insulin-stimulated glucose uptake with PPT, suggesting an important role for ERα in mediating skeletal muscle insulin sensitivity [90]. While ob/ob mice treated with PPT for 7 days demonstrated no increase in glucose uptake in soleus or EDL muscle [91], this could be attributed to the significantly lower dose of PPT used in the latter study. It can be somewhat difficult to parcel out the effects of ERs in skeletal muscle glucose regulation, however, given the fact that estrogen stimulates both ERα and ERβ.

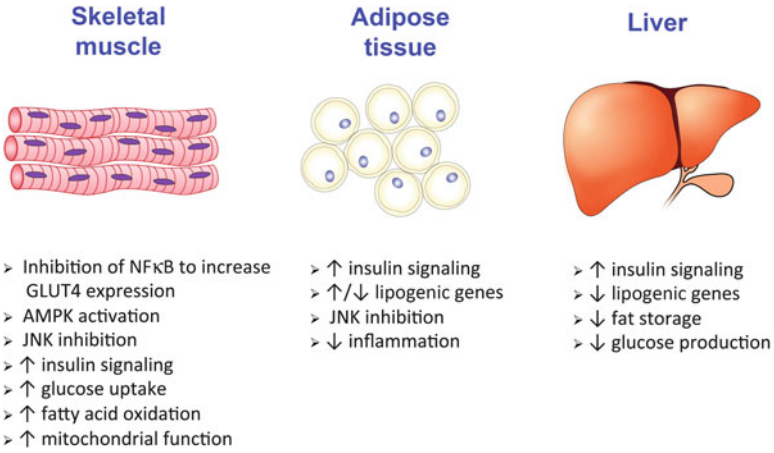

Fig. 5.2 Differential effects of ERα activation on glucose and fatty acid metabolism in skeletal muscle, adipose tissue, and liver. *AMPK* AMP-dependent kinase, *NFkB* nuclear factor kappa B, *JNK* c-Jun N-terminal kinase. Image credit to Stanton Fernald, KIDDRC Illustration &Imaging Center, University of Kansas Medical Center

It is likely that estrogens and estrogen receptor modulators produce distinct phenotypes depending on whether a tissue expresses predominately ERα or ERβ [92]. It has been suggested that ERα is more highly expressed in insulin-sensitive tissues [51, 89, 93, 94]. However, other accounts indicate that ERβ expression predominates in skeletal muscle [95]. ERα and ERβ regulation of function varies with the target tissue and activation of ERβ could oppose the action of ERα in the regulation of glucose metabolism [47, 72, 96]. Distinct tissue-specific effects of estrogen and estrogen receptor modulators occur with the recruitment of different coregulatory proteins to ERs [92, 97]. Differences in coregulatory protein recruitment can result in a modulator expressing agonist or antagonist properties. The coregulatory proteins involved in ERα activation by estrogen and PPT in skeletal muscle have not yet been identified, but could have an important role in determining the regulation of glucose metabolism by estrogen in vivo.

While studies demonstrate that estrogen results in increased insulin signaling, this does not appear to have a functional effect on glucose uptake in vivo. Phosphorylation of Akt [98] and AMPK [99] occurs with estrogen treatment in C2C12 muscle cells, and acute incubations (5 and 10 min) with estrogen increase phosphorylation of Akt, AMPK, and TBC1D1/4 in soleus muscle [100]. However, insulin-stimulated glucose transport is not increased with acute in vivo estrogen incubation. In addition, 3 days of estrogen treatment in vivo demonstrated no effect on insulin-stimulated glucose uptake in soleus or EDL muscles [90]. This is in contrast to an increase in glucose uptake with 3 days of with PPT treatment. The activation pattern of ERs with estrogen in vivo remains to be determined in skeletal muscle.

When ERα is stimulated via PPT, direct activation of the insulin-signaling pathway occurs as shown by increased phosphorylation of Akt [90]. In C2C12 myotubes, stimulation of the insulin-signaling pathway with resveratrol was also shown to be dependent on ERα activation [101]. ERα also has an effect on insulin-independent pathways of glucose regulation with PPT increasing phosphorylation of AMP-dependent kinase (pAMPK) in soleus and EDL muscles [90]. ERα-KO mice demonstrate decreased pAMPK in skeletal muscle [89] and a recent study demonstrates that estrogen-induced AMPK activation is mediated by ERα [100]. These findings suggest that ERα acts as a positive modulator of AMPK activation. AMPK can phosphorylate both AS160 [102, 103] and TBC1D1 [104, 105], although with phospho-specific sites distinct from those activated by Akt, to stimulate an increase in glucose uptake. The ability of ERα to stimulate both insulin-dependent and non-insulin-dependent pathways has important implications for the regulation of glucose uptake in vivo.

ERα may also regulate glucose metabolism through direct modulation of GLUT4. GLUT4 is decreased in the gastrocnemius muscle of male ERα knockout (ERα-KO) mice [72]. However, a more recent report shows no change in GLUT4 levels in the quadriceps or soleus muscles in female ERα-KO mice [89]. It is possible that the ability of ERα to regulate GLUT4 may be fiber type specific as Gorres et al. demonstrated that activation of ERα results in increased GLUT4 in the EDL (fast-twitch) but not in the soleus (slow-twitch) muscle [90]. While numerous transcriptional pathways regulate GLUT4 [106], acute ERα activation may be an additional and lesser known mechanism for modulating GLUT4.

Estrogen Regulation of Glucose Metabolism in Adipose Tissue

Studies show that estrogen treatment in adipocytes increases insulin-stimulated glucose uptake and activation of the insulin-signaling pathway more than insulin alone [107, 108]. Furthermore, Muraki et al. found that the beneficial effects of estrogen were abolished when adipocytes were co-treated with methylpiperidinopyrazole (MPP), a specific ERα inhibitor. The beneficial effects were restored with PPT treatment to activate ERα [107]. These studies suggest that activation of ERα can potentiate the insulin-signaling pathway and glucose uptake in cultured adipocytes.

In adipose tissue, ERα KO mice have increased body weight and white adipose tissue weight compared to WT mice. ERα KO mice also have increased adipocyte size and number, although food intake does not differ [54]. Similarly, aromatase KO mice, in which androgens cannot be converted to E_2, have increased body weight [109] and adipose tissue weight [110] compared to WT mice. In contrast, ERβ KO mice do not have increased adipose tissue weight or percent body fat compared to WT mice [111]. Therefore, estrogen/ERα signaling appears to be an important regulator of body weight and adipocyte regulation. Ovariectomy of ERα KO mice improves insulin resistance after reducing adipocyte size, indicating a role of ERβ in negative regulation of insulin action. The relative ratio of ERα/ERβ expression

seems to be directly associated with obesity as well as serum level of leptin in omental adipose tissue of women [112].

Estrogen has the potential to regulate fat storage and triacylglyceride accumulation by altering transcription of lipogenic proteins such as SREBP-1 and its downstream targets, ACC, and FAS [56, 113–116]. The effects of estrogen on lipogenic pathways have primarily been assessed in response to estrogen treatment or replacement. For example, Phrakonkham et al. [117] demonstrated that estrogen treatment increased FAS expression in cultured adipocytes. However, other studies have demonstrated opposite effects, with estrogen treatment in mice shown to decrease ACC and FAS mRNA in adipose tissue [114, 118]. As has been previously shown, physiological estrogen levels may positively modulate glucose metabolism, while high or low estrogen levels have a different effect [107, 108]. More studies are needed to assess the role of estrogen and ER expression in modulating lipogenic pathways in cycling, OVX, and estrogen- treated animals.

Increased lipid intermediates and oxidative stress in insulin-responsive tissues can result in activation of stress kinases [119–123]. We [119, 120] and others [89, 124] have previously shown that increased stress kinase activation and decreased heat shock protein (HSP) expression contribute to decreased insulin signaling and glucose uptake in skeletal muscle. Further evidence suggests that ERα may be involved in stress kinase activation and HSP expression. Ribas et al. demonstrate increased activation of JNK in skeletal muscle and adipose tissue of ERα knockout mice [89]. When challenged with a high-fat diet, ERα knockout mice display greater JNK activation and decreased HSP72 expression in adipose tissue compared to high-fat fed wild-type mice [89]. These data suggest that ERα may contribute to glucose regulation by positively modulating stress kinase activation and HSP expression.

Estrogen Regulation of Glucose Metabolism in the Liver

With respect to the liver, ERα KO mice show hepatic insulin resistance during the euglycemic-hyperinsulinemic clamp test [56]. While hepatic glucose production decreases in wild-type (WT) mice, insulin is not able to decrease hepatic glucose production in ERα KO mice. Gene analyses of liver tissue from ERα KO mice exhibit downregulation of genes regulating lipid transport and upregulation of genes for hepatic lipid synthesis. Estrogen treatment reduces hyperglycemia, oxidative stress, and ameliorates liver dysfunction in diabetic rats via increased expression and signaling of insulin receptors [125].

ERs have a profound role in lipid metabolism in the liver. In liver, ERα has direct binding sites on promoters of genes involved in lipid and glucose metabolism, including PPARα, PDK4, PCK1 [78]. The anti-diabetic effects of estrogen treatment to diabetic ob/ob mice are associated with reduced lipogenic genes in the liver [126]. On the other hand ERβ KO mice have normal hepatic glucose output and insulin secretion [56], suggesting that ERα likely plays the predominant role in regulation of hepatic glucose homeostasis, possibly due to the much greater expression of ERα in the liver compared to ERβ. But another report showed that ERβ KO fed a high-fat

diet have decreased liver fat and increased insulin sensitivity, possibly due to the reduction in triglyceride accumulation in the liver [86].

Estrogen treatment to ovariectomized mice protects against fatty liver disease when fed a high fat diet [127]. The insulin-resistant liver faces a paradox for insulin action because insulin resistance impairs the ability of insulin to suppress gluconeogenesis but increases insulin's ability to promote lipogenesis. Estrogen treatment selectively inhibits the lipogenic aspect of insulin signaling and yet promotes the glucose suppressing-action of insulin [127], maintaining overall glucose homeostasis in the body in the face of a HFD.

Recent studies indicate that amount of fat accumulation in the liver is a better predictor of complications of obesity than visceral fat including insulin resistance and diabetes. Estrogen and ERα agonists have been associated with reduced fatty liver disease in mouse models [128] and women [129]. Women with fatty liver disease have hyperinsulinemia and insulin resistance, but also have less estrogen levels than women without fatty liver disease, concurrent with the observation that fatty liver disease is more prevalent in postmenopausal and women with polycystic ovarian syndrome [129].

In summary, estrogen mediates its metabolic effects on tissue via its two receptors, ERα and ERβ; and many of these effects are dependent on the expression levels and activation of the two receptors in various tissues. In insulin-sensitive tissues like skeletal muscle, adipose tissue, and liver, ERα is the predominant mediator of estrogen action. Activation of ERα increases energy metabolism pathways, enhances efficient fatty acid oxidation and improves insulin sensitivity. Estrogen's metabolic effects are achieved via both genomic mechanisms, for example increasing expression of genes for mitochondrial biogenesis, and via non-genomic mechanisms, for example prevention of NFκB-mediated GLUT4 inhibition. In skeletal muscle, estrogens increase insulin signaling and glucose uptake, while in the liver, estrogens reduce fat storage and suppress glucose production. In contrast, estrogens may promote fat storage in adipose tissue in order to secure excess fatty acids which could be harmful in the circulation.

Conclusion

Estrogen's regulation of glucose metabolism is a delicately balanced process in the body. While several reports show strong evidence of protective effects of estrogens, other studies clearly indicate that estrogen levels out of the physiological range, either lower or higher, are related to increased insulin resistance [130, 131]. Given the potentially contrasting roles of ERα and ERβ in regulating glucose homeostasis, ER isoform-specific agonists may have more beneficial effects in preventing insulin resistance in postmenopausal women than estrogen treatment alone. Future research and long-term clinical trials are required to verify the therapeutic benefits of ER modulators on glucose metabolism. The rapid increase in obesity and diabetes worldwide warrants extensive investigation of ERs to improve glucose homeostasis.

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Chapter 6
The Impact of Estrogen Receptor α Expression in the Pathogenesis of the Metabolic Syndrome

Andrea L. Hevener and Brian G. Drew

Abstract Considering the trends in life expectancy, women in the modern era are challenged with facing menopausal symptoms including hot flashes, sleep and mood disorders, and sexual dysfunction as well as heightened disease risk associated with increasing adiposity and metabolic dysfunction for up to three decades of life. Treatment strategies to combat menopausal symptoms and associated pathologies have been hampered by our lack of understanding regarding the biological causes of these clinical conditions and our incomplete understanding of the effects of estrogens and the tissue-specific functions and molecular actions of its receptors. In this chapter we provide evidence supporting a critical and protective role for the estrogen receptor α in the maintenance of metabolic homeostasis and insulin sensitivity. Studies identifying the ER-regulated pathways required for disease prevention will lay the important foundation for the rational design of novel and better-targeted therapeutic strategies to improve the health of women while limiting complications that have plagued traditional hormone replacement interventions.

Keywords Metabolic syndrome • Estrogen action • Insulin resistance • Obesity

Overview: Menopause, Sex Hormones, and Metabolic Disease

The National Vital Statistics report from the Centers for Disease Control indicates that life expectancy has increased for white females from 48 years in 1900 to 80.6 years in 2011, with a noted gender difference of almost 5 years compared to male counterparts.

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Considering that menopause on average occurs at 51 years (National Institutes of Health, NIA www.nia.nih.gov), women in the modern era are challenged with facing menopausal symptoms including hot flashes, sleep and mood disorders, and sexual dysfunction as well as heightened disease risk associated with increasing adiposity and metabolic dysfunction for up to three decades of life. Arming women with knowledge about the health consequences of ovarian failure as well as furthering our understanding of the biological actions of estrogens in reproductive and nonreproductive tissues have become critical endeavors if we wish to improve the health of women around the world. Although many researchers and clinicians have focused on the impact of replacement estrogens to ameliorate clinical symptoms and provide protective health benefit, an incomplete understanding of the tissue-specific effects of hormone action, and estrogen receptor distribution and function contributes to our confusion and failure to advance therapeutic strategies to combat female-associated pathologies.

In this chapter we will first present a brief history of hormone replacement highlighting observational studies and findings from the largest randomized clinical study conducted in the United States to date, the Women's Health Initiative. The remainder and bulk of the chapter will focus on the biological and tissue-specific actions of the estrogen receptor in regulating metabolic homeostasis, as metabolic dysfunction is identified as a major underpinning in the pathobiology of many chronic diseases that plague our society today.

In 1941, the U.S. Food and Drug Administration approved the use of estradiol as a hormone replacement therapy to exclusively treat postmenopausal symptoms. In 1966, the book *Feminine Forever* was published fueling the argument and general belief that hormone replacement was beneficial and the answer to preserving a woman's health and beauty. (Robert A Wilson. Feminine Forever, New York: M Evans. 1966). In the 1970s, estrogen replacement monotherapy was linked to endometrial cancer which led to progesterone addition to the replacement formulation for treatment of women with a uterus [1, 2]. Despite these known cancer links, by the 1990s, approximately 40 % of postmenopausal women in the U.S. were prescribed hormone replacement therapy, i.e. estrogen with or without progestin, to treat menopausal symptoms [3]. Additionally, as a result of several human observational and rodent-based studies showing a benefit of HRT in preventing or ameliorating complications associated with atherosclerosis and diabetes, many physicians were prescribing HRT for off-label uses not approved by the FDA. Not until the halting of the Women's Health Initiative (WHI) study due to determination of increased risk of coronary heart disease events, stroke, and breast cancer, were the benefits of HRT experimentally scrutinized [4]. In the wake of reports published from the WHI findings, HRT prescriptions abruptly declined by ~68 %. Unfortunately, it appears that many overestimated disease risk associated with postmenopausal hormone replacement, as the overall conclusions from the WHI studies in many cases do not apply to the vast majority of menopausal women starting HRT in their fifties closer to the menopausal transition [5–8]. Considering that the mean age of WHI study participants was 63 years and the mean time since menopause

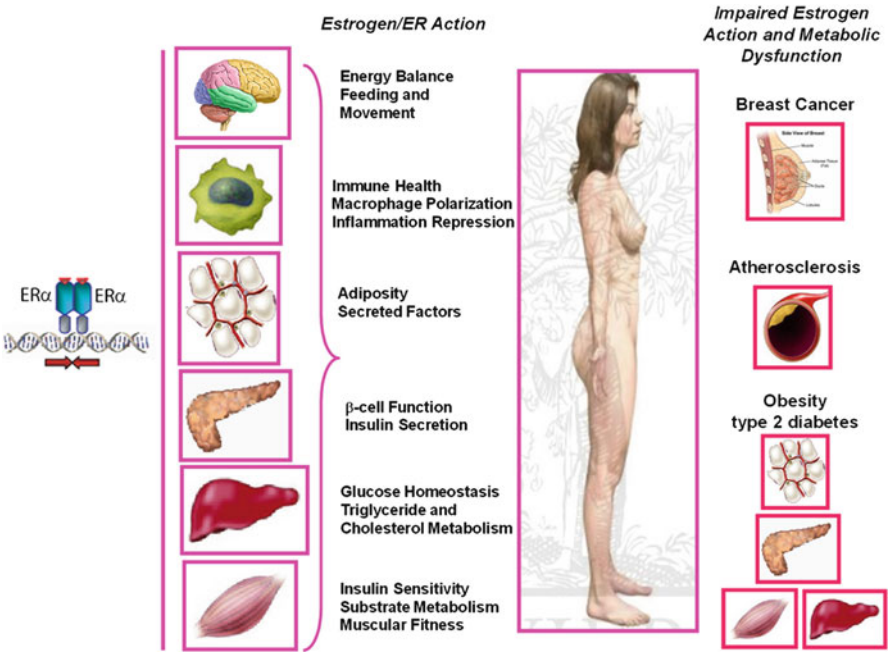

Fig. 6.1 Schematic overview of the chapter discussion pertaining to the role of estrogen receptor (ER) α action in the maintenance of glucose homeostasis and insulin action for the protection against obesity and chronic diseases associated with metabolic dysfunction including atherosclerosis, type 2 diabetes, and certain forms of cancer

was more than a decade, many have questioned whether the timing of hormone administration may determine clinical benefit [8, 9]. Evidence suggests that for symptomatic menopausal women younger than age 60 years or within 10 years of menopause, the benefits of HRT outweigh the risks [10]. Because of the dramatic increase in life expectancy since the turn of the century, women will now spend several decades after menopause in estrogen deficiency, a condition shown to provoke metabolic dysfunction predisposing to obesity, type 2 diabetes (T2D), cardiovascular disease, and certain forms of cancer [11, 12]. Therefore, the contribution of sex hormone deficiency to the pathogenesis of metabolic associated diseases has become a therapeutic challenge of the twenty-first century. Understanding how estrogens and estrogen receptor expression contribute to energy balance, glucose homeostasis, and adipose tissue development promises to yield novel therapeutic applications. Here we review evidence in humans and rodents supporting a role of estrogens and their receptors in regulating fuel homeostasis and insulin sensitivity for the preservation of disease-free health (Fig. 6.1). We will also present basic research suggesting that the estrogen receptor (ER), specifically the α form of the receptor, is an important target to combat metabolic dysfunction.

Molecular Mechanisms of Estrogen Receptor (ER) Action

Early studies in reproductive tissues investigating the actions of estradiol led to the paradigm of classical nuclear ERs as ligand-activated transcription factors (Fig. 6.2) [13]. Although ERs exist in two main forms, α and β, which have multiple splice variants of unknown function, ERs exhibit tissue specificity in expression and function [14]. The classical, or genomic mechanism of ER action, includes the ligand-activated dissociation of ER from its chaperone, receptor dimerization, and receptor binding either to estrogen response elements (ERE) in target genes promoters or to AP-1 or SP-1 response elements through association/tethering with other transcription factors bound to DNA (Fig. 6.2) [15]. After binding, ER dimers interact with basal transcription factors leading to activation or repression of target gene expression. Overlap in binding sites for E_2-liganded ERα and ERβ is observed when receptors are expressed individually; however, when both ERs are present, few sites are shared. Each ER restricts the binding site occupancy of the other, with ERα dominating [16]. Moreover, ligand-activated ER promotes transcription in a cyclic fashion. The repeated cycling of the receptor complex on and off target promoters in the presence of continuous E_2 stimulation may represent a mechanism of continuous sensing and adaptation to the external hormonal milieu to yield the appropriate transcriptional response [17].

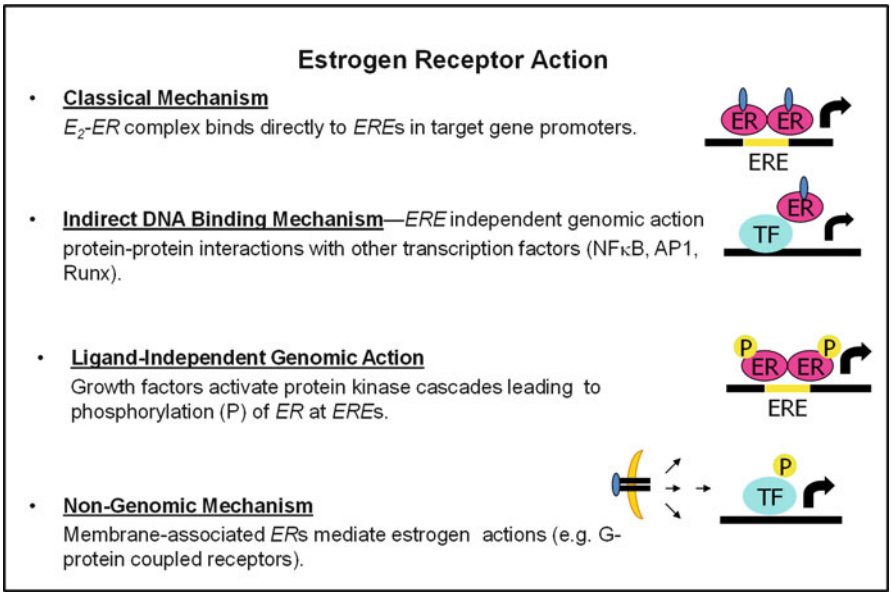

Fig. 6.2 Molecular actions of ERα to activate or repress target genes by classical, DNA binding or nongenomic actions. *ERE* estrogen response element in target gene promoters, *P* phosphorylation, *TF* transcription factor

In addition to classical signaling, E_2-ERα can act within minutes or seconds via extranuclear and membrane-associated forms of the receptor (Fig. 6.2) [18]. Membrane associated receptors are localized to caveolae where they congregate with other signaling molecules, including G proteins, growth factor receptors, tyrosine kinases (Src), linker proteins (MNAR), and orphan G-protein coupled receptors (GPCRs). In a variety of cell types, membrane and extranuclear pools of ERs activate protein kinases that phosphorylate transcription factors to promote their nuclear translocation and transcriptional action [18, 19]. The G protein-coupled estrogen receptor (GPER), or GPR30, has been reported to respond to E_2 however its role as an ER is still controversial and thus will not be discussed in this chapter. Although it is thought that estrogen effects on reproductive function are almost exclusively mediated via classical nuclear ERs acting as ligand-activated transcription factors, a large component of ER action related to energy metabolism also involve extranuclear ERs (Fig. 6.2) [20]. A central question in the field pertaining to the tissue-specific sites of ERα action and the molecular mechanisms by which the receptor selectively activates or represses target genes persists.

The Impact of Brain Estrogen Action in the Regulation of Energy Intake and Expenditure

Studies in humans and animal models have established important roles for estrogens in the regulation of metabolism. As estrogen levels decline during and following menopause, the prevalence of obesity escalates markedly [11, 21, 22]. Ovariectomy (OVX), i.e. removal of the ovaries, leads to increased adiposity in rodents [23–25], that is prevented by estrogenization typically by subcutaneous implantation of a time-release estrogen pellet [26]. Although OVX induces a transient increase in food intake, prevented by E_2 normalization [27, 28], hyperphagia cannot solely account for the changes in metabolism and the development of obesity [25]. Furthermore, mice of both sexes lacking the enzyme CYP19, aromatase (aromatase promotes the synthesis of estradiol), develop obesity in the absence of hyperphagia. Instead, aromatase-deficient mice exhibit reduced physical activity and diminished lean body mass [29]. Similarly, mice of both genders with a homozygous null mutation for Esr1 (ERα) develop obesity in the absence of hyperphagia [30, 31]. This work suggests that although endogenous E_2 favors body weight homeostasis by increasing energy expenditure [32], exogenous estrogens may promote energy balance by influencing both energy intake and expenditure. Thus, loss of ERα action produces a predominant decrease in energy expenditure, while conversely, increasing ERα signaling by raising serum E_2 concentrations both suppresses energy intake and increases energy expenditure as illustrated in Fig. 6.3 and discussed below.

For over a century we have known that specific regions of the CNS control food intake, energy expenditure, and weight gain, as lesioning of specific hypothalamic nuclei within the ventral medial hypothalamus (VMH) [33, 34] or lateral hypothalamus (LH) caused dramatic changes in these biological processes [35, 36].

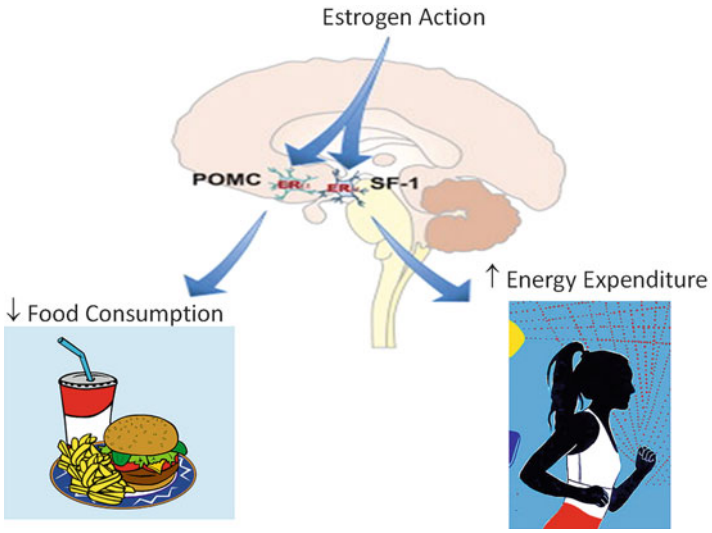

Fig. 6.3 The effects of ERα action in POMC and SF-1 neurons to control feeding and energy expenditure. Findings from Xu et al. Cell Metabolism 14, 453–465, 2011

ERα is highly expressed in the rodent brain including the ventrolateral portion of the VMN, the ARC, the medial preoptic area (MPOA), and the paraventricular nuclei (PVN) [37–43]. ERβ is expressed in the same hypothalamic nuclei but at significantly lower levels by comparison. The effects of E_2 on energy balance are thought to be primarily mediated by ERα, as women or female mice with mutations in the ERα gene become obese [30, 44]. Moreover, ERα homozygosity in animals prevents the anti-obesity effects of estrogen replacement [26]. These gene deletion studies are consistent with pharmacological interventions in ovariectomized (OVX) mice in which the selective ERα agonist PPT was shown to suppress food intake, in contrast to the selective ERβ agonist DPN which failed to influence feeding behavior [45–47].

The signaling mechanisms of ER action in hypothalamic neurons are not fully defined, but evidence suggests the involvement of both classical and extranuclear ER actions [27, 48]. In the arcuate nucleus, ERα is highly expressed in pro-opiomelanocortin (POMC) neurons shown to modulate food intake, energy expenditure, and reproduction (Fig. 6.3) [49]. POMC neurons secrete α melanocyte stimulating hormone (αMSH), which acts in the PVN and lateral hypothalamus on melanocortin 3 and melanocortin 4 (MC3/MC4) receptors to produce catabolism by reducing food intake and increasing energy expenditure [50–53]. ARC POMC mRNA levels fluctuate over the course of the estrous cycle, with a marked increase occurring coincident with proestrous when E_2 concentration is the highest [27, 54–57]. Conversely, POMC levels are reduced in ERα knockout mice [58]. These E_2-induced synaptological rearrangements in POMC neurons are tightly paralleled with the effects of estrogens on food intake, energy expenditure, and body weight [27], and

appear mediated by MC4R [59]. Indeed, deletion of ERα in POMC neurons of mice leads to hyperphagia without affect to energy expenditure or adipose tissue distribution [49].

The ventral medial hypothalamus, VMH, is also thought to be an important neural circuit responsible for the homeostatic regulation of body weight and food intake, as estrogens have been shown to directly alter the electrophysiological properties of VMH neurons [60]. Small hairpin (sh) RNA-mediated ERα gene silencing and selective ablation of ERα from SF-1 containing neurons of the VMH blunts E_2-induced weight loss, promoting increased visceral fat deposition, and reductions in energy expenditure in the absence of hyperphagia (Fig. 6.3) [61]. These findings support the notion that ERα signaling in VMH neurons plays an important role in regulating physical activity, thermogenesis, and fat distribution.

Since leptin was first described in 1994 [62], it has proven to be a powerful catabolic signal in the brain, inhibiting food intake and increasing energy expenditure [52, 63, 64]. Leprb is localized in several brain areas including the VMH and the ARC, and colocalizes with several neuropeptides known to control food intake and reproduction [65–67]. Leprb also binds ERα in the ARC [68], which is consistent with estrogens inducing leprb mRNA [69], possibly by direct genomic action mediated by ERE binding in the promoter of the leptin receptor gene [70]. The extensive hypothalamic colocalization of these two receptors suggests a closely coupled interaction between peripheral derived signals in the regulation of energy homeostasis. Indeed, increased E_2 is associated with enhanced central leptin sensitivity in rodents [71–73]. Although circulating leptin levels do not change appreciably during the estrous cycle, ARC leprb expression is highest during estrous and metestrous and is inversely correlated with neuropeptide Y (NPY) mRNA expression [69]. Moreover, OVX lowers the sensitivity to central leptin when compared to normally cycling females, and this leptin resistance is prevented by E_2 replacement [73]. Analogously in male rodents, exogenous E_2 administration also increases sensitivity to central leptin [73]. The differences in central leptin sensitivity caused by the presence or absence of estrogens may occur downstream of leprb transcription and translation, possibly at the level of STAT3, as E_2 decreases food intake and increases energy expenditure, resulting in a reduction in body weight in leptin-deficient (ob/ob) and leptin resistant (db/db) mice of both sexes [27].

NPY is an effective anabolic peptide considering that central administration of NPY potently increases food intake and decreases energy expenditure and fat oxidation [74–77]. ARC neurons coexpress NPY mRNA and leprb protein. Leptin administration decreases, while leptin deficiency or leptin resistance increases NPY (and AgRP) mRNA, demonstrating that leptin is a critical determinant of ARC NPY function [78]. NPY neurons in the hypothalamus not only affect feeding, but also influence reproduction, therefore, E_2 can modulate both of these neuroendocrine systems by regulating NPY gene expression, NPY Y1 receptor expression [79], and NPY release [80]. Additionally, NPY/AgRP neurons are required to mediate the anorexigenic effects of E_2 and hypothalamic expression of NPY and AgRP is tightly regulated across the estrus cycle, with the lowest levels during estrus concomitant with the E_2 peak and feeding nadir in wild-type mice [81]. Importantly, the cyclic changes

in food intake and E_2-induced anorexia are blunted in mice with degenerated NPY/AgRP neurons [81], indicating that neurons coexpressing NPY and AgRP are functionally required for the cyclic changes in feeding across estrous cycle and that NPY/AgRP neurons are essential mediators of the anorexigenic function of E_2. Interestingly, it was shown that ERα is completely excluded from NPY/AgRP neurons in the mouse hypothalamus [81], suggesting that E_2 may regulate these neurons indirectly via unknown presynaptic neurons expressing ERα.

ERα Expression in the Control of Adipose Tissue Distribution and Obesity Susceptibility

There is a well-described sexual dimorphism in body fat distribution as men have less total body fat but increased intra-abdominal adipose tissue compared with women who have more total fat but a higher proportion of gluteal/femoral subcutaneous (SC) adipose tissue which is shown to be less pathogenic [82–86]. Following menopause and the decline in circulating E_2, women gain intra-abdominal fat and develop male-pattern adiposity ameliorated by estrogen replacement therapy [87–89]. Additionally, adipose tissue redistribution is reported in male–female transsexuals receiving estradiol supplementation in which increased SC fat relative to intra-abdominal adipose tissue is observed [90]. Thus, estrogens are thought to regulate fat distribution [90, 91], and this may contribute to improved metabolic profile in women receiving HRT.

Accumulation of central intra-abdominal or visceral fat, ("android," or male-pattern obesity) is correlated with increased risk and mortality from diabetes and atherosclerosis [92]. Intra-abdominal adipose tissue is thought to be metabolically and functionally different from SC adipose tissue. Indeed, compared the SC fat, intra-abdominal adipose tissue has more efferent sympathetic axons and capillaries, per unit volume, and these capillaries drain into the hepatic portal system [92]. Surgical removal of intra-abdominal adipose tissue in humans results in decreased insulin and glucose levels [93] and prevents the onset of insulin resistance and glucose intolerance in male rodents [94]. In contrast, surgical removal of SC fat tissue of equal weight has no appreciable impact on the same parameters [94]. Teleologically, males may preferentially deposit adipose tissue in the intra-abdominal depot because of its ability to be rapidly mobilized providing a quick and abundant energy source during times of increased energy demand. In contrast, SC adipose tissue allows for efficient storage of maximal calories per unit volume of tissue. Lipid deposition into SC adipose tissue may provide an evolutionary advantage for females by extending protection during limited caloric supply in order to maintain reproductive function. Importantly, females also mobilize adipose tissue stored in SC depots to meet the caloric demands placed on the body during breast feeding. In contrast to android or male-pattern adiposity, "gynoid" or female-pattern fat deposition is only weakly correlated with metabolic dysfunction and disease risk [95–99]. In fact, transplantation of SC fat from donor mice into

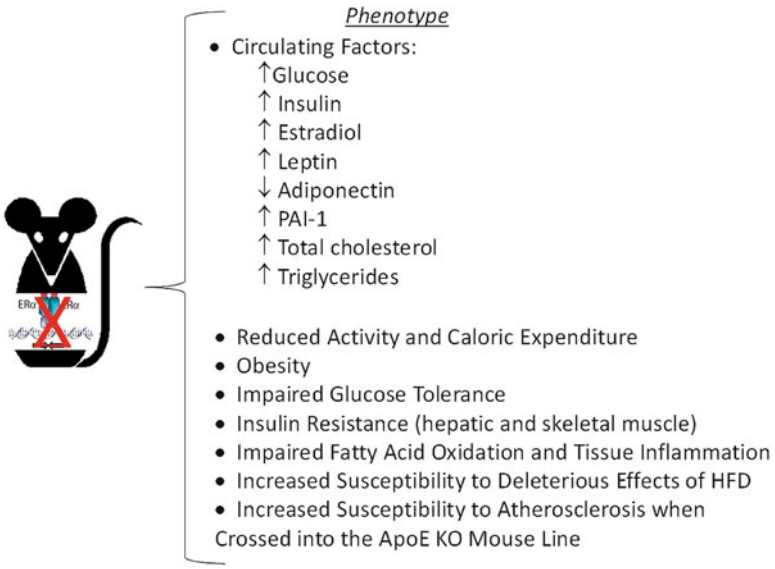

Fig. 6.4 Metabolic dysfunction in ERαKO mice. Animals develop insulin resistance, impaired glucose tolerance and obesity as early as 3–6 months of age and are more susceptible to the deleterious effects of HFD than age-matched WT counterparts

visceral regions of recipient mice decreases total fat and improves glucose homeostasis suggesting that adipose-secreted factors may act systemically to improve metabolism [100].

In addition to ovarian production, estrogens are also synthesized in adipocytes (via aromatization of androgenic precursors by CYP19), and circulating levels are elevated in proportion to total body adiposity [101, 102]. ERα is highly expressed in adipose tissue [103, 104], and targeted global deletion of the ERα gene (ERαKO) in mice of both genders promotes increased adiposity, with a near doubling of the visceral depots compared with age-matched wild-type (WT) mice (Fig. 6.4) [30]. In contrast, mice with a homozygous deletion of ERβ (βERKO) show a body composition identical to WT mice, suggesting that ERβ may play a limited role in adipose tissue development and metabolism [105]. Reduced ERα expression or impaired ERα function due to genetic alteration has been linked with increased prevalence of specific features of the metabolic syndrome including obesity in both male and female humans and rodents [106–113]. The role of ERα in adipocytes and the phenotypic outcomes of adipose-specific ERα deletion in mice are currently under investigation by several laboratories around the world. Whether the obesity phenotype observed in whole body $Esr1^{-/-}$ mice or women harboring an *ESR1* inactivating mutation is explained by loss of ERα from adipose tissue specifically or as a result of a secondary phenotype of ERα deletion from other metabolic tissues remains unknown.

Estrogen Action and Insulin Sensitivity

Insulin resistance is a central disorder in the pathogenesis of type 2 diabetes and a defining feature of the Metabolic Syndrome, a clustering of metabolic abnormalities including obesity, hypertension, glucose intolerance, and dyslipidemia [114, 115]. Normally cycling women show enhanced insulin sensitivity compared to men when normalized to lean mass, and this is a likely contributor to the reduced incidence of type 2 diabetes in pre-menopausal women [116, 117]. Moreover, although a 40–50 % reduction in insulin-mediated glucose disposal is consistently observed in male mice following high fat feeding [118, 119], E_2-replete females, humans and rodents, are typically protected against a high fat diet and acute fatty acid-induced insulin resistance [120–123]. Following menopause or OVX, a precipitous decline in insulin sensitivity coincides with increased fat mass, and elevated circulating inflammatory markers, LDL, triglycerides, and fatty acids [11, 124, 125]. OVX mice and rats are insulin resistant, show impaired exercise-stimulated glucose disposal into muscle [126], and are more susceptible to the deleterious effects of high fat diet or lipid oversupply, and these physiological consequences of OVX are prevented by restoration of circulating estradiol within a physiological concentration [127].

Although chronic administration of E_2 is shown to improve insulin sensitivity, at least in rodents, the acute action of E_2 to promote insulin-stimulated glucose uptake into muscle remains disputed; this despite consistent observations of E_2-induced activation of Akt and AMP-activated Protein Kinase (AMPK) [128, 129]. Furthermore, although administration of intravenous conjugated estrogens and E_2 to postmenopausal women or OVX rats, respectively, is shown to stimulate a significant increase in glucose disposal during hyperinsulinemic-euglycemic clamp studies [130, 131], ex vivo treatment of skeletal muscle with E_2 failed to recapitulate the same increase in insulin-stimulated glucose disposal [128]. This is also in contrast to short-term estradiol effects on insulin action in myotubes from postmenopausal women and age-matched men [132]. Thus, two questions remain; does E_2 enhance insulin sensitivity and what are the critical tissues of E_2 action that confer protection against insulin resistance induced by nutrient oversupply?

Similar to findings for ovarian failure in women and rodents, a reduction in circulating estrogens resulting from rare inactivating mutations or experimental deletion of Cyp19 (aromatase) in mice confers an obesity-insulin resistance phenotype [29, 106, 133–139]. The physiological and genetic evidence argues that E_2 and ER favor insulin sensitivity in rodents and humans of both sexes when E_2 is maintained within a tight physiological concentration. Indeed, replacement or augmentation of E_2 to supraphysiological levels or over-stimulation of ER is thought to induce insulin resistance secondary to hyperinsulinemia and or a reduction in total GLUT4 expression in muscle [140, 141]. In fact, two studies have reported that in postmenopausal women, higher plasma levels of E_2 were prospectively associated with increased risk of developing T2D [142, 143]. Clearly, additional studies in rodents and humans using a dose response strategy are necessary to better understand the interplay of steroid hormones including E_2, testosterone, and progesterone on the regulation of metabolism and insulin action in glucoregulatory tissues.

Although, several laboratories have characterized the whole body ERαKO mouse (Fig. 6.4 Phenotype Overview), many questions still remain including the tissues responsible for conferring the severe insulin resistance-obesity phenotype. Does obesity arise from loss of ERα within adipocytes specifically or can it be driven as a secondary phenotype resulting from a loss of ERα in brain, skeletal muscle, liver, or even selective immune cells? Furthermore, does a loss of ERα specifically from myocytes drive skeletal muscle insulin resistance, or does this phenotype arise as a secondary consequence of increased adiposity and altered adipokine/cytokine release?

Although two forms of the receptor are expressed in many of the glucoregulatory tissues, ERα is found in much higher abundance than ERβ, as ERβ transcript is nearly undetectable in human and rodent muscle [132, 144, 145]. Consistent with these observations, homozygous deletion of ERβ failed to produce insulin resistance [44] in contrast to the marked skeletal muscle insulin resistance observed in ERαKO animals (Fig. 6.4) [146, 147]. The underlying mechanism contributing to impaired insulin action in ERαKO animals remains disputed as findings reported by Bryzgalova et al. [148] suggesting reduced total GLUT4 levels in muscle as an underlying cause for the ERαKO insulin resistance phenotype was not supported by Ribas et al. [146]. Furthermore, despite maintenance of GLUT4 mRNA and protein, Ribas et al. reported more dramatic skeletal muscle insulin resistance in ERαKO mice than Bryzgalova et al. work by Hevener and colleagues suggests that the skeletal muscle insulin resistance observed in ERαKO mice is predominantly due to direct effects of ERα deletion on pro-inflammatory signaling and proximal insulin signal transduction.

Indeed, emerging findings in muscle from muscle-specific ERα knockout mice and myotubes with ERα knockdown show no alteration in GLUT4 mRNA or protein despite a marked impairment in insulin-stimulated glucose disposal. These observations in the muscle-specific ERα mouse are entirely consistent with those of whole body ERαKO mice (Figs. 6.4 and 6.5) [146, 149]. Furthermore, additional studies by Barros et al. [141, 150] investigating alteration in GLUT4 expression in response to ovariectomy and E_2 supplementation are in conflict with other published studies of similar design [132, 145, 151–153]. Given the lack of consensus ERE in the GLUT4 promoter [154] and absence of confirmatory findings in cellular reporter and chromatin immunoprecipitation assays, the regulation of GLUT4 expression by ERα requires further investigation. GLUT4 is impacted by several redundant transcriptional pathways [155, 156], and this redundancy in regulation likely underlies the maintenance of total GLUT4 transcript and protein levels in the absence of ERα as well as in humans or rodents of both genders in the context of insulin resistance, obesity, and type 2 diabetes [157–160]. Considering the concomitant increase in ERα and GLUT4 expression observed in muscle of exercise-trained humans and mice, it is conceivable that while ERα deletion fails to impact GLUT4 expression, increased ERα action may mediate in part the training-induced increase in total GLUT4 content [157, 161–166].

Myocyte enhancer factor 2 (MEF2) expression and a functional MEF2 element in the GLUT4 promoter are critical for GLUT4 gene expression [167]. Furthermore,

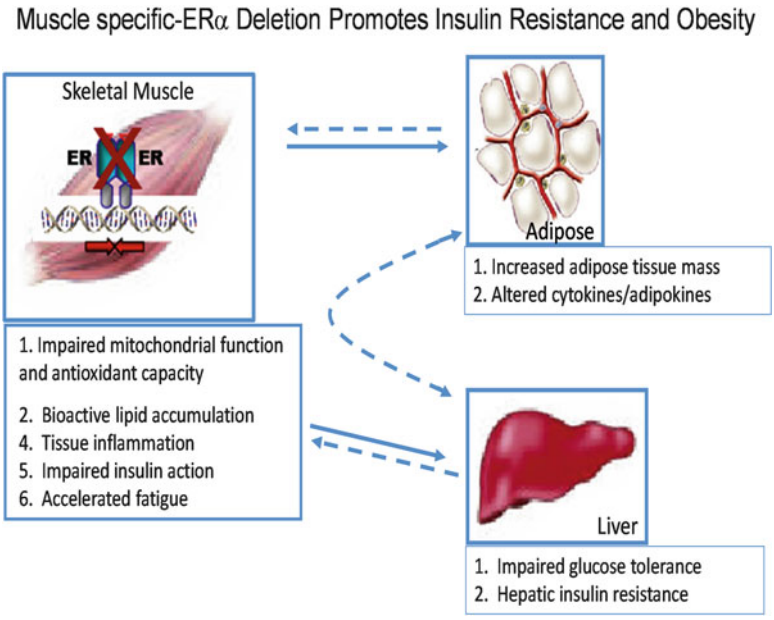

Fig. 6.5 The impact of skeletal muscle-specific ERα deletion on insulin sensitivity and adiposity in female mice (Ribas et al. [149])

reciprocal regulation between ERα and MEF2 can be observed in cardiomyocytes via ERα interaction with class II HDAC [168]. Despite complex transcriptional signal integration in the regulation of GLUT4 expression [155, 156, 169–172], it is reasonable to speculate that elevated ERα action could promote increased GLUT4 transcription via heightened protein tethering with MEF2 on the GLUT4 promoter or by indirect action via AMPK [128, 173]. It is important to note that transcriptional activity of the GLUT4 promoter is quite low under basal conditions and other ovarian hormones, e.g. progesterone, are shown to play an antagonistic role in the regulation of GLUT4 expression [126]. These issues as well as the dose of E_2 administration during interventional studies (presumably off-target effects of supraphysiological doses of E_2 are deleterious to metabolism), the age and hormone status of the human subjects and rodents used are important considerations when interpreting the literature. Given the varying roles that muscle and adipose tissue play in controlling whole body metabolic homeostasis, it is likely that the interplay of transcriptional regulators of GLUT4 vary markedly between tissues. Taken together, these data would suggest a potential role for ERα as an enhancer of GLUT4 transcription in muscle under certain conditions, but not necessarily obligatory in the direct regulation of GLUT4 expression under basal conditions.

Collectively, work by Ribas et al. suggests that the skeletal muscle insulin resistance observed in whole body ERαKO mice and animals with a muscle-specific deletion of ERα is predominantly the result of impaired insulin signal transduction [149].

A role for ERα in the regulation of proximal insulin signal transduction has been suggested previously as E_2 administration to insulin resistant rodents increases insulin receptor substrate (IRS)-1 abundance and insulin-stimulated tyrosine phosphorylation and as well as the phosphotylation of Akt at Ser473 [147, 174]. Akt serves many functions in myocytes including ERα-induced regulation of myogenic differentiation [175], suppression of muscle-atrophy associated ubiquitin ligases via FOXO1 inhibition [176], and induction of genes associated with myocellular proliferation [175, 177–180]. In breast cancer cell lines, endothelial cells, and cortical neurons, ERα-specific binding and activation of PI3kinase as well as suppression of the tumor suppressor and PI3kinase inhibitory protein, PTEN, is well-established [181–185]; however, studies on these direct interactions in skeletal muscle are limited. Additionally, E_2 acting via ERα is also shown to promote phosphorylation of p38 MAPK [186, 187], a signaling cascade shown to enhance GLUT4 intrinsic activity and glucose uptake [188–190]. Furthermore, it is possible that ERα activation of Akt and MAPK pathways may underlie in large part the E_2-mediated protection of muscle against age-induced sarcopenia [191–197], exercise-induced muscle damage [179, 193, 198, 199], and myocyte apoptosis in the face of a variety of cellular perturbations [200–203]. Thus, ERα stimulation of muscle growth and insulin sensitivity via direct and indirect interaction with these pathways requires further exploration.

In contrast to the protective effects of ERα, it is suggested that ERβ may promote insulin resistance in skeletal muscle when the ERβ:ERα is elevated. In OVX mice, while ERα-specific activation by PPT improved muscle insulin action [129], conversely, ligand-specific activation of ERβ by DPN failed to ameliorate insulin resistance [129]. Moreover, ovariectomy of hyperestrogenic female ERα-deficient mice was shown to improve glucose tolerance and insulin sensitivity presumably though elimination of ERβ activation by endogenous E_2 [204]. Similarly, administration of an ERβ-selective agonist to male E_2-deficient ArKO mice decreased glucose uptake [150]. Finally, evidence indicates that ERβ-deficiency protects against diet-induced insulin resistance in male mice by increasing PPARγ signaling in adipocytes, which indirectly improves skeletal muscle insulin action by promoting lipid accumulation in adipose tissue and diminishing ectopic lipid deposition in muscle [205, 206]. A role for ERβ in the pathogenesis of human insulin resistance remains unknown and there is still much work to do in determining the tissue-specific interactions of these transcription factors under more physiological conditions.

ERα and Skeletal Muscle Fatty Acid Metabolism and Inflammation

Normally cycling women are protected against acute lipid-induced insulin resistance compared with estrogen-deficient women and men [120, 207]. Furthermore, muscle from premenopausal women shows enhanced insulin sensitivity despite 47 % higher triglyceride content compared with age-matched men [158]. These observations are

consistent with a reduced respiratory quotient and greater reliance on the oxidation of fatty acids as a fuel source in women [208]. Interesting similarities between E_2 replete women and exercise trained subjects including elevated muscle ERα expression [144, 166, 209], heightened insulin sensitivity [210], elevated muscle lipid tolerance [211], and enhanced oxidative capacity [212, 213] are consistently observed. Estrogen supplementation is shown to enhance lipid oxidation in men during acute endurance exercise [214] as well as palmitate oxidation in myotubes from male subjects ex vivo [132]. The effect of E_2 to increase the expression of fatty acid transport protein FAT/CD36 and FABP as well as transcription factors and key enzymes that regulate oxidative metabolism [151, 157, 215] likely underlie these observations in male subjects. In addition, exercise and E_2 are shown to rapidly stimulate AMPK phosphorylation in both muscle and myotubes [128, 216]. AMPK is considered a central regulator of many cellular processes including growth, mitochondrial biogenesis, and oxidative metabolism [217, 218]. Similar to the effects of E_2, the ERα-selective agonist PPT stimulates AMPK phosphorylation in muscle of female rats [129] while OVX or whole body ERα deletion is associated with reduced skeletal muscle levels of phosphorylated AMPK [146, 219]. Muscle PPARα, PPARδ, and UCP2 expression are also reduced in whole body ERαKO mice suggesting that ERα is essential in the regulation of a coordinated program regulating oxidative metabolism. Importantly, although the impairment in muscle fatty oxidation was recapitulated in the muscle-specific ERαKO mice (MERKO), no alteration in basal p-AMPK, PPARα, PPARδ, or UCP2 was observed [149], thus suggesting that these specific alterations in muscle gene expression in ERαKO mice are secondary to the loss of ERα in other metabolic tissues, likely the CNS, adipose, or liver. Despite these model differences, the skeletal muscle insulin resistance and bioactive lipid accumulation (triacylglycerol, diacylglycerol, and ceramides) was surprisingly similar between ERαKO and MERKO. Consistent with these observations, oxygen consumption rates in C2C12 myotubes with ERα knockdown were reduced significantly. In addition, mitochondria from muscle cells depleted of ERα produced high levels of reactive oxygen species thus precipitating increased cellular oxidative stress. Collectively these data support the notion that ERα is critical for maintaining fatty acid oxidation in skeletal muscle by mechanisms including the regulation of: (1) fatty acid transport into the cell, (2) activation of intermediary signaling critical for shifting substrate metabolism, (3) transcriptional regulators of fatty acid metabolism, and mitochondrial function. Thus, ERα expression in skeletal muscle may be a central regulator of adiposity by indirect action as MERKO mice reproduced the obesity phenotype observed in the whole body ERαKO (Fig. 6.5).

Moreover, E_2 treatment reduces HFD-induced insulin resistance in skeletal muscle by 50 % (assessed by hyperinsulinemic-euglycemic clamp) in an ERα-dependent manner [147]. The mechanistic link between the accumulation of lipid intermediates, activation of inflammatory signaling cascades, and impaired insulin action is shown in myocytes and rodent muscle, and indeed these factors are observed concurrently in obese, type 2 diabetic subjects [220–223], as well as muscle from whole body and muscle-specific ERαKO mice [146]. Bioactive lipid intermediates

including diacylglycerol and ceramides are believed to activate stress kinases including IKKβ, c-Jun-N-terminal kinase (JNK), and certain nPKCs [221, 224–226]. Indeed, muscle from normal chow-fed whole body ERαKO mice showed heightened inflammatory signaling as reflected by markedly increased JNK phosphorylation and TNFα transcript [146]. In addition to the increase in bioactive lipid intermediates found in ERαKO muscle, the production of reactive oxygen species as well as the possible ERα de-repression of selective inflammatory targets within the nucleus are likely mediators of heightened muscle inflammation.

Markers of inflammation and oxidative stress are elevated in rodent models of obesity and in patients with type 2 diabetes [227, 228]. Myotubes and skeletal muscle with ERα deletion showed a marked reduction in Gpx3 expression, an antioxidant enzyme reported to scavenge hydrogen peroxide and diminish oxidative stress [145, 146]. E_2 replacement in OVX animals also increased Gpx3 expression in skeletal muscle [145]. Given that Gpx3 expression levels in skeletal muscle are elevated in females compared to male [229], reduced in T2DM patients [230], are associated with insulin resistance and metabolic dysfunction [230], and the gene is now identified as a causal candidate for obesity [231], additional work studying the direct role of estrogen action in the regulation of anti-oxidant enzymes appears warranted.

Although reductions in mitochondrial number and function have been implicated in the pathobiology of insulin resistance [232–235], and indeed gender dimorphisms in mitochondrial biology have been described [236], whether E_2/ERα preserves insulin action by maintenance of mitochondrial integrity remains unknown. Emerging unpublished findings from the Hevener laboratory indicate that skeletal muscle ERα is critical for the maintenance of mitochondrial function and the turnover of damaged organelles. However, the mechanisms underlying these observations remain incompletely understood at the present time.

ERs and Hepatic Insulin Sensitivity

Hepatic insulin resistance contributes to impaired glucose tolerance and fasting hyperglycemia of type 2 diabetes by unrestrained hepatic glucose production. Although a direct role of the liver in the insulin resistance phenotype induced by E_2 deficiency or tissue-selective ablation of ERα remains unclear. Bryzgalova et al. showed that the global insulin resistance of female mice with a homozygous ERα null mutation was due almost exclusively to impaired suppression of hepatic glucose production (HGP) during euglycemic-hyperinsulinemic clamp studies in anesthetized mice [148]. In contrast, Ribas et al., reported in the same ERα-deficient female mice only modest reduction in liver insulin sensitivity during euglycemic-hyperinsulinemic clamp studies in conscious animals [31]. Thus, the possibility exists that anesthesia artificially contributed to the severe hepatic insulin resistance phenotype observed by Bryzgalova et al. in ERα-deficient mice [148]. Studies in liver-specific ERα-deficient mice should provide the definitive findings required to determine the role of liver ERα in controlling hepatic insulin action and glucose

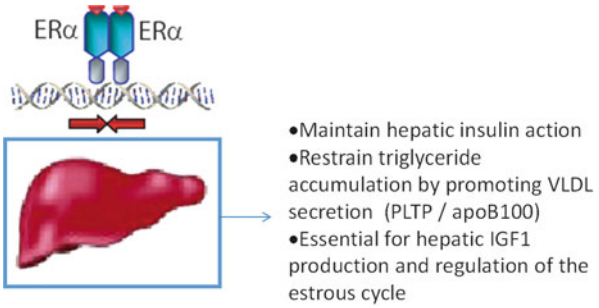

Fig. 6.6 Proposed actions of estrogen and ERα in the protection against hepatic triglyceride accumulation and insulin resistance

tolerance. Still, E_2 and PPT treatments ameliorate insulin resistance in genetically obese leptin resistant and high fat diet-fed mice [147, 237]. Numerous studies are in agreement that E_2 suppresses lipogenic gene expression, triglyceride accumulation, and steatosis in liver of HFD-fed [237] and leptin-resistant female mice (Fig. 6.6) [238]. Interestingly, this effect is not always reproduced by the ERα, selective agonists PPT [239]. A significant limitation of studies inducing chronic estradiol elevation is the inability to ascribe specific actions of estradiol or ERα agonism to a given tissue as circulating estradiol impacts all metabolic tissues producing numerous secondary phenotypes due to extensive tissue crosstalk. Moreover, E_2, and possibly ERα, cyclicity appears critical in the regulation of gene expression and cellular function, and this cyclicity is eliminated during chronic E_2 or ERα agonist administration. Together, these data suggest that ERα activation protects from hepatic insulin resistance by preventing ectopic lipid accumulation in liver (lipotoxicity), but the direct involvement of ERα and the molecular mechanisms of action in hepatocytes require further clarification.

Estrogen Action, Immunity, and the Metabolic Syndrome

Estrogens affect many immune and inflammatory conditions including autoimmune diseases [240–245] as well as immuno-modulatory responses to parasitic and bacterial infection [246–251]. Following OVX, immune cell infiltration and increased tissue inflammation (TNFa, iNOS, and CD11c) coincided with a ~4 fold increase in perigonadal and inguinal fat. The T-cell marker CD3 and the Th1 cytokine interferon γ were also elevated in perigonadal fat from ovariectomized female mice [32] suggesting that the absence of E_2 promotes immune cell inflammation. Indeed, similar to findings in rodents, circulating levels of pro-inflammatory cytokines are elevated in women following natural or surgical menopause [124]. In line with these studies, work by the laboratory of Pierre Gourdy showed that E_2 heightens the inflammatory response to intraperitoneal injection of thioglycollate or lipopolysaccharide, and that ERα is critical in mediating these actions as well as reducing bacterial burden through phagocytosis [248]. Taken together, these data

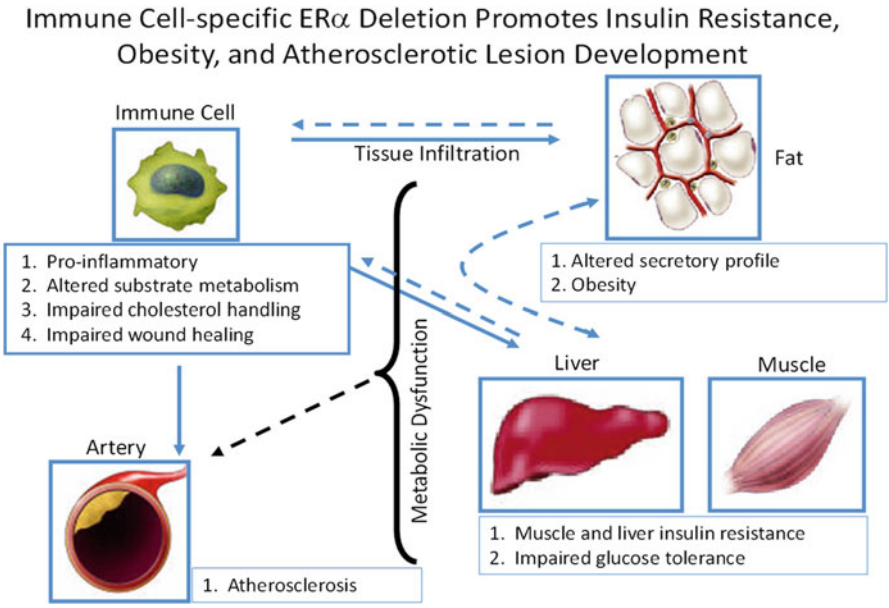

Fig. 6.7 Myeloid-specific ERα deletion promotes obesity, insulin resistance, and atherosclerosis susceptibility in female mice

suggest that ERα expression in immune cells is critical for mediating a variety of cellular responses necessary for normal innate and adaptive immunity.

ERα is expressed in macrophages and other immune cells known to exert dramatic effects on glucose homeostasis. Macrophages are central effector cells of innate and adaptive immunity, and over the past decade their role in modulating whole body metabolism and insulin sensitivity has taken on increasing interest [252, 253]. Recently Ribas et al. investigated the impact of ERα expression on macrophage function to determine whether hematopoietic or myeloid-specific ERα deletion manifests an obesity-induced insulin resistance phenotype in mice [254]. This group sought to determine how much of the whole body ERαKO phenotype could be recapitulated by deletion of ERα specifically from myeloid cells. Indeed, altered plasma adipokine and cytokine levels, glucose intolerance, insulin resistance, and increased adipose tissue mass were observed in animals harboring a hematopoietic or myeloid-specific deletion of ERα (Fig. 6.7) [254]. A similar obese phenotype with increased atherosclerotic lesions was produced in LDLR-KO mice transplanted with ERαKO bone marrow. In isolated macrophages, Ribas et al. found that ERα is necessary for repression of inflammation, maintenance of oxidative metabolism, IL4-mediated induction of alternative activation, full phagocytic capacity in response to LPS, and oxidized LDL-induced expression of ApoE and Abca1 (Fig. 6.7) (301). Moreover, bone marrow-derived macrophages lacking ERα secrete factors that induce skeletal muscle and adipocyte insulin resistance in

culture [254]. A major limitation in the field is the failed identification of these pro-inflammatory insulin-resistance producing substance secreted from immune cells. Thus, metabolomic and proteomic analyses will be necessary to move the field forward in this regard. It is likely that these macrophage-secreted factors include a combination of cytokines, fatty acids, and reactive oxygen/nitrogen species that act to alter metabolic function of adjacent cells in contact or in close proximity with tissue resident macrophages.

Taken together, these data suggest that ERα expression in immune cells is critical for mediating a variety of cellular responses necessary for normal innate and adaptive immunity. When E_2 levels are low or ERα action is impaired, disease susceptibility increases as the functionality and responsiveness of critical immune cell types become compromised. Although a few direct ERα targets in myeloid cells have been identified, considering the intricate and diverse signaling by ERα as well as the complex nature and crosstalk between cell types, the impact of sex steroids on immunometabolism requires further and more sophisticated dissection.

Estrogen Action and Pancreatic β-Cell Function

The role of estrogens and ERs in β-cell function and the protection of β-cell mass has been recently reviewed [19] and for the purpose of this chapter, we will focus on the most recent developments. In rodent models, treatment with E_2 protects pancreatic β-cells against various injuries encountered in both T1DM and T2DM, including oxidative stress, amyloid polypeptide toxicity, lipotoxicity, and apoptosis [19]. Three receptors—ERα, ERβ, and GPER—have been identified in rodent and human β cells. Unlike the classical nuclear ERs acting as ligand-activated transcription factors in breast or uterine cells, β-cell ERs reside mainly in extranuclear locations. They promote their effect via cytosolic interactions with kinases such as Src, ERK, and AMPK or transcription factors of the STAT family [19, 255–258]. Activation of ERα enhances glucose-stimulated insulin biosynthesis [255, 259] though a pathway involving Src, ERK, and the stimulation of the nuclear translocation and binding to the insulin promoter of the insulintropic transcription factor NeuroD1 (Fig. 6.8) [255]. Activation of ERα reduces de novo synthesis of fatty acids and restrains lipogenesis and accumulation of toxic lipid intermediates in islets (Fig. 6.8) [256–258]. This anti-lipogenic action involves extranuclear ERα activation and promotes the nuclear translocation of STAT3 leading to an inhibition of the master regulator of lipogenesis the liver X receptor (LXR)β and its transcriptional targets, sterol regulatory element-binding protein 1c (SREBP1c) and carbohydrate response element binding protein (ChREBP). The suppression of LXRβ and SREBP1c mRNA may be mediated via a membrane associate ERα working through Src and STAT3 [258]. In β-cells, chronic LXR activation provokes lipogenesis associated with lipotoxicity and apoptosis [260]. Thus, ERα suppression of LXR expression in β-cells may account for the inhibition of lipogenesis and prevention of islet lipotoxicity (Fig. 6.8) [258]. Moreover, ERα can also activate AMP-kinase to suppress SREBP-1c gene

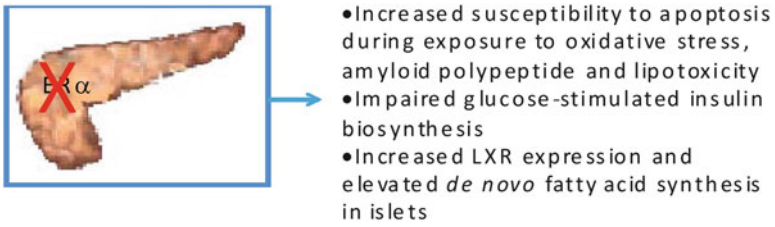

Fig. 6.8 The effects of islet-specific ERα deletion on pancreatic function and glucose homeostasis [19]

and protein expression [258]. Thus, taken together, ERα acts via STAT3 and AMPK pathways to decrease expression and activity of the master effector of FA synthesis under conditions of glucose surplus [256].

Additionally, ERα promotes β-cell survival from most pro-apoptotic stimuli encountered in the diabetic condition [261–263]. Anti-apoptotic mechanisms involve a combination of rapid ERα actions mediated by protein phosphorylation [262, 263], and a more classical genomic mechanism inducing an anti-inflammatory cascade via liver receptor homolog-1 (LRH-1) [264]. Activation of ERβ seems to predominantly enhance glucose-stimulated insulin secretion [265, 266] via a membrane pathway and activation of the ANF receptor promoting closure of KATP channels [265]. GPER activation, however, protects β-cells from lipid accumulation [257] and promotes cell survival [262, 267, 268]. GPER activation also enhances glucose-stimulated insulin secretion [267, 269] via activation of the epidermal growth factor receptor and ERK [269], but has no effect on insulin biosynthesis [255]. However, it has been proposed that GPER induces the expression of ERα36, a splice variant of the classical long isoform of ERα66 [270], as both ERα66 and ERα36 are expressed in β-cells [256]. Thus, it is unclear whether GPER effects on β-cells are due to intrinsic GPER action or indirect effects of GPER collaborating with ERα36 at the membrane. Importantly, the beneficial effects of ER ligands on β-cell survival, function, and nutrient homeostasis described above are all observed in human β-cells [19, 256, 262, 268, 271].

Perhaps the translational potential of E_2 therapy in β-cell protection would be best achieved in the context of pancreatic islet transplantation (PIT). Fertile women with type 1 diabetes (T1D) show E_2 deficiency compared to nondiabetic women [272], suggesting that islet survival in T1D women undergoing islet transplantation could be enhanced by short-term augmentation of circulating E_2. To explore this hypothesis, Mauvais-Jarvis and colleagues used a T1D model with xenotransplantation of human islets in nude mice rendered insulin-deficient by streptozotocin. In this model a 4 week E_2 treatment protected β-cell mass and enhanced islet revascularization and engraftment [273]. Thus, transient E_2 treatment provided an immediate therapeutic impact to improve PIT and achieve insulin independence with fewer islets. Studies in human subjects are warranted and considering the short-term duration of E_2 administration, the risk of secondary complications arising in reproductive tissues is minimal.

Conclusions and Perspectives

Over the past decade, a vast literature of molecular targets has emerged promising the prospect of pharmacological intervention for the restoration of metabolic homeostasis and insulin action to ameliorate complications associated with diabetes and obesity in humans. The inherent simplicity and elegance of using estrogens or ER agonists as therapeutic agents, at least in women, is underscored by decades of research and in depth knowledge related to biological/clinical efficacy and toxicity profiles obtained by in vivo studies in preclinical animal models and humans. Estradiol and ERα-specific agonists are shown to promote energy homeostasis, improve body fat distribution, and diminish insulin resistance, β-cell dysfunction and inflammation. The challenge with estrogen supplementation, however, is the relatively narrow therapeutic index when used chronically. Thus, although successful in rodents, the translation of basic science advances showing amelioration of complications associated with metabolic dysfunction by E_2 described in this review becomes problematic when extending to clinical practice. However, 10 years after the WHI concluded that the risks of hormone therapy outweigh benefit, reevaluation of the WHI findings and determination that the risks of breast cancer, coronary heart disease, stroke, and pulmonary embolism with estrogen–progestin treatment were overstated, a revised position statement issued by the North American Menopause Society [274], stating that HRT has a role in short-term treatment of menopausal symptoms has led to the renewed interest in investigating the therapeutic benefits of HRT. Thus, moving forward, it will be important to determine whether short-term treatment with HRT during early menopause offers protection against metabolic dysfunction.

Additionally, it is imperative that we determine mechanistically how best to modulate specific ERα-regulated pathways involved in energy balance and glucose homeostasis and develop targeted estrogen mimetics yielding metabolic benefit without unwanted side effects. This could be achieved possibly by fusion peptides [275, 276] or through novel SERMs that retain the beneficial metabolic effects of E_2 in desired tissues, while exerting minimal or antagonistic effects on ERs in breast and uterus. With regard to whole body metabolism and obesity, future studies should focus on identifying the critical brain sites where ERs regulate body weight homeostasis and delineate the intracellular signaling pathways that are required for estrogen action. Additionally, determining the functional role and molecular mechanisms ER action in islets and immune cells, skeletal muscle, liver, and adipose tissue as well as the metabolic crosstalk between these tissues may reveal additional pharmacological targets for therapeutic intervention. Furthermore, a major limitation in our understanding and interpretation of E_2 action is the lack of information regarding the contribution of extranuclear vs. nuclear ER actions, as well as ligand vs. non-ligand-mediated functions of estrogen receptors in metabolic tissues. Delineation of these pathways and the tissue specificity with which these signaling pathways are engaged will be critical in moving the field forward and advancing therapeutic strategies to improve women's health.

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Chapter 7
Metabolic Health in the Aging Female: Human Perspective

Alice S. Ryan

Abstract The incidence rates of overweight and obesity has increased dramatically over the last several decades and women with increased body fat and a sedentary lifestyle have a greater risk for cardiovascular disease, type 2 diabetes mellitus, hypertension, dyslipidemia, and certain cancers. This chapter provides a human perspective of obesity and aging, describes the cardiometabolic profile of postmenopausal women, presents a view of racial disparities in older women, and discusses the effects of aerobic and resistive exercise training on metabolic health in aging women. Finally, the chapter imparts some future research directions in the aging female.

Keywords Postmenopausal women • Obesity • Exercise • Metabolism

Overweight and Obesity in Aging Women

Body mass index (BMI), a measure of an individual's weight in relation to height is used to define overweight and obesity. Overweight in adults is defined by a BMI between 25 and 29 kg/m^2. Obesity is defined as a BMI $\geq$ 30 kg/m^2. Further classifications for obesity by BMI include Class I obesity (BMI: 30.0–34.9 kg/m^2), Class II obesity (35.0–39.9 kg/m^2), and Class III or morbid obesity (BMI $>$ 40 kg/m^2). Numerous adverse health conditions and diseases are associated with overweight and obesity such as insulin resistance, dyslipidemia, and risk for type 2 diabetes, stroke, arthritis, and cardiovascular disease. Obesity is also a well-established risk

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factor for most common cancers including cancer of the breast (postmenopausal), colorectal, esophagus, liver, gallbladder, and uterine cancer. There is also evidence that being overweight increases the risk for cancer recurrence and may reduce likelihood of survival [1–4].

The National Center for Health Statistics began tracking the prevalence and trends of overweight US adults in 1960 with the National Health Examination Survey (NHES I) and completed additional National Health and Nutrition Examination Surveys (NHANES I, II, III, continuous) in 2000 [5]. During this 40-year period, the prevalence of obesity increased significantly from 18.5 to 37.8 % in 40–59-year-old women and from 26.3 to 35 % in women >60 years of age between 1960 and 1999–2000. From 1999 to 2004, there was no increase in the prevalence of obesity in women. Trends in obesity from 1999 to 2008 are the most recently reported NHANES data [6] which indicated a 68 % overall prevalence of overweight and obesity and slightly lower prevalence of 64 % among women only. The prevalence of obesity varies by age and racial and ethnic groups for women such that the likelihood of being obese was significantly greater in both non-Hispanic black women and Mexican American women than non-Hispanic white women [6]. In particular, the likelihood of being obese was significantly higher in women aged 40–59 years as well as in the age group 60 years or older compared to the 20–39 year age group which suggest that the prevalence of obesity did not appear to be growing at the same rate as it had over the last 10 years for women [6]. Given this evidence, it could be inferred that women may be taking steps to comply with current nutrition and physical activity advice to modify overweight and obesity.

An obvious concern of severe obesity is that it is associated with increased mortality [7]. Reduced physical fitness is also associated with increased risk of all-cause and CVD mortality [8–10]. This is also true in postmenopausal women wherein there is a graded inverse relationship between physical activity and all-cause mortality risk, indicating that increasing frequency and the intensity of exercise results in a reduced mortality rate [11]. Middle-aged women who maintained a healthy lifestyle by not being overweight, not smoking, exercising moderately or vigorously 30 min/day, and eating a healthy diet, had more than an 80 % reduction in the incidence of coronary events compared to those women without these traits [12]. Furthermore, brisk walking and vigorous exercise are associated with considerable and similar risk reductions in the incidence of coronary events among women [13]. These investigations provide evidence that a program of regular moderate intensity exercise can have substantial health implications in the aging women.

Cardiometabolic Profiles in Postmenopausal Women

Body Composition

The increase in body fat in women as just described above not only effects disease risk and mortality but an increase in obesity in postmenopausal women may have a profound effect on the cardiometabolic profile. More specifically, regional

adiposity may confer a higher metabolic risk. We investigated whether there was a critical visceral fat level associated with elevated cardiovascular risk factors in over 200 older women [14]. A woman with a visceral fat of ≥106 cm^2 was five times more likely to have a reduced HDL-cholesterol and about four times more likely to have a high LDL/HDL cholesterol ratio than women whose visceral fat was below this level [14]. In addition, women in the highest quintile of visceral fat (>163 cm^2) were also at a higher risk of impaired glucose tolerance, whereas those postmenopausal women in the lowest quintile (≤105 cm^2) had lower fasting glucose and insulin concentrations and better lipid profiles than all other quintiles of women with higher visceral fat levels. As shown in other populations, insulin sensitivity determined by a hyperinsulinemic-euglycemic clamp is negatively correlated with waist circumference, waist-to-hip ratio, and visceral fat in postmenopausal women [15]. These results suggest that increased abdominal adiposity and an upper body fat distribution are associated with insulin resistance in older women.

Lower body fat may also be important in cardiovascular risk and the insulin resistance observed in postmenopausal women. Mid-thigh intramuscular fat was associated with fasting insulin, leptin, triacylglyccerol, total cholesterol, and low-density lipoprotein (LDL)-cholesterol [16]. We also found in an early study that insulin sensitivity was negatively associated with mid-thigh low-density lean tissue or intramuscular fat (Fig. 7.1) [15]. Our data was recently confirmed wherein increased thigh intermuscular fat was associated with insulin resistance in healthy early postmenopausal women [17]. In addition, insulin sensitivity was positively associated with thigh subcutaneous fat independent of total body fat [17]. Postmenopausal women with levels of low muscle attenuation (increased intramuscular fat) had worse metabolic risk including elevated fasting and 2-h glucose and reduced insulin sensitivity by the glucose clamp [18]. Furthermore, women with high muscle attenuation (lowest mid-thigh intramuscular fat) as well as increased visceral fat had the worst profiles [18], suggesting that visceral fat may be more important than fat within the muscle in terms of metabolic risk.

In the Kronos Early Estrogen Prevention Study, healthy early postmenopausal women ($n=650$) underwent computed tomography (CT) imaging to study cardiac and intra-hepatic fat and their relationship to metabolic risk factors [19]. Increased epicardial adipose tissue was associated with high LDL-cholesterol, triglycerides, glucose, insulin, hs-C-reactive protein (CRP), and low HDL levels. Likewise, higher pericardial adipose tissue was associated with increased triglycerides, insulin, hs-CRP, and low HDL-cholesterol. Results of these associations of cardiac fat, in general, persisted after adjustment for total obesity by BMI and abdominal fat (waist circumference). In addition, hepatic fat was associated with a worse profile of the above risk factors in this large group of postmenopausal women [19]. In another study, uric acid, BMI, waist circumference, alanine aminotransferase, triglycerides levels, and HDL-cholesterol were associated with insulin resistance measured by Homeostasis model assessment (HOMA) in postmenopausal women without diabetes [20]. In summary, there is evidence in postmenopausal women that subcutaneous abdominal fat and ectopic fat (visceral fat, thigh intramuscular fat, and hepatic fat) are associated with increased disease risk.

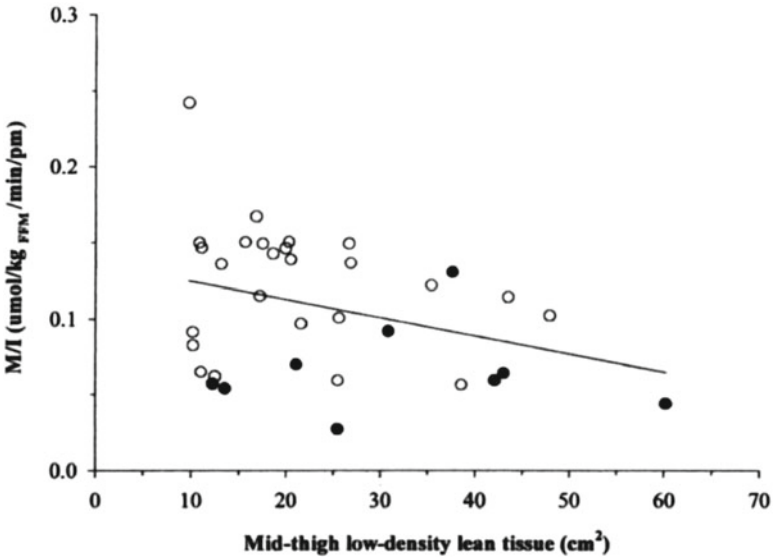

Fig. 7.1 Relationship between mid-thigh low-density lean tissue and insulin sensitivity, M/I ($r = -0.33$, $P = 0.05$) in African American and Caucasian women

In a comprehensive longitudinal examination of changes in body composition during menopause, Lovejoy et al. [21] found that women who became postmenopausal by the fourth year of the study had gained weight and body fat. In addition, only women who became postmenopausal had an increase in visceral fat, whereas all women gained subcutaneous abdominal fat over time. The group also reported that not only did energy expenditure by accelerometry decline as the women aged but 24-h energy expenditure and substrate oxidation measured with a whole-room calorimeter decreased over time, with greater decreases in the women who became postmenopausal [21]. This study was the first longitudinal investigation to substantiate the cross-sectional observations of increases in abdominal fat in women during the perimenopausal years. It is thought that fat distribution changes may be influenced by sex hormone concentrations.

Hormonal Status

Several reports have examined hormones or metabolites such as insulin, sex hormone-binding globulin (SHBG), testosterone, leptin, and adiponectin to investigate hormonal status in postmenopausal women. A higher fasting insulin is associated with a worse lipoprotein lipid profile (increased triglyceride and reduced HDL-cholesterol) in postmenopausal women [22]. Insulin was also associated with

apolipoprotein A–I after adjustment for abdominal adiposity, estrone, and SHBG [22]. When body fat is similar between perimenopausal and postmenopausal women, fasting insulin is not different between groups [23]. Fasting insulin increased with an associated weight gain in women studied over a 3-year period of menopause [24].

SHBG is a serum glycoprotein that binds testosterone with high affinity and estrogen with lower affinity and is considered an indirect marker of androgenicity. Serum SHBG is negatively associated with obesity [25] and visceral fat [26]. SHBG is also associated with insulin resistance and glucose tolerance in postmenopausal women [27, 28] and is an independent marker of risk for type 2 diabetes [29]. In over 750 postmenopausal women who were lean to obese (BMI range 15–53 kg/m^2), HOMA-IR, BMI, and diastolic blood pressure were inversely related to serum SHBG and combined, explained approximately 34 % of the variation of SHBG [30]. Furthermore, there was a positive relationship between serum SHBG and serum total testosterone; however, the relationship between SHBG and insulin resistance was independent of circulating testosterone [30], suggesting that androgens do not help explain this relationship. Others have shown that serum SHBG is negatively correlated with metabolic syndrome ([31] E) and markers of inflammation including CRP [32–34] in women. Moreover, serum SHBG is inversely correlated with serum CRP concentrations even after adjustment for age, components of the metabolic syndrome, insulin resistance, LDL-cholesterol, serum sex hormones, estradiol, and total testosterone [33], suggesting that SHBG may be independently associated with inflammation in Asian postmenopausal women.

Leptin, a product of the OB gene in humans, has been studied in the context of obesity and glucose homeostasis given that the OB protein regulates body weight and fat deposition through alterations in appetite and metabolism [35, 36]. Leptin is highly correlated with percent fat, subcutaneous abdominal fat, and visceral fat in women [37]. Moreover, leptin is associated with fasting insulin even after controlling for body fat. We also found that plasma leptin levels changed little from basal during hyperglycemic (approximately 10 mmol/L0 or hyperinsulinemic-euglycemic (400–3,000 pmol/L) clamp studies in women athletes and controls [37]. In ~150 postmenopausal women, serum leptin levels were significantly higher in women with the metabolic syndrome than those without the metabolic syndrome even after adjustment for BMI, WHR, and visceral fat [38]. Leptin is also associated with BMI and total fat mass in Japanese postmenopausal women with knee osteoarthritis [39]. Menopausal status is a significant predictor of both leptin and adiponectin in premenopausal and postmenopausal Tunisian women [40]. In another cross-sectional study, plasma leptin was associated with BMI and percent body fat in physically active postmenopausal women between the ages of 50 and 85 [41].

Adiponectin, a peptide expressed specifically and abundantly in adipose tissue [42, 43], is considered an anti-inflammatory and insulin-sensitizing adipokine which is lower in obesity [44]. In approximately 150 women, adiponectin and leptin levels were measured to examine the relationships among those adipocytokines, total and central obesity, and insulin sensitivity across the adult age span in women [45]. Adiponectin was negatively associated with BMI, percent fat, waist

circumference, visceral fat, subcutaneous abdominal fat, and leptin [45]. In healthy postmenopausal Korean women, adiponectin was significantly negatively correlated with waist circumference, high-density lipoprotein cholesterol, diastolic blood pressure, and insulin resistance by HOMA model [46]. In the Women's Health Initiative Observational Study, adiponectin levels were associated with stroke risk factors including obesity and systolic blood pressure [47]. We also determined peripheral tissue sensitivity to exogenous insulin using the hyperinsulinemic-euglycemic clamp and showed that in a multivariate analysis, only insulin sensitivity or M was a significant independent predictor of adiponectin [45]. The mechanism by which adiponectin exerts its insulin-sensitizing effects is through a decrease in muscle and liver triglyceride content [48]. In skeletal muscle, adiponectin increases fatty acid oxidation [48, 49] by inactivating acetyl CoA carboxylase (ACC) and activating AMP-activated kinase [50], thereby regulating glucose metabolism.

Racial Disparities in Postmenopausal Women

Several studies from our group suggest that race may differentially affect body composition in the older female. Postmenopausal African American women have significantly more subcutaneous abdominal fat and greater waist circumference than Caucasian postmenopausal women [15, 51]. Mid-thigh low-density lean tissue (a marker of intramuscular fat) is almost 35 % higher in postmenopausal African American than Caucasian women [15]. Studies are conflicting with regard to racial differences in visceral fat. Caucasian postmenopausal women had approximately 22 % higher visceral fat area but similar subcutaneous fat area in one study [14] and no differences in visceral fat area in others [15, 51, 52]. Subcutaneous abdominal fat at L2–L3 was lower in African American than Caucasian postmenopausal women so that the ratio of visceral to subcutaneous fat was lower in African than in the Caucasian women [52].

We have also studied postmenopausal women who are overweight or obese and sedentary and made several metabolic comparisons between African American and Caucasian women. Postmenopausal African American women had similar circulating levels of SHBG even after adjustment for body fat and fasting insulin as Caucasian postmenopausal women [51]. Furthermore, levels of testosterone did not differ between groups [51]. The associations of body composition, insulin, and lipids with SHBG differ between African American and Caucasian obese postmenopausal women. In African American women, SHBG is not related to central obesity, insulin, or HDL-cholesterol, but SHBG is related to these factors in Caucasian women. These results suggest that after menopause, sex steroid metabolism may affect regional fat distribution, lipid and glucose metabolism differently in African American and Caucasian women [51, 53, 54]. Postmenopausal African American women have higher insulin levels [15, 51], higher insulin area under the curve [51] and higher leptin concentrations [51] than Caucasian postmenopausal women. Leptin concentration was 20 % lower in obese postmenopausal African American than Caucasian women matched for level of body fat [55]. In an early study, we

reported that postmenopausal African American women had 60 % lower glucose uptake, assessed during a 240 pmol/m^2 per minute hyperinsulinemic-euglycemic clamp due to a 98 % lower nonoxidative glucose disposal [15]. Insulin sensitivity was negatively correlated with mid-thigh low-density lean tissue, suggesting that increased fat deposition in the muscle in African American women decreases insulin sensitivity. Rates of basal and insulin-suppressed lipolysis in abdominal fat were higher in African American than Caucasian postmenopausal women [52]. The decline in sex steroids with menopause may contribute to this finding. More studies are needed to investigate the mechanisms that may contribute to the body composition and metabolic differences between African American and Caucasian postmenopausal women.

Exercise Training in the Aging Female

Exercise training has beneficial effects on cardiometabolic health and traditional cardiovascular risk factors including obesity, hypertension, dyslipidemia, and glucose intolerance. Studies in highly competitive athletes offer a unique perspective to compare to healthy but sedentary older women. In comparisons of athletes and sedentary women, we have reported that women athletes (swimmers, runners, triathletes) between 18 and 69 years of age have lower percent body fat, fat mass, visceral, and subcutaneous abdominal fat than normal BMI age-matched controls [56]. Of particular interest to this chapter, the postmenopausal athletes had half the amount of visceral fat than the older controls as well as similar total body fat and subcutaneous abdominal fat to the young athletes implying that the competitive nature and increased endurance training enabled the older athletes to maintain a reduced adiposity despite their age [56]. We also studied glucose metabolism in these older women athletes and reported β-cell sensitivity to glucose and peripheral tissue sensitivity to insulin was preserved in women athletes as a function of age [57]. The older sedentary women had a 70 % greater first-phase insulin response, a 103 % greater second-phase insulin response during hyperglycemia, and utilized significantly less glucose than the older athletes, indicating increased insulin sensitivity in older athletes [57]. HDL-cholesterol is greater and LDL-cholesterol is lower in women athletes than sedentary women [58]. Moreover, total and LDL-cholesterol increased with age even after adjustment for fitness (VO_2max) and visceral fat suggesting that changes in lipoproteins are due to primary aging [58]. These studies highlight the benefits of high-intensity training in older competitive women athletes.

Exercise, Body Composition, and Cardiometabolic Outcomes

Improvements in physical fitness and changes in body weight are important to changes in body composition. In sedentary obese postmenopausal women who participated in a walking and weight loss program, a 10 % increase in VO_2max results

in a 20 % decrease in visceral fat, whereas a lack of change in VO_2max decreases visceral fat by about half that amount (10 % decrease) [59]. In several of our studies of different groups of postmenopausal women, we have examined changes in body composition after weight loss alone, or weight loss combined with aerobic training over a 6-month period. We report that an 8–10 % decrease in body weight results in a 13–18 % decrease in visceral fat and a 12–17 % decrease in subcutaneous abdominal fat [60–63]. Weight loss and walking also reduced intramuscular fat by ~4–18 % in postmenopausal women [60–62, 64]. To study the effects of exercise intensity on visceral fat loss, Nicklas et al. [65] conducted a randomized clinical trial in overweight and obese postmenopausal women enrolling in either 20 weeks caloric restriction alone, calorie restriction plus moderate exercise, or calorie restriction plus vigorous exercise. All groups lost significant amounts of weight with similar losses in visceral fat and subcutaneous abdominal fat [65]. Changes in the metabolic parameters (decreases in triglycerides, LDL-cholesterol, fasting glucose and insulin, and areas under the curve) did not differ by group. In this same trial of caloric restriction versus caloric restriction + moderate intensity, versus caloric restriction + vigorous intensity exercise in obese postmenopausal women, pericardial fat decreased slightly more than 15 % among all groups without a significant difference between groups [66]. These studies all suggest that reduction in weight by a decrease in energy intake is critical to the loss of fat in the various depots.

We have shown that resistive training alone does not result in a loss of body weight or total body fat but total body FFM and thigh muscle area increase after resistive training in postmenopausal women [67]. Others have also shown gains in muscle mass after resistive training postmenopausal women [68, 69]. After 10 weeks of unilateral strength training, muscle volume increased without changes in intermuscular fat or mid-thigh subcutaneous fat in women aged 50–85 years of age [70]. The women over the age of 65 then completed a 12-week program of whole-body strength training and had significant increases in FFM [71]. Melnyk et al. [72] reported increased proximal, middle, and distal quadriceps muscle areas of the thigh after a 9-week one-legged strength training program in older (65–75 years) women, suggesting that the increases in muscle area occur across the entire thigh muscle. In contrast, one study has shown that 6 months of progressive whole-body resistive training did not change lean mass in older aged [65–74] year women [73]. Visceral fat has been reported to decrease after resistive training in older women [74]. On the contrary, visceral fat and subcutaneous abdominal fat did not change but total body FFM increased by ~1 kg in community-dwelling frail elderly (>78 years of age) women after a 3-month progressive resistance training program that followed an aerobic exercise training intervention [75]. In a randomized control trial, Kemmler et al. [76] showed that appendicular skeletal muscle mass and total lean body mass increased and abdominal and total body fat by DXA decreased after an 18-month high-intensity exercise (aerobic, balance, strength components) in 250 elderly community-dwelling women. Thus, in contrast to aerobic exercise that results in minimal changes in FFM, resistive training alone may increase total body and regional muscle mass but the population studied may be important with respect to body composition results.

Endogenous sex hormones were studied in overweight postmenopausal women with impaired glucose tolerance who underwent an intensive lifestyle modification program or metformin of the Diabetes Prevention Program [77]. SHBG levels increased and dehydro-epiandosterone (DHEA) decreased without changes in estradiol or testosterone in the lifestyle group. Furthermore, changes in SHBG and DHEA were associated with reductions in both fasting and 2-h glucose levels independent of changes in waist circumference and fasting insulin [77]. An 8-week cycle exercise program decreased fasting insulin and insulin-like growth factor (IGF)-1 levels and did not change IGF binding protein-3 in a small sample of overweight and obese postmenopausal women [78]. In sedentary postmenopausal women from Brazil, a 16-week resistive training program increased IGF-1 levels but total testosterone, estradiol, and cortisol remained unchanged [69].

Exercise and Energy Expenditure

Postmenopausal women had a reduction in daily physical activity energy expenditure on the days they participated in center-based moderate or vigorous exercise [79]. The authors suggest that postmenopausal women may compensate for the increased energy expended during exercise sessions by reducing their activity/energy levels outside the structured program [79]. This could have important implications for weight control and obesity in postmenopausal women, especially for women who are trying to maintain their current weight or prevent weight regain after weight loss.

Sedentary overweight and obese postmenopausal women who have higher levels of physical activity energy expenditure have better lipoprotein and lipid profiles and lower circulating inflammation than those women with lower activity levels [80]. In a post hoc analysis combining two cross-sectional studies in inactive postmenopausal women, Lavoie et al. [80] reported that the interaction of physical activity energy expenditure by doubly labeled water and diet quality by the Canadian Healthy Eating Index were associated with higher HDL-cholesterol, apoB, LDL-cholesterol/apoB ratio, and lower hs-CRP levels, suggesting that physical activity and dietary habits work synergistically to create a favorable cardiometabolic risk factor profile.

Exercise and Glucose Metabolism

Cross-sectional and longitudinal studies consistently demonstrate the benefits of exercise on glucose homeostasis. In a large population of elderly men and women, physical activity, by self-administered questionnaire decreased with increasing glucose intolerance and persisted after adjustment for age, BMI, waist–hip ratio, family history of diabetes, and smoking [81]. This was confirmed in a similar recent study

where insulin sensitivity by a euglycemic clamp was not different in younger women athletes versus older endurance-trained athletes [82]. Since the normal-weight younger subjects have similar insulin sensitivity to the older subjects, it could suggest that the obesity and physical inactivity are more important in insulin resistance than aging per se [82]. Insulin sensitivity as estimated by the homeostatic metabolic assessment for insulin resistance (HOMA-IR) was determined in over 750 athletes representing 33 different sports [83]. Those athletes with the lowest HOMA-IR values were rowers and short-distance track athletes, whereas archery and field-throwing athletes had higher HOMA-IR values than the control group. Weight-lifting athletes may not confer the same metabolic benefits in terms of glucose metabolism as aerobically trained athletes.

Aerobic exercise training can improve glucose metabolism in overweight and obese postmenopausal women by reducing glucose and insulin levels during oral glucose tolerance tests and increasing glucose utilization during glucose clamps [61, 64]. The exercise effects may be long lasting in older women given that insulin sensitivity increased approximately 20 % even when glucose clamps are performed 72 h after the last training session [84]. When whole-body insulin resistance is estimated by HOMA-IR, it decreases with diet alone and exercise + diet but not exercise alone compared to control in overweight and obese postmenopausal women [85]. We recently conducted a large clinical trial of caloric restriction alone and combined with 6 months of aerobic training. Postmenopausal women underwent skeletal muscle biopsies prior to and during a hyperinsulinemic-euglycemic clamp both before and after the interventions to examine the mechanisms responsible for improved insulin sensitivity with these lifestyle interventions [62]. The effect of in vivo insulin to increase glycogen synthase (GS) fractional activity is strongly correlated to whole-body glucose utilization (Fig. 7.2). In addition, GS fractional activity was significantly lower in women with impaired glucose tolerance than women with normal glucose tolerance which likely contributes to the insulin resistance in women with impaired glucose tolerance. As a result of the interventions, the change in glucose utilization was associated with the change in insulin-stimulated GS fractional activity. In examining this further, we found that in women with impaired glucose tolerance, there is an enhanced insulin-stimulated GS activity following aerobic exercise + caloric restriction and the effect of insulin to increase GS total activity is greater after the combined intervention than caloric restriction alone [62]. Our results would suggest that adding aerobic exercise to caloric restriction improves insulin sensitivity in overweight and obese postmenopausal women at risk for diabetes and that changes in GS activity may be one contributing mechanism.

Resistive training has also been studied as a mode of exercise to improve glucose homeostasis in postmenopausal women. Resistive training does not change fasting plasma glucose [86] regardless of age or sex [87]. Yet, resistive training does improve glucose metabolism in women [86–88]. A 4-month resistive training program alone and resistive training plus weight loss increased insulin action and reduced hyperinsulinemia as assessed by hyperglycemic clamps in middle-aged postmenopausal women [88]. Furthermore, a 6-month resistive training program

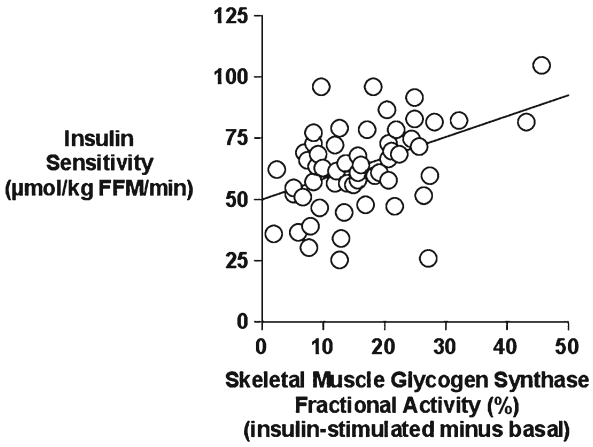

Fig. 7.2 Relationship between skeletal muscle glycogen synthase (GS) fractional activity (insulin-stimulated minus basal) and insulin sensitivity (M, glucose utilization) in postmenopausal women ($r=0.45$, $P<0.005$)

tended to improve insulin action in older postmenopausal insulin-resistant women, and the change in glucose utilization was a function of initial glucose utilization [86]. Even though there are a limited number of metabolic studies after resistive training in postmenopausal women, the current evidence would imply that this type of exercise is advantageous in the aging female.

Additional Benefits of Exercise Training

Physical activity is also important to women during and after menopause for physical and psychological reasons. The benefits of exercise training on increasing bone mineral density or reducing the loss of bone density with aging in older women are beyond the scope of this chapter. Readers are encouraged to peruse other chapters or reviews on this topic [89–91]. There are a few studies which report the importance of physical activity in enhancing quality of life among menopausal women [92], reducing frequency of hot flashes [93, 94], and controlling body weight or reducing a gain in body fat [95]. Since weight gain during menopause is associated with increases in total cholesterol, LDL-cholesterol, triglyceride levels, and blood pressure [24], weight reduction programs during and after menopause may be particularly important to reduce cardiovascular risk. Women (premenopausal, perimenopausal, postmenopausal) who have an increase in physical activity over an 8-year period have improved quality of life compared to women whose physical activity declined [96]. Quality of life deteriorated in more women who were in the menopausal transition than postmenopausal women [96]. This indicates that increased physical activity levels in this period may be critically important.

Summary and Future Directions in the Aging Female

It is clear that obesity remains a significant concern for the aging female. The cardiovascular risk factors that accompany obesity such as dyslipidemia, hypertension, and glucose abnormalities, warrant the continued emphasis on initiating and maintaining exercise programs in the aging female. New nontraditional types of exercise should be explored in healthy women and older women with disabilities. As well, more studies should focus on elderly postmenopausal women as less scientific evidence and information is available for this group. The cellular mechanisms that underlie metabolic improvements after exercise training in postmenopausal women and the interaction with sex steroids remain fruitful areas of investigation.

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Chapter 8
Transitions Across a Lifetime: Unique Cardiovascular Physiology of Women and Relationship to Cardiovascular Disease Risk

Juliana M. Kling, Virginia M. Miller, and Sharon L. Mulvagh

Abstract Mortality from cardiovascular disease for women exceeds that of men. This health disparity reflects differences in economic, environmental and psychosocial factors but more importantly the lack of basic knowledge in differences of cardiovascular *physiology* between men and women that drives surveillance and treatment guidelines for women. Sex differences in disease frequency and presentation are due to sex chromosomes, as well as activational and organizational effects of the sex steroid hormones. This chapter addresses how two conditions unique to women, pregnancy and menopause, affect cardiovascular function and therefore, types, frequency, and expression of cardiovascular pathologies in women.

Keywords Cardiovascular disease • Estrogen • Hormone • Menopause • Pregnancy

Introduction

Mortality from cardiovascular disease for women exceeds that of men [1]. Factors contributing to this difference in mortality include exposures to psychosocial stressors, environmental toxins, access and utilization of heath care, lack of appropriate surveillance and treatment guidelines for women, and lack of utilization of validated guidelines where appropriate. These latter two factors may reflect the absence of basic knowledge of the differences in cardiovascular physiology between men and women.

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Sex differences in disease frequency and presentation are due to sex chromosomes, as well as activational and organizational effects of the sex steroid hormones. In women, biological sex is defined by two X chromosomes, one of which undergoes partial inactivation. Activational hormonal effects are reversible phenotypes which disappear with the loss of gonadal hormones such as estrogen, but reappear with reinstitution of the gonadal hormones such as with menopausal hormone treatments. Organizational hormonal effects, on the other hand, are those characteristics that are determined by sex steroid hormones but remain after the loss of gonadal function. Changes in the cardiovascular system associated with two conditions unique to women, pregnancy and menopause, demonstrate the activational and organizational effects of sex steroid hormones [2]. Thus, understanding the mechanisms contributing to disease variations based on sex chromosomal compliment and hormonal status may lead to more informed surveillance and treatment options for women including higher risk stratification and earlier monitoring of women with a history of hypertensive (and perhaps other) pregnancy disorders and use of menopausal hormone therapy (MHT) in perimenopause or early in the postmenopausal state for alleviation of menopausal symptoms. The following chapter will describe the established sex and gender differences in cardiovascular physiology while exploring the possible etiologies for these differences through recent scientific findings.

Regulatory Mechanisms Contributing to Sex Differences in Cardiovascular Physiology

Genetic Mechanisms

When considering genetic compared to environmental factors influencing cardiovascular disease, genetic factors influence cardiovascular risk profiles for coronary heart disease and stroke more in women than men, whereas environmental and lifestyle factors contribute more to risk for men than women [3, 4].

In men, the Sry locus of the Y chromosome contributes to development of hypertension through regulation of tyrosine hydroxylase, a critical enzyme in the synthesis of norepinephrine [5, 6]. In experimental animals, transfection of the Sry locus from hypertensive male animals to normotensive animals led to increased systolic blood pressure, plasma catecholamines, and tyrosine hydroxylase activity [7, 8]. Therefore, greater activity of tyrosine hydroxylase in men secondary to Sry expression predisposes men to hypertension differently than women. However, estrogen also modulates activity of tyrosine hydroxylase as well as other aspects of sympathetic nerve activity subsequently affecting vascular smooth muscle tone and peripheral resistance including: (a) the synthesis and release of the neurotransmitter, norepinephrine, (b) reuptake and degradation of norepinephrine, and (c) post-junctional activation of adrenergic receptors on the vascular smooth muscle and cardiac muscle [9].

These complex processes demonstrate the multiple genetic influences to blood pressure regulation and cardiovascular function. Although specific studies have identified ways in which estrogen affects peripheral sympathetic adrenergic neurotransmission in the peripheral circulation, similar data does not exist regarding cholinergic transmission. Estrogen, however, affects both sympathetic and parasympathetic function through central regulation. This is evidenced by the expression of estrogen receptors on central neurons. For example, injection of 17β-estradiol into the central nucleus of the amygdala decreased sympathetic nerve activity [10–12]. Vasomotor symptoms of menopause are most likely mediated centrally in the hypothalamic region of the brain. Similarly, parasympathetic and sympathetic tone can be altered by decreasing levels of estrogen during menopause. This has been demonstrated in oophorectomized women whose heart rate variability was lower when compared to women of the same age with ovaries [13]. Estrogenic effects on cholinergic neurotransmission in the brain are also implicated in hippocampal function associated with memory [14, 15].

Early in the female developmental process, part of one of the two X chromosomes is inactivated in order to provide X equivalence between males and females. This process is dependent on the X-inactive-specific transcript (Xist) gene being transcribed from the X chromosome that is being inactivated, as well as a protein that assists in X chromosome silencing. These regulatory processes are complex. It is unclear whether inactivation of one particular X is permanent throughout life and exactly what X inactivation means for the physiology of women [16]. Moreover, it is unknown exactly how women compensate for the loss of the Y-linked genes, although it is believed that it is through up-regulation of their X-linked homologs [17]. Evidence demonstrates that the genes that miss X inactivation, which is about 15 % in humans, may contribute to phenotypic sex differences [18]. In fact, many of these genes are expressed more strongly in females. Sexual dimorphism is impacted by the sex chromosome complement independent of hormone influence [19]. The importance of escape genes after X inactivation is demonstrated in Turner's syndrome, which is a disorder of a single X chromosome, characterized by congenital cardiovascular malformations of the heart valves and large arteries as well as short stature, webbed neck, and ovarian dysgenesis [20]. Furthermore, genes on the X chromosome that escape inactivation are important in brain function [21] and possibly in aging [22]. It may be through these pathways, as well as their overall impact on gene regulation contributing to sexual dimorphisms, that produce sex differences in cardiovascular diseases. Although no specific escape genes linked to cardiovascular disease have been described, there are genes that exist on the inactivated X that affect metabolism and lead to oxidative stress because of the genetic material in the mitochondria. Oxidative stress can lead to chronic inflammation and subsequently progression of cardiovascular disease [23, 24].

The importance of genetic components of immunity pathways in development of cardiovascular disease in women is supported by SNP analysis of pathways associated with anticoagulant, procoagulant, fibrinolytic, and innate immunity in menopausal women who were within 3 years of menopause. In these estrogen-depleted women, carotid intima medial thickening (CIMT) was positively associated with

Table 8.1 Sex differences in cardiovascular diseases

Female	Male
Hypertensive disorders of pregnancy	Erectile dysfunction
Coronary artery dissection of pregnancy	Systemic hypertension
"hot flashes" of menopause	Myocarditis
Pulmonary hypertension	
Raynaud's disease	
Microvascular angina	
Migraine	
Postural orthostatic tachycardia syndrome (POTS)	
Heart failure with preserved ejection fraction (HFpEF)	
Apical Ballooning (Tako-Tsubo)	

Examples of cardiovascular diseases that are sex-specific or have differential prevalence in one sex compared to another

SNPS of the MAP4K4 gene and the IL5 gene. These genes mediate gene TNFα signaling and the differentiation of B cells and eosinophils, respectively. However, CIMT was negatively associated with CCL5 gene that modulates RANTES, a chemo-attractant for monocytes, eosinophils, and memory T-helper cells [25].

Hormonal Mechanisms

Activational and organizational effects of the sex steroids contribute to sex differences in cardiovascular function through specific receptors located in all tissues of the cardiovascular system. Activational effects of the sex steroids alter production of endothelium-derived factors, regulation of intracellular calcium and calcium-sensitivity of the smooth muscle, synthesis, and degradation of norepinephrine as well as the expression of adrenergic receptors of the vascular smooth muscle [2, 26, 27].

Organizational effects include regulation of cell proliferation and migration that may contribute to development of vascular lesions and cardiac remodeling [27, 28]. Thus, the predominance of testosterone in men and cyclic variation of ovarian hormones, estrogen and progesterone in women, will affect different structural and regulatory aspects of the heart and blood vessels. In women the increase in estrogen and progesterone with pregnancy allows the cardiovascular system to accommodate increased blood volume and cardiac output to sustain blood flow and nutrient requirements of the developing fetus. Therefore, sex differences in prevalence and expression of cardiovascular disease between men and women (Table 8.1) result from the combined effect of sex chromosomes, activation and organizational effects of the sex steroids. At menopause, activational effects will be lost with decline in ovarian hormones; thus, it would be expected that in women, a less adaptive cardiovascular system would become more "male-like" with age.

Phenotypic Expression of Sex Differences in Cardiovascular Disease

Hypertension

Although men may present with systemic hypertension at an earlier age than women, the incidence of pulmonary hypertension is greater in women than men [29–31]. Alterations in sympathetic activity affects vascular tone and subsequently arterial pressure and blood flow to end-organs such as the heart, brain, liver, and skeletal muscle [9]. As discussed above, regulation of adrenergic neurotransmission is affected by the presence of both the sex chromosomes and sex steroid hormones. In men, increases in resting sympathetic vasoconstrictor activity increases total peripheral vascular resistance [32] which may contribute to the manifestation of hypertension in men at younger ages than in women [33]. However, in young women, increases in sympathetic nerve activity do not cause a linear increase in total peripheral resistance or cardiac output [34]. Additional evidence demonstrating effects of hormones on sympathetic-mediated changes in blood flow are observed in changes in skin blood flow during a woman's menstrual cycle [35, 36]. These changes suggest that female sex steroids may modulate the response of the cutaneous circulation of the head, limb, and trunks that are controlled by adrenergic and cholinergic neurons that lead to vasoconstriction and vasodilatation, retrospectively [37]. Relationships between sympathetic nerve activation and total peripheral resistance are just beginning to be explored in menopausal women [38], but the peripheral and central factors controlling mechanisms of vasomotor disturbances (hot flashes/flushes) of menopause remain unknown.

Other examples of cardiovascular disease having a female predominance most likely reflecting differences in autonomic function are vasomotor disorders such as Raynaud's and postural orthostatic tachycardia syndrome (POTS) [9].

Stroke incidence, on the other hand, demonstrates a male predominance. For men, stroke incidence rates have been reported at 20.7 per 10,000, whereas for women rates are 9.6 per 10,000 [39]. Systemic hypertension is a major risk factor for stroke. Therefore, as the rates of hypertension in women go up after menopause, so too does this chance of stroke. Treating systolic hypertension can significantly lower the incidence of stroke [40], including hemorrhagic, ischemic, and lacunar stroke.

Sex differences in underlying physiological mechanisms will affect not only manifestations of disease (symptoms) but also response to treatment. For example, presentation of symptoms for myocardial infarction may differ in men and women with more nonspecific or abdominal-related symptoms seen in women and treatments for hypertension while effective in women may not always result in reduction of blood pressure in women to treatment targets [41].

Heart Failure with Preserved Ejection Fraction

Heart failure can manifest with depressed or preserved ejection fraction. In women, it is more common to find heart failure with preserved ejection fraction (HFpEF). The major risk factors for development of heart failure in women include hypertension and diabetes. Moreover, women have a higher risk for mortality than men if they suffer from left ventricular hypertrophy [42]. Evidence indicates that estrogen receptors in the heart modulate hypertrophy and subsequently, progression of heart failure. The composition of the vascular and cardiac extracellular matrix also contributes to the greater incidence of HFpEF in women, as well as Tako-Tsubo cardiomyopathy, a transient apical ballooning syndrome [43, 44]. Activational and organizational effects of hormones on cardiac remodeling that are adaptive to increases in cardiac output during pregnancy may be maladaptive in other circumstances, and the usual treatments directed towards increasing ejection fraction may not be appropriate for conditions when ejection fraction is preserved. Furthermore, it is unclear whether treatment guidelines for heart failure are applied to HFpEF, meaning women are not being started on renin–angiotensin inhibitors as often as those with depressed ejection fraction heart failure.

Ischemic Heart Disease and Microvascular Disease

Differences in response to myocardial injury between males and females are multifaceted and involve differences in contractile function, function of mitochondria, calcium metabolism, and cardiac growth [45]. Similar to hypertension, epidemiologic studies demonstrate that compared to men, premenopausal women have a reduced risk for ischemic heart disease. After menopause, this risk increases [46]. Ischemic heart disease in women is becoming recognized as having different etiology compared to obstructive disease characterized by occlusion of a large coronary artery at a single site [47]. Obstruction in women tends to be diffuse extending along the length of the artery [48, 49]. The risk factor profile for predicting development of ischemic disease may include factors other than those defined in the Framingham Risk Score (body mass index, total cholesterol, high density lipoprotein, systolic blood pressure, and smoking) [50] and include factors affecting ovarian function and pregnancy history [51–53]. Experimentally it has been shown that female sex favorably affects cardiac remodeling after reperfusion for ischemic injury. Sex steroid hormones are implicated in these differences. Interestingly though, cardiogenic shock is more common in women and women are less likely to present to revascularization-capable sites [54]. Moreover, women receive evidence-based therapy and coronary intervention less frequently than men demonstrating discrepancies in access or availability of care [46]. More research is needed to identify specific therapeutic strategies for women given identified underlying sex and gender differences.

Atherosclerosis and Other Inflammatory Associated Cardiovascular Condition

Propensity for inflammation may explain some of the sex differences in cardiovascular disease presentation. Atherosclerosis is an inflammatory disease provoked by biochemical sources such as lipid peroxidation and oxidative stress associated with metabolic syndrome [55], infection [56–58], or immunity-associated cellular activation [59, 60]. In general, inflammatory conditions in the general population demonstrate an increased propensity in women. For example, rheumatoid arthritis carries a 3.6 % prevalence in women and 1.7 % in men and Lupus has a 10:1 predominance in women. Most autoimmune disorders including Lupus are associated with increased risk of CV disease. However, some conditions, such as myocarditis, specifically fatal myocarditis, and myocardial fibrosis, have a male prevalence [61]. Additional research is needed to understand the mechanisms linking inflammatory conditions, immunity and cardiovascular disease, and preventive strategies in both men and women.

Thrombosis

Factors contributing to thrombosis are multifactorial and reflect not only genetic components but also the presence of infection, cancers, and mechanical injury [62, 63]. Biologically, these interactions were explained by Virchow as a triad of interaction of coagulability of the blood, blood flow, and anatomy of the vascular wall [63]. Coagulability of the blood reflects the type and amount of soluble proteins of the coagulation cascade and formed elements, that is, platelets, leukocytes, red blood cells, and cell-derived microvesicles [53]. Sex steroid hormones will affect production of proteins of the coagulation cascade and also the content of vasoactive and mitogenic factors in platelets by way of genomic actions in the liver and bone marrow megakaryocytes, respectively. Thus, it might be expected that hormonal shifts associated with pregnancy and menopause would affect risk for thrombosis. Indeed, incidence of thrombosis in the general population is age related and may be slightly greater in women of reproductive age compared to age-matched men and greater in older men than age-matched menopausal women [64]. Pregnancy may increase the risk for venous thrombosis but the factors contributing to risk may vary with each trimester reflecting hormonal changes on the blood components [65] and mechanical issues related to fetal and positional obstruction of venous flow.

Estrogenic menopausal treatments carry a black box warning for thrombotic risk. However, oral products may increase risk more than transdermal products due to the first pass effect on the liver and potential effects on the production of coagulation and inflammatory proteins in the liver apart from direct effects on platelets [66]. In ovariectomized experimental animals, estrogen treatments

decrease platelet aggregation and secretion of mitogenic, vasoactive factors [67]. Similar observations need to be confirmed in postmenopausal women as data are emerging to support relationships between activated platelets and pro-thrombotic microvesicles in development of carotid intima medial thickening (associated with stroke risk) and development of white matter hyperintensities in the brain of postmenopausal women [68, 69].

Sex-Specific Conditions for Women

Pregnancy

Cardiovascular diseases associated with reproductive function are sex-specific. In men, erectile dysfunction is a sex-specific condition which is not life threatening but may be indicative of systemic atherosclerosis which could lead to myocardial infarction [70]. On the other hand, some cardiovascular-related diseases of pregnancy such as hypertensive disorders and spontaneous coronary artery dissection can predispose the affected woman to increased life-long risk for major adverse cardiovascular events (i.e., myocardial infarction, stroke, death) [71–75].

Given the major physiological adaptations of the cardiovascular systems needed to support fetal health, pregnancy has been described as a stress test on the cardiovascular system of women [76]. While most women "pass the test" without difficulty, others develop hypertensive disorders during the gestational period that are classified by time of gestation, magnitude of systolic blood pressure and the presence of protein in the urine such as gestational hypertension, preeclampsia, and eclampsia (Table 8.2) [53].

It remains to be clarified as to whether pregnancy "unmasks" preexisting cardiovascular pathologies leading to hypertensive disorders that may increase a women's life-long risk for cardiovascular disease or whether particular circumstances of the pregnancy such as ischemic-induced injury to the placenta, angiogenic growth factors, maternal endothelial dysfunction, and/or immunologically related cytokines cause the hypertensive disorder [71] (Fig. 8.1). In a retrospective cohort study, a modest association was found between a history of hypertensive pregnancy and increased frequency (odds ration [OR], 1.62; 95 % CI, 1.00–2.63), severity and duration of vasomotor symptoms in women suggesting perhaps an underlying physiological condition that may predispose a women to both conditions. However, after correction for potential confounders such as age, socioeconomic status, sedentary lifestyle, smoking, hormone use, body mass index, and hypertension, the significant association was lost indicating the need to further investigate the causal relationships between pregnancy-associated hypertension, hypertension development in women as they age and menopausal symptoms that have an autonomic regulatory component [77].

Table 8.2 Definitions of hypertensive pregnancy disorders

<20 GW	≥20 GW	Delivery	≥12 weeks post-partum	Diagnosis
Normotensive	Hypertension without Proteinuria		Resolution of hypertension	Transient hypertension
	Hypertension without Proteinuria		Persistent hypertension	Chronic (incident) hypertension
	Hypertension with Proteinuria		Resolution of proteinuria and hypertension	Preeclampsia[a]

[a]Eclampsia is a convulsive form of preeclampsia and is based on new onset seizures, in the absence of a previous history of a seizure disorder. *GW* gestational weeks; Table is redrawn from fig. 3 of reference [53]

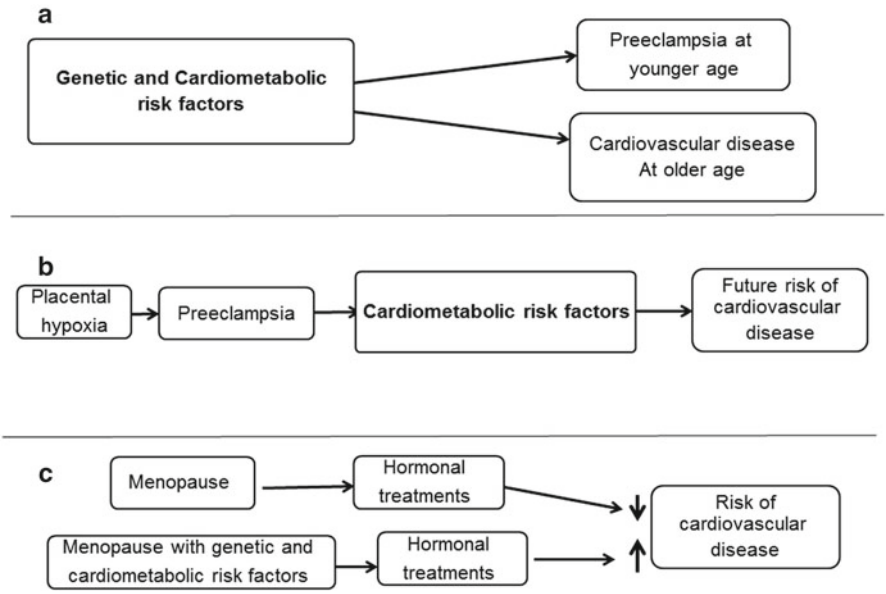

Fig. 8.1 Schematic of interaction of sex-specific conditions in women with cardiometabolic risk factors in establishing risk for development of cardiovascular disease. Cardiometabolic parameters include obesity, hypertension, diabetes, hyperlipidemia, inflammation, and infection. (**a**) The cardiometabolic factors predispose to development of preeclampsia, while in (**b**) preeclampsia initiates changes in the cardiometabolic profile that persists to increase life-long risk of cardiovascular disease. (**c**) Depicts two possibilities for disparate findings of effects of menopausal hormone treatments and risk for cardiovascular disease

Menopause

Menopause is the manifestation of decreased production of ovarian hormones and, thus, the loss of activational hormonal effects on the cardiovascular system. It would be reasonable, then, to propose that replacement or treatment of women with

ovarian hormones would restore these activational effects and reduce risk of cardiovascular disease in menopausal women. Indeed, estrogenic treatments reduce vasomotor symptoms of menopause and multiple observational studies demonstrate reduced incidence and all-cause cardiovascular disease mortality in menopausal women using such treatments for symptom relief [78–85]. However, observational studies are criticized for demonstrating healthy user bias.

The Women's Health Initiative (WHI) was designed as a prospective randomized, placebo controlled trial to evaluate risk of cardiovascular events in postmenopausal women. Treatments were conjugated equine estrogen alone or in combination with medroxyprogesterone acetate. The results of this trial were surprising in that the number of cardiovascular events was greater rather than fewer in the treated groups compared to placebo [86]. Important to note is that women in the WHI were greater than a decade past menopause (mean age about 63 years) and not representative of women using hormone treatments for menopausal symptoms as was the case in many of the observational studies [83, 85].

Results from basic science studies support the hypothesis that timing of initiation of hormonal treatments affects the outcomes. In studies of nonhuman primates, hormone treatments started at the time of ovariectomy reduced coronary artery atherosclerosis, whereas initiation of treatment 2 years later did not [87]. Thus, timing of initiation of hormonal treatments may contribute to their biological effects [88]. In terms of the classification of hormonal effects, there may be a limited period in which activational hormonal effects can be reversed. This period of opportunity may vary by cell type and the temporal manifestation of the physiological effects may exceed the duration of the treatment itself.

There are several studies which support these hypotheses. The Danish Osteoporosis Prevention Study randomized recently postmenopausal women to hormone treatment or placebo and found that after 10 years women receiving hormonal treatments had a significantly reduced risk (hazard ratio 0.48, 95 % confidence interval 0.26–0.87; $P=0.015$) of cardiovascular events such as myocardial infarction and heart failure with no increased risk of venous thromboembolism, cancer, or stroke [89]. However, in a subgroup analysis of women closer to menopause (50–59 years of age), cardiovascular events were fewer in the treated groups compared to placebo. In addition, in long-term follow-up of women in the WHI, decreases in coronary heart disease and stroke in women randomized to conjugated equine estrogen were not observed until about 7 years after cessation of treatment [90, 91].

Meta-analysis of epidemiological studies of women who had early menopause by oophorectomy and used hormonal treatments suggest that reversible activational effects of hormonal treatments might be tissue specific. Treatments initiated and used until the age of natural menopause seem to be protective against stroke but use of the treatments for years beyond the time of natural menopause increased the risk of stroke [92].

Hormonal treatments may interact with other metabolic parameters that contribute overall cellular viability and thus influence disease risk (Fig. 8.1c) [93]. For example, in the WHI, women meeting criteria for metabolic syndrome had an

increased risk for adverse cardiovascular events with estrogenic treatments [94]. Furthermore, as estrogens affect gene methylation, genetic variants that may associate with disease parameters in hormone replete or deplete conditions may not do so in the reverse situation.

Conclusion

Cardiovascular disease mortality for women exceeds that of men for many reasons including, but not limited to, biological differences due to sex chromosomes and both activational and organization effects of the sex steroid hormones. Underlying physiological regulatory mechanisms of the cardiovascular system differ in women compared to men resulting in sex differences in prevalence and presentation of cardiovascular conditions including those associated with autonomic regulation, development of hypertension, and conditions associated with vascular and cardiac remodeling. Hormonal mechanisms drive cardiovascular changes in two conditions unique to women, pregnancy and menopause. However, much remains to be learned regarding potential causal relationships among cardiovascular challenges associated with pregnancy and menopause and progression of cardiovascular disease in women across their life span in order to better define risk stratification, monitoring and treatments to reduce the disparity in cardiovascular mortality between women and men.

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Chapter 9
Estrogen, Cardiac Protection and Aging

Anne A. Knowlton

Abstract Estrogen loss and aging are inexorably linked. 17β-Estradiol (E2) can protect the heart from injury, but its effects are reduced in aging. Aging and estrogen loss contribute to a pro-inflammatory state and a decreased ability to handle ROS. This is further complicated by loss of the protective heat shock response with aging. E2 has many protective properties, including reducing cardiac injury and protecting the mitochondria, but the confounding effects of aging have not been well studied. E2 has a positive impact on pathologic cardiac hypertrophy and there are distinct differences in the roles of ERα and ERβ in hypertrophy, but whether this persists with aging is unknown. Clinically, postmenopausal women have an acceleration of atherosclerosis. Unexpectedly, double-blind randomized clinical trials of hormone replacement therapy (HRT) showed increased cardiovascular events and cancer with HRT. However HRT was initiated on average 10 years postmenopause and this likely contributed to the increase in cardiovascular events. Although our understanding of estrogen has come far over the last 20 years, much more basic research is needed to understand the consequences of aging and estrogen loss.

Keywords Aging • Cytokines • ROS (reactive oxygen species) • Heat shock proteins • Mitochondria • Gene expression • SERMs

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Overview

The dual processes of aging and estrogen loss impact the heart in ways we are just beginning to understand. Aging is recognized as being accompanied by the development of a pro-inflammatory state (Fig. 9.1) [1, 2]. This is compounded in females by the concomitant loss of estrogens, which are anti-inflammatory, cardioprotective steroid hormones. Hormone replacement therapy (HRT) was common until the first double-blinded randomized clinical trials of HRT were done in the 1990s [3, 4]. These studies demonstrated not only no cardiac benefit for HRT, but in some studies an increase in cardiac events. Not surprisingly these reports led to a dramatic drop in the use of HRT and to the rise of the idea that estrogens were toxic and must be avoided. However, given that women prior to menopause have a much lower incidence of cardiovascular disease than age-matched men, the story is of course much more complicated. As ideally with all new observations, the Women's Health Initiative and other clinical trials led to new thinking about estrogens and to new ideas. These are discussed as part of this chapter on estrogen, cardiac protection, and aging.

Although estrogen loss predominantly occurs later in life, basic studies on estrogen overwhelmingly have focused on young models such as 6–8-week-old mice. Because of this dearth of studies in aged models, studies on younger models will be discussed as well as those on aged models. This does not imply that there are no differences with aging, rather that much more investigation in aged models is needed.

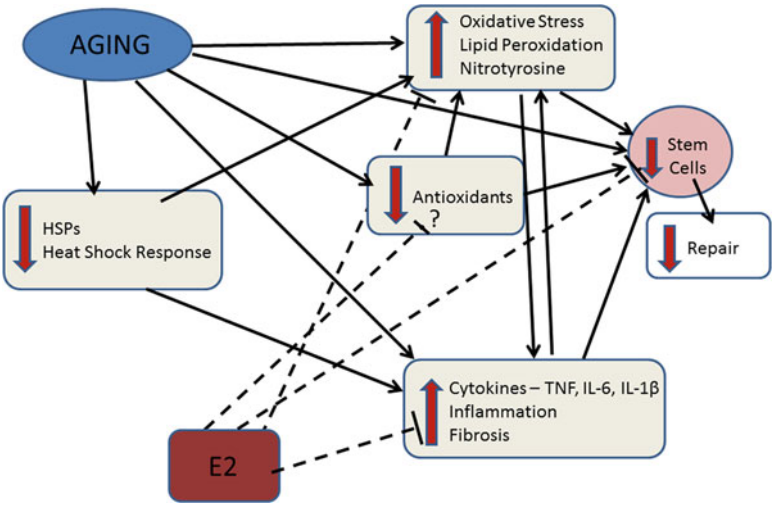

Fig. 9.1 Diagram summarizes known effects of aging and estrogen on HSPs and inflammatory stimuli in the heart. *Dashed line*—E2 effects; *solid line*—aging effects; *arrow*—stimulated; *blunted line*—inhibition

Aging, Estrogen, and Cardiac Function—It has been unclear if aging and loss of estrogen results in a decline in cardiac function. Subtle changes in cardiac function were reported in postmenopausal women compared to age-matched controls [5]. However, the mean age of these women was only 47. A careful study of cardiac function in intact Fischer 344 rats measured cardiac functional parameters at 4, 13, 22, and 30 months of age [6]. Fractional shortening decreased significantly at 22 and 30 months from 54.8 ± 8 % to 47 ∀ 7 at 22 months and 43 ∀ 9 at 30 months. Estrogen levels were not measured in this study, but levels are known to decline with age in the rat. Investigation of diastolic function demonstrated that isovolumic relaxation time increased by 30 % at 30 months [6]. At the same time there was evidence of LV hypertrophy and increased LV volume with advancing age. Others have reported a drop in fractional shortening in aged ovariectomized Norway Brown (NB) rats at 22 months compared to ovariectomized rats treated with 17β-estradiol (E2, the most potent human estrogen) replacement [7]. Studies of isolated cardiac myocytes from 10-week-old SD rats 10 weeks post ovariectomy (ovx), with half receiving E2 replacement, found that the ovx myocytes had decreased +dL/dt compared to sham surgery and ovx + E2 replacement [8]. Multiple other parameters were abnormal in the ovx group including peak shortening and −dL/dt. Calcium handling was also impaired with increased resting calcium in the ovx group. This was accompanied by a reduced SERCA2a/phospholamban ratio in the ovx group [8]. Thus, several studies support loss of cardiac function with aging and estrogen loss and this has implications for the aging population.

Cardiac Myocyte Changes with Aging

Clinical trials, although very important, are limited in their ability to provide insights into underlying mechanisms. Thus, basic studies on appropriate models, such as the aging vs. adult rodent with and without estrogen, are important for understanding differences related to aging vs. estrogen loss, in order that we can better elucidate the mechanisms of cardiovascular changes in aging females. Unfortunately, work in this area is very limited. Basic studies on cardiac myocytes from aged and adult (22 vs. 6 months) ovx NB rats with and without immediate E2 replacement demonstrated disparate responses with aging and estrogen loss [7]. In the intact rats, serum inflammatory cytokines, interleukin (IL)-6, and tumor necrosis factor alpha (TNF) did not differ among the adult and aged rats with and without E2 [7]. Cultured cardiac myocytes derived from the aged ovx rats had much higher expression of IL-6 and TNF at the mRNA level. This increase in IL-6 and TNF could be attenuated by treatment with E2 for 6 h. Furthermore, the aged ovx cardiac myocytes had a greatly impaired ability to handle reactive oxygen species (ROS), the production of which is known to increase with age [9, 10]. E2 treatment in culture had no effect on the inability of the aged ovx cardiac myocytes to handle ROS. Expression of antioxidant genes did not differ between young and aged groups. Thus for the aged cardiac myocytes there was an increase in inflammatory cytokine expression and a

markedly reduced ability to handle ROS with aging and loss of estrogen (Fig. 9.1). *The results suggest that tissue levels of cytokines may be more revealing than plasma levels.* In vivo E2 replacement improved many of these changes, and cytokine expression in culture was blunted by the addition of E2 to the media.

The Heat Shock Response, Aging, Estrogen, and Cardiac Protection

The heat shock response is a protective response that occurs with a diverse set of cell and tissue injuries and stresses including ischemia, hypoxia/reoxygenation, stretch (including angioplasty), and heat [11–13]. The heat shock response was originally described in heat-shocked drosophila in the 1960s. Heat and other stresses led to activation of heat shock factors (HSF), the predominant one for the heat shock response being HSF1. HSF1 is phosphorylated, trimerizes, and moves to the nucleus where it binds to heat shock elements in the promoters of the heat shock protein (HSP) genes, stimulating their transcription and translation. HSP72, the inducible HSP70, increases several fold with ischemia/reperfusion as well as with simulated ischemia in isolated adult cardiac myocytes, and inhibition of the increase in HSP72 with antisense, increases cell injury [14–16]. Thus, the endogenous heat shock response is protective. Further studies have shown convincingly that over expression of HSP72 in the heart protects against ischemic injury [17–19]. Thus, the heat shock response is vital to protection of the heart from injury.

E2 indirectly regulates cardiac HSP 72 expression in both male and female adult rat cardiac myocytes [20–22]. Cardiac HSP72 levels in female Sprague Dawley (SD) rats are twice the levels of males [22]. Ovariectomy leads to a drop in cardiac levels of female SD rats, but this change takes 9 weeks, suggesting that estrogen indirectly regulates the expression of HSP72 and that loss of estrogen leads to a cascade of changes. This finding has implications for the timing of studies looking at changes post ovariectomy. E2 replacement at the time of ovariectomy prevented the drop in HSP72 [22]. In addition to its role in protein folding, HSP72 has cardioprotective effects in transgenic models, reduces apoptosis by stabilizing the mitochondrial membrane, and prevents apoptosome formation [18, 23–25]. In isolated adult SD female cardiomyocytes, E2 treatment increased HSP 72 expression through the consecutive activation of the transcription factors, NFκB and HSF1 [26]. E2 pretreatment also protected against hypoxia/reoxygenation in cell culture [20, 27]. Recently we have shown that E2 activates NFκB via simultaneous activation of Akt, P38, and JNK followed by activation of ERK 1/2 and that inhibition of Akt. P38 or JNK prevents activation of NFκB [28]. Two synthetic estrogen receptor modulators, tamoxifen and raloxifene, also rapidly activated NFκB, but neither of these SERMs activated JNK [28]. Both tamoxifen and raloxifene mediated activation of NFκB via Akt and P38 leading to ERK 1/2 activation and cardiac myocyte protection. However, NFκB activation by each of these SERMs was less than that seen with E2. Although E2 can induce cardiac HSP 72 expression in adults, little is

known how the loss of estrogen that occurs naturally during menopause in aging female rats affects the cardiac HSP response and the adaptive response to stress.

HSPs, the Heat Shock Response and the Aging Heart—The response of the aged heart to estrogen differs from the younger heart. Cardiac myocytes derived from aged (22 months) and adult (6 months) NB rats 9 weeks post ovx with and without immediate E2 replacement were studied to determine the role of E2 in ameliorating the inflammatory changes of aging. The adult cardiac myocytes, regardless of E2 replacement status, showed activation of NFκB and increased HSP72 expression with estrogen treatment in culture [7]. However, neither aged group had NFκB nor increased HSP72 in response to E2, and in fact NFκB was activated at baseline in the aged ovx cardiac myocytes. Hypoxia and reoxygenation induce the heat shock response with activation of HSF1 and increased HSP expression, a protective response. As expected, both groups of adult cardiac myocytes had activation of the heat shock response, but the aged cardiac myocytes regardless of estrogen replacement had no activation of HSF1 and no increase in HSP72 [7]. HSF1 expression levels did not differ among the groups, but in the aged ovx phosphorylation at serines 303/307, which inhibits activation of transcription, but not binding to the promoter by HSF1, was present [7]. Thus with aging, in the female heart there was inhibition of the protective heat shock response secondary to posttranslational modification of HSF1. Estrogen replacement had no effect on this response. Similar loss of the heat shock response and loss of activation of HSF1 have also been found in male models of aging with different mechanisms proposed [29–31]. One mechanism of inhibition of HSF1 is acetylation of the transcription factor at lysine 80, which prevents activation of HSF1 by preventing binding of HSF1 to the heat shock element [32]. In the aged female rat cardiac myocytes, there was no evidence of acetylation of HSF1 [7]. These differences in mechanisms of inactivation of HSF1 in aging female cardiac myocytes and other tissue types and cell lines may reflect that there are multiple mechanisms regulating HSF1 activation or true gender/aging/tissue differences. Given the recent interesting link of DNA-damage-associated cell senescence with a drop in HSF1 as well as the importance of loss of the heat shock response in aging, clearly more investigation is warranted in this area [33, 34].

Ischemia and other injuries induce activation of the heat shock response with activation of HSF1 and increased expression of HSPs, particularly HSP72. Simulated ischemia failed to induce activation of HSF1 and increased expression of the cardioprotective heat shock protein (HSP)72 in aged female cardiac myocytes from aged ovariectomized NB rats, regardless of the presence or absence of estrogen replacement. In contrast, cardiac myocytes from ovariectomized adult NB rats had HSF1 activation and increased HSP72 with simulated ischemia regardless of estrogen status. In the aged cardiac myocytes only, regardless of E2 replacement, HSF1 was inactivated by phosphorylation at serines 303/307, and there was no increase in the cardioprotective HSPs after simulated ischemia [7]. As discussed above, E2 replacement in vivo prevented many of the increase in cytokines and ROS with aging and OVX, and in vitro addition of E2 to aged ovx-cultured cardiac myocytes reduced cytokine expression; however, neither in vivo nor in vitro E2 replacement prevented the inactivation of HSF1 associated with aging. This has important implications for

patients, as ROS and inflammatory cytokines, such as TNF, are an important causes of cardiac injury. The heat shock response mediated by activation of HSF1 is important for cardiac protection, and loss of the heat shock response makes the aging heart more vulnerable to injury.

E2 and Mitochondrial Protection

The mitochondrion, despite its importance for the generation of high energy phosphates to provide energy to fuel contraction, is also an important contributor to cardiac injury. Mitochondria produce a large amount of ROS as a by-product of energy production. In normal tissue, there is an extensive system of antioxidants and scavengers to prevent damage from these ROS. However, ischemic injury alters the balance of antioxidants and ROS. E2 is cardioprotective in the setting of ischemic injury, and amelioration of mitochondrial damage is likely a key component of this protective response. E2 treatment inhibited cytochrome c release from isolated cardiac mitochondria exposed to high calcium levels [35]. Likewise, E2 prevented cytochrome c release in global ischemia in the isolated perfused heart; furthermore E2 protected mitochondrial respiration and inhibited DNA fragmentation [36]. In isolated neonatal myocytes subjected to hypoxia and reoxygenation E2 reduced apoptosis and ameliorated ROS production mediated by prevention of p53 phosphorylation and translocation to the mitochondria [37]. In the isolated perfused heart the protective effects of E2(100 nM) during global ischemia/reperfusion were markedly decreased by inhibition of protein kinase G [38]. Similarly, inhibition of either Akt or eNOS reduced E2's protective effects [39]. E2 was also protective in trauma-hemorrhage (T-H), and this protection was mediated by ERβ, which lessened the reduction in cardiac mitochondrial ATP, inhibited lipid accumulation, and protected protein expression [40]. Inhibition of PGC-1α blocked E2's protective effects in a Tfam-dependent manner [40]. Thus protection of the mitochondria and reduction of ROS production are critical protective effects of E2 (Fig. 9.1).

Mitochondrial Proteomic Changes—E2 influenced the mitochondrial phosphoprotein profile in young rat hearts [41]. Aldehyde dehydrogenase 2 (ALDH2), which detoxifies ROS, has increased phosphorylation and increased activity in the female heart [41]. This increase in ALDH2 phosphorylation and activity is blocked by Wortmannin treatment. α-Ketoglutarate dehydrogenase (αKDGH), which is a major source of ROS with high NADH/NAD+ ratios, as found with ischemia/reperfusion, also had increased phosphorylation, but evidence suggests the increased phosphorylation may decrease activity and thus decrease ROS [41]. Whether these effects would occur in aged mitochondria is unknown.

Aging, Estrogen, and Mitochondrial Protection—Studies in aged hearts on estrogen and mitochondrial function have been quite limited. Aging, regardless of estrogen status, was associated with a small decrease in the respiratory control index (RCI, state 3/state 2) for complexes I and II [42]. Pretreatment with the selective estrogen agonist, propyl pyrazole triol (PPT), increased RCI for complexes I and II,

but also increased Ca^{2+} sensitivity in aged female mitochondria, regardless of estrogen status, suggesting ER signaling could be a double-edged sword in the aged mitochondria. [42]

Estrogen, Ischemia, and Cardiac Protection

There is a substantial literature demonstrating that estrogen can protect the heart from ischemic injury and several first-rate reviews have recently addressed this topic [43–45]. Activation of either ERα or ERβ has been shown to protect the heart from ischemia/reperfusion injury using knockout models as well as selective agonists [43, 46–49]. More recently a third, membrane associated ER, G-protein-coupled estrogen receptor (GPER) has been identified [50]. Pretreatment with G-1, a selective GPER agonist, decreased infarct size and enhanced functional recovery in a Langendorff perfused-heart model. Like ERα, GPER activated both Akt and ERK 1/2 [51]. Although some disagreement persists, the literature supports that ERα and ERβ each can activate PI3K, Akt, and a anti-apoptotic, protective signaling cascade [47].

Estrogen and Protection in Trauma Hemorrhage—A number of studies have investigated the benefit of E2 treatment in models of trauma hemorrhage (T-H) and neural injury. In T-H models reduction in organ neutrophil infiltration and improved cardiac function with a single treatment of E2 have been found [52, 53]. A one time treatment with high dose of E2 reduced mortality and improved recovery in a rat model of trauma [54]. A wide array of endpoints are improved with E2 treatment in basic studies of trauma hemorrhage and changes associated with E2 treatment included reduction in inflammatory cytokine levels and enhanced expression of heat shock proteins [55]. AKT activation by E2 led to increased heme oxygenase (HO)-1 expression in trauma hemorrhage [56]. Likewise, others have reported that female rats at peak estrus were had improved morbidity and mortality after trauma [57]. Similarly, female rats in proestrus/estrus, when estrogen levels would be high, had less red blood cell damage and less lipid peroxidation compared to males and low estrogen females after T-H-hemorrhage [58]. E2 just prior to cerebral ischemia increased heat shock protein expression in brain arteries, as well as glia and neurons [58]. Others found no difference in a spinal cord contusion model between females at low and high estrogen levels [59]; however, these studies were based on vaginal smears as an indicator of estrogen levels and compared proestrus and estrus female rats. Depending on the exact hour given the 4-day rat cycle, there may not have been striking differences in the estrogen levels, which were not measured, between groups. In a prospective clinical study of polytrauma, female patients less than 50, suggesting the presence of estrogen, have been shown to have better outcomes and less complications, including less days with multiorgan dysfunction syndrome (MODS), lower cytokine levels and less sepsis than age-matched males [60]. A weakness of the study was the assumption that women 50 or younger were pre-menopausal and estrogen levels were not measured. However, overall the studies on

trauma-hemorrhage are intriguing and raise the possibility that the benefit of a single high dose of E2 in the laboratory can be applied to trauma victims in the emergency room.

Heart Failure

Systolic Heart Failure—The prevalence of systolic heart failure (sHF) has increased over the last 10–15 years as a result of improvements in the treatment of acute heart disease. SHF, with dilation of the ventricle and loss of systolic function, occurs as a result of coronary disease and myocardial infarction (ischemic heart failure) as well as secondary to a diverse set of causes such as toxins and viruses, which are referred to as dilated cardiomyopathy or more recently as nonischemic cardiomyopathy. Gender differences have been reported in systolic heart failure characterized by an improved course and better survival in females [61, 62]. A retrospective analysis conducted using the Vesnarinone database found better survival in women taking estrogen. In contrast, analysis of the CHARM database demonstrated improved survival for women, but the presence or absence of HRT had no effect on survival [61, 63]. Given that there can be significant variation in the drugs used in HRT, it is not surprising that no difference was observed. Other studies have reported no gender difference in heart failure survival [64]. The pooled analysis of five heart failure trials again suggested longer survival for female patients [65]. Overall, the trial data suggests a possible gender variation, but underlying weaknesses preclude the drawing of strong conclusions. For some of the studies follow-up was only a year, too short to discern survival differences. All of these papers are retrospective analyses of databases collected with other aims, the number of female patients is limited and only two databases had data on the use of estrogen. Another issue is that gender and estrogen are used interchangeably for many of these studies, but women with heart failure may be premenopausal, postmenopausal, or postmenopausal on HRT. Thus these patients may have widely varying estrogen levels. Ergo, comparing the outcomes for all female patients vs. all males will be misleading unless the estrogen status of the female population is established. The type of estrogen replacement is also important, and benefit might not be seen with conjugated equine estrogen (CEE), which has been the most commonly used source of estrogen for HRT. CEE is derived from the urine of pregnant mares and contains no 17β-estradiol, which is the most potent form of estrogen found in women [44]. The type of estrogen(s) present matters as different estrogens will have different binding affinities and different selectivity for the estrogen receptors. CEE does contain estrones, and estrone levels have been linked to the generation of thrombin, which is a key step in the activation of coagulation [66]. Furthermore, most HRT has been via oral replacement and this leads to high hepatic concentrations of estrogens, secondary to first pass metabolism. High hepatic estrogen levels will alter gene expression, as a major function of ERα and ERβ is as transcription factors, and may increase the expression of proteins involved in the clotting cascade. Transdermal HRT is thought to be safer as it avoids

the high hepatic levels of estrogen seen with oral replacement. The heart failure databases contain little or no data on actual estrogen therapy, as the original intent of these studies was not focused on the effects of estrogen.

Overall the data on gender, estrogen, and morbidity and mortality is insufficient to draw any definitive conclusions with regards to a gender benefit or estrogen benefit in heart failure. Certainly there are many basic studies suggesting that estrogen has protective effects in the heart. Clinical studies directly investigating the effects of gender and/or HRT are needed to determine whether estrogen or gender improve survival in heart failure. The development of synthetic estrogen receptor modulators (SERMs, discussed in more detail below) raises the possibility of having a gender neutral estrogen analogue that can activate protective responses in the heart as E2 does.

A number of more focused studies have reported gender benefits in sHF. For example, female patients with sHF show greater benefit with cardiac resynchronization therapy (CRT) than male patients, but whether this is a gender-based difference or hormonal, remains unknown [67]. Explanted failing hearts removed during heart transplants have been extensively studied. It is known that the rate of apoptosis is increased in the failing heart, but in addition it has been found the end-stage failing female hearts have half the rate of cell death as male hearts [68]. Others have identified some differences in cardiac gene expression between females and males with new onset heart failure [69]. Females had higher expression PDE6b, which is cardioprotective, GLUT12, involved in glucose transport, and GATAD1, which is involved in the regulation of adrenergic and angiotensin receptor trafficking. Males had higher expression of CD24, which is involved in regulation of immunity.

Basic investigations on gender and heart failure have been quite limited. ERα is nearly double in nonischemic cardiomyopathy independent of gender [70]. In the normal heart ERα is found in the cytosol, sarcolemma, intercalated discs, and nuclei. In contrast, in end-stage failing hearts, neither ERα or connexin 43 was present in the intercalated discs. The Knaub group has made the interesting observation that in heart failure cyclic nucleotide regulatory binding protein (CREB) was disproportionately downregulated in female hearts. Female hearts were very susceptible to downregulation of CREB with a dominant negative transgene construct, while male littermates showed little response to this reduction in CREB [71]. As early as 4-week female hearts had increased ROS, decreased MnSOD, and decreased glutathione peroxidase. These changes were accompanied by loss of mitochondrial cristae, decreased cardiac function, and markedly increased mortality by 21 weeks. In contrast, males had far less evidence of cellular injury and much greater survival. CREB has been found to decrease in heart failure, it is possible that this decrease in CREB makes the course of heart failure more severe in females, though there is uncertainty as to whether females have better or worse survival in heart failure, as discussed above, and many questions remain to be answered [71].

Diastolic Heart Failure—Approximately half of heart failure is diastolic heart failure (dHF), which in contrast to sHF, is characterized by preservation of ejection fraction but impaired relaxation. Diastolic heart failure occurs secondary to pathologic hypertrophy, most commonly occurring as a result of hypertension [72, 73].

In pathologic hypertrophy there is inadequate capillary proliferation, leading to decreased capillary density and fibrosis. On the other hand, normal/physiologic hypertrophy occurs in response to exercise, pregnancy, and normal growth. Physiologic hypertrophy has normal capillary density without fibrosis. DHF is thought more prevalent in women and the incidence increases with aging. Therefore, women with dHF will tend to be postmenopausal and likely not on HRT, as HRT use has dropped dramatically in the last 10 years [74, 75]. Abnormal diastolic relaxation results in an increased left ventricular end-diastolic pressure, and this leads to elevated pressure in the pulmonary vasculature, which can lead to pulmonary congestion. Hence, despite good cardiac contractility, patients with dHF become dyspneic. Notwithstanding the preservation of ejection fraction, diastolic heart failure has a prognosis that is as grim as that for systolic heart failure. Investigators at the Mayo Clinic reported a 50 % 5-year survival in patients with dHF [76]. DHF can be more difficult to manage than sHF, where multiple medications are available to reduce symptoms and prolong survival. Treatment for dHF relies on beta blockers and calcium channel blockers to reduce the heart rate and possibly improve diastolic relaxation. In addition diuretics are used to reduce breathlessness. There have been no advances in treatment of diastolic heart failure in many years, and the effectiveness of current treatment is quite unsatisfactory.

Estrogen and Pathologic Cardiac Hypertrophy

Cardiac hypertrophy is more common in males. Transverse aortic constriction (TAC) has frequently been used as a model to investigate the effects of gender, E2 and ERα and ERβ on cardiac hypertrophy. WT, ERα, and ERβ knockout mice were ovariectomized and half were begun on standard sustained-release E2 replacement. E2 replacement reduced TAC-induced cardiac hypertrophy in ERα knockout but not in ERβ knockout mice [77]. E2-mediated reduction in TAC-associated hypertrophy was not associated with a change in cardiac function. In contrast, uterine hypertrophy was inhibited by ERα knockout but not by ERβ knockout. In ER∀ knockout and WT mice, but not ERβ knockout mice, E2 replacement prevented increased phosphorylation of p38, increased ANP, and decreased hypertrophy. In studies of intact female mice compared to males, males had more hypertrophy and heart failure post TAC. TAC led to increased matrix and mitochondrial gene expression, and again the changes were more pronounced in males than females. After ER∃ knockout, greater hypertrophy, including increased cardiac myocyte diameter ensued in both males and females [78]. ERβ knockout mice had increased expression of pro-apoptotic genes, and this was more marked in males than females. These studies are consistent with ERβ mediating the protective effects of E2 in pathologic hypertrophy.

Some very intriguing work has been done linking estrogen to increased calcineurin degradation, and this is mediated by ER receptor pathway, and is possibly specific to ERβ [79, 80]. E2 signaling promotes increased degradation of calcineurin via ubiquitination and the 26 s proteasomal system. In calcineurin knockout mice, E2

treatment did not inhibit LV hypertrophy [80]. Calcineurin knockout prevents downstream signaling by the NFAT family of transcription factors, which are key targets of calcineurin. These findings suggest that calcineurin degradation may be a major pathway for E2-mediated inhibition of hypertrophy. This work again implicates ER∃ as critical in mediating the protective responses to E2 in cardiac hypertrophy.

Quite interesting basic investigation has shown that E2 can modulate the pathologic hypertrophic response through several other mechanisms. There are three known estrogen receptors: ERα, ERβ, and GPER (also known as GPR30). In models of pathologic hypertrophy, E2 suppressed MMP2 expression, fibrosis, and hypertrophy through activation of ERβ [77–79, 81–83]. Nevertheless, the typical model for much of this work is 6–8-week-old mice, and these findings may not translate to older models or to aging humans. Others have demonstrated that GPER activation attenuates diastolic dysfunction as well as ventricular remodeling post ovariectomy in an aging hypertensive rat model [84]. These findings are very promising as GPER activation is not known to be associated with the adverse effects of estrogen, and dHF remains a difficult problem. New insights into the underlying mechanisms leading to dHF are needed to help develop better therapies. The use of aged models, to determine if E2 has similar effects in aging, is essential to develop new therapies for dHF.

Gene Expression

The unexpected finding that HRT postmenopause resulted in an increase in cardiovascular events as well as the expected increase in cancer led to a dramatic drop in the use of HRT. This decrease in HRT use has been followed by a drop in the incidence of breast cancer, which was not unexpected; however, the risk of cancer with HRT remains controversial [3, 74, 75, 85–87]. Nonetheless, the increase in cardiovascular events with HRT was surprising since there were so many reports of benefits. Careful analysis of the trials data led to the development of the timing hypothesis [88, 89]. In an effort to make sure patients were postmenopausal, the average patient enrolled in the trials was 10 years postmenopause. The timing hypothesis proposed that the long delay between menopause and reinitiation of estrogen lead to changes that were not corrected by late estrogen replacement. The timing hypothesis promotes the idea that the ideal time for initiation of HRT would be during perimenopause rather than postmenopause.

The mechanism of the increase in cardiovascular events with essentially late HRT initiation in the clinical trials was not evident, although as estrogen is known to increase thrombosis, this was considered a possible contributor to the greater number of coronary events in women on HRT. We hypothesized that late replacement of estrogen would have unexpected, pro-inflammatory effects on gene expression.

We investigated *late* replacement of E2 in an aging NB rat model, and found that late E2 replacement, with a sustained-release subcutaneous capsule, led to increased expression of genes associated with inflammation and/or monocyte adhesion to the

endothelium [90]. STAT3, fibronectin, iNOS, and MADD expression all increased with late E2 replacement. Interestingly, although TNF mRNA was not increased with late E2 replacement, the TNF protein levels were increased. The genes induced by late E2 replacement are pro-inflammatory and would have a negative affect on the vasculature promoting atherosclerosis and the development of ischemic heart disease. Furthermore, CD11b, which is a membrane receptor on the endothelial cell for monocyte binding, also had significantly elevated expression with late E2. Hence, delayed estrogen replacement resulted in increased expression of pro-inflammatory genes and genes promoting monocyte adhesion to the vessel wall in the aging cardiovascular system.

Certainly, ovx without E2 replacement was associated with an increase in pro-inflammatory and pro-apoptotic gene expression. Ovx without E2 replacement increased caspase 3, caspase 9, calpain 2, matrix metalloproteinase (MMP)9, and TNF-α expression [90]. Most of these changes were prevented by immediate E2 replacement. Changes in gene expression with late E2 replacement were even more compelling, as discussed above. Thus, ovx in the aged heart was associated with increased expression of many potentially deleterious genes, but delay in E2 replacement led to a marked increase in pro-inflammatory gene expression. This could be avoided by immediate E2 replacement.

SERMs

Estrogen is a steroid with a wide-ranging effects on the cardiovascular system. Estrogen protects cardiac myocytes and other cells through PI3K, Akt, and the MAP kinase pathways activation [43]. In addition estrogen modifies the plasma lipid profile, reducing LDL and raising the cardioprotective HDL, and this accounts in part for the lower levels of cardiovascular disease in premenopausal women vs. age-matched men [91, 92]. Certainly HRT has complex consequences, which are both beneficial and deleterious as evidenced by the increase in cancer and thrombosis risk in those on estrogen replacement. Moreover late estrogen replacement had unanticipated negative effects. However over the last 20 years a number of SERMs have been synthesized. The SERMs have the potential to provide selective activation of estrogen receptors. SERMs have been shown to have beneficial effects such as lowering cholesterol levels, improving endothelial function and decreasing smooth muscle tone through increased NO production [93, 94]. SERMs also have direct cardioprotective properties, and treatment with either tamoxifen or raloxifene decreased cardiac myocyte injury in a hypoxia/reoxygenation model [28]. SERMs were also found to increase expression of eNOS and MnSOD [28]. However, SERMs both differ from estrogens and from each other. Careful study will be needed to elucidate the spectrum of cardioprotective properties for different SERMs.

At this time, there are two SERMs in clinical use: raloxifene, which has mixed agonist/antagonist properties, and tamoxifen, which is mainly an estrogen receptor antagonist. Raloxifene is used to treat osteoporosis (estrogen is important in bone formation) and tamoxifen is used in the treatment of hormone-dependent cancers.

Many other SERMs are in development with different properties and these raise the prospect of selectively exploiting estrogen's protective effects to the benefit of patients [74].

Conclusions—There are well-established gender differences in the heart, such as the much lower incidence of myocardial infarction in premenopausal women compared to age-matched men. With loss of estrogen women undergo an acceleration of atherosclerosis, but this is only one aspect of cardiac disease. Clearly there are cardiac differences secondary to estrogen loss and aging. Aging and estrogen loss contribute to a pro-inflammatory state and increased basal production of ROS in the heart. E2 can protect the heart and cardiac myocytes from injury, but its effects are reduced to abolished in aging, depending on the end-point. The protective heat shock response is lost with aging, regardless of estrogen status, even though in younger models HSP72 levels in female hearts are twice the levels in males. Mitochondrial injury is reduced by E2 treatment, but studies in aging models are limited. E2 treatment has a positive impact on pathologic cardiac hypertrophy and there appear to be distinct differences in ERα and ERβ's roles in hypertrophy. Whether there are gender differences or benefits from E2 treatment in heart failure is not clear at this time. Studies directly addressing this issue are needed. GPER, the third estrogen receptor, so far appears to primarily activate the PI3K/Akt protective pathway, and not other effects of estrogen, such as cell proliferation and changes in gene expression, a primary function of ERα and ERβ. There are selective GPER agonists, and these have possible benefits, but more work is needed on GPER signaling. Double-blind randomized clinical trials of HRT showed increased cardiovascular events and cancer with HRT. However it is now clear that HRT was initiated very late and this may have contributed to the increase in cardiovascular events. Insights into the types of estrogen used for HRT as well as the gene changes evoked by late estrogen replacement (pro-inflammatory, pro-atherosclerotic) has helped explain the unexpected results of these clinical trials. In the last 20 years SERMs have been developed, which raise the possibility of selective activation of the estrogen receptors to activate protective responses to estrogens in a controlled manner. We know far more than we did 20 years ago, but clearly there are many more important questions to answer, especially with regards to the consequences of aging and concomitant estrogen loss.

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Chapter 10
Diet and Exercise Are Potent Modulators of Cardiovascular Disease in Women

Kristen K.B. Barthel, Pamela A. Harvey, and Leslie A. Leinwand

Abstract Cardiovascular disease (CVD) is the number one killer of women in developed countries and its incidence is increasing in women in developing nations (Gholizadeh and Davidson, Health Care Women Int 29:3–22, 2008; Mosca et al., Circulation 123:1243–1262, 2011). Many factors influence a woman's likelihood of developing CVD throughout her lifetime. These include intrinsic variables such as genetic background, hormonal status, and age as well as extrinsic variables such as smoking, diet, and level of physical activity. While the potential for intervention regarding the intrinsic factors can be limited and challenging, women can have a lot of control over the extrinsic factors, which may ultimately influence cardiovascular health more powerfully than pharmacologic interventions and can potentially counteract genetic predisposition (Stampfer et al., N Engl J Med 343:16–22, 2000).

Keywords Cardiovascular disease • Diet • Exercise • Estrogen • Menopause

Introduction

Cardiovascular disease (CVD) is the number one killer of women in developed countries and its incidence is increasing in women in developing nations [1, 2]. Many factors influence a woman's likelihood of developing CVD throughout her lifetime. These include intrinsic variables such as genetic background, hormonal

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status, and age as well as extrinsic variables such as smoking, diet, and level of physical activity. While the potential for intervention regarding the intrinsic factors can be limited and challenging, women can have a lot of control over the extrinsic factors, which may ultimately influence cardiovascular health more powerfully than pharmacologic interventions and can potentially counteract genetic predisposition [3].

Although overall age-adjusted mortality rates associated with CVD have been decreasing in the USA since the 1980s, analysis of age-specific groups has revealed a disturbing trend towards an increase in mortality among women aged 35–44 since 2000, potentially foreshadowing an even greater healthcare crisis as these women continue to age [4]. This has occurred despite great strides in treatments since the 1980s. Since this is a relatively short time period, genetic changes cannot explain this increase. Rather, it is presumed that this change is most likely due to an increase in risk factors attributable to deterioration in lifestyle, including dietary patterns and levels of physical activity. It has been suggested that 64 % of coronary heart disease (CHD) deaths in women could have been prevented through lifestyle modification [5]. Moreover, results from the Nurses' Health Study (NHS) indicate that 82 % of coronary events in women may have been caused by a high-risk lifestyle [3]. Given these striking observations, this chapter dissects how diet and exercise influence cardiovascular health and disease in women.

Diet

General Dietary Guidelines for Good Cardiovascular Health in Women

Although the American Heart Association (AHA) has been publishing dietary guidelines since 1957 [6], the first specific to women did not appear until 1999 [7]. The necessity for specifically addressing women in the prevention of CVD was apparent from studies revealing decreased awareness of the threat of CVD in women compared to men and thus less focus on the improvement of risk factor profiles in women. In 1997, only 8 % of women surveyed perceived heart disease as the biggest threat to their health [8]; in 2003, this number had only increased to 13 % [9]. In addition, there are unique aspects of female physiology that may directly relate to CVD risk, including pregnancy, menopause, and postmenopausal hormone replacement therapy (HRT). Current (2011) dietary recommendations encompass both macro- and micronutrients [2]. Fruits and vegetables, oily fish, fiber, and whole grains are emphasized, while excessive sugar, saturated fat, *trans*-fats, cholesterol, and sodium intakes are cautioned against. These dietary suggestions have arisen from numerous large clinical studies, some considering both sexes and some exclusive to women.

Major Women's Health Studies Focused on Diet

Women's Health Initiative Dietary Modification Trial

The Women's Health Initiative Dietary Modification (WHI DM) Trial was a large-scale study designed to explore the effects of a low-fat, high fiber, fruit, and vegetable diet on cancer and, secondarily, CVD [10]. 48,835 postmenopausal women not currently consuming a low-fat diet were assigned to either a control or intervention group, where specific dietary guidelines regarding total fat, fiber, and fruits and vegetables were given along with intensive behavioral modification sessions. Healthy women were initially enrolled in 1993–1998 and followed for 8 years. Surprisingly, the intervention group showed no significant reduction in CVD risk factors or development of CVD. However, several criticisms suggest that these findings may be inconclusive. First, the trial was designed primarily to focus on cancer prevention; CVD was a secondary outcome. Thus, it focused on reducing overall fat intake but did not make specific recommendations regarding reduction of saturated fatty acids (SFAs) and *trans*-fats and incorporation of mono- and polyunsaturated fatty acids (MUFAs and PUFAs), which are more specific to cardiovascular health. When the study was designed, the nuances in type of fat in relation to cardiovascular health were poorly understood: that SFAs and *trans*-fats may be particularly harmful, while MUFAs and PUFAs may be particularly beneficial was not yet appreciated. In addition, the intervention group did not achieve as great a reduction in fat intake and increase in fruit and vegetable consumption as the study had intended while the control group had a lower than expected total fat intake, further contributing to the smaller than expected difference in total fat between groups. There was also no specific recommendation for fish intake. Moreover, CVD can take a long time to develop, and the mean follow-up time of 8.1 years has been criticized as being too short to observe a statistically significant change in an endpoint such as CVD or cardiac death (although follow-up continued for 5 years after the official trial end date [11]). However, there was a trend towards risk reduction in those women who achieved the highest levels of fruit and vegetable consumption and the lowest levels of saturated and *trans*-fat in their diets, which has spurred further research.

Nurses' Health Study

The NHS is one of the largest, longest running studies devoted to the investigation of women's health issues. Since 1976, over 80,000 female nurses have been regularly assessed by questionnaires that probe various lifestyle factors, including smoking, menopausal status, and HRT. In 1980, assessments of physical activity and dietary patterns (food frequency questionnaires: FFQs) were added to the study. Unlike the WHI DM Trial, the NHS is not an interventional trial but rather a

prospective study designed to understand how changes in lifestyle trends affect trends in disease, including CVD. Many dietary factors have been considered over the years, including (but not limited to) fruits, vegetables, and carbohydrates [12] along with glycemic load [13], whole grains [14], alcohol [15], dietary fat [16], and *trans-fatty* acids in particular [17], fish and *n*-3 fatty acids [18] as well as α-linolenic acid (ALA) [19], vitamins B [20], E [21], and C [22]. Some of these studies will be addressed in more detail below.

In 2000, the NHS defined a low-risk lifestyle for the primary prevention of CHD in women [3]. This report focused on five risk factors, which at ideal levels are healthy diet (low for *trans*-fat intake and glycemic load; high for cereal fiber consumption, marine *n*-3 fatty acids, folate, and PUFA to SFA ratio), moderate daily physical activity, body mass index <25, smoking abstention, and moderate alcohol consumption (compared to none). Women in the highest quintile for healthy risk factor profile had a relative risk (RR) of 0.17 compared to all other women. Unfortunately, even in 1994 when the final data were collected for this NHS report, only 3 % of these women fit these criteria. Risk factor profiles, particularly BMI, have continued to deteriorate since that time [23]. Various organizations, including the AHA, have relied heavily on the results of the NHS to make recommendations regarding heart-healthy lifestyles for women. To quote a 2002 Scientific Statement released by the AHA and commenting upon the results of the NHS, "Clearly, the majority of the causes of CVD are known and modifiable" [24].

Bioactive Dietary Compounds

The interplay between diet and cardiovascular health and disease is complex. In attempts to deconstruct the contributions of various dietary factors, individual bioactive dietary compounds have been the subject of numerous trials that assess their effectiveness in the prevention of primary or secondary cardiovascular events. Dietary interventions in the form of supplements or foods that have consistently shown strong inverse correlations with CVD and are recommended as part of a heart-healthy diet include marine sources of very long-chain *n*-3 fatty acids (eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)) and plant-derived stanols and sterols. Conversely, certain compounds that have previously been proposed for primary or secondary prevention of CVD are now recommended against, either because they have been shown to be ineffective in CVD prevention or because they may actually be harmful. These include soy isoflavones, antioxidant supplements (such as vitamins E, C, and beta-carotene), and folic acid (in post-childbearing years), with or without vitamins B_6 and B_{12}. These recommendations are specific to CVD prevention as some of them may be beneficial in other settings. In addition, there are a number of dietary components that are actively being pursued in numerous clinical studies but whose effectiveness in the prevention of CVD remains uncertain. These include vitamin D, α-linolenic acid, and resveratrol. Table 10.1 summarizes the recommendations regarding these compounds [2, 25, 26]. We discuss the scientific basis for these recommendations below.

Table 10.1 AHA recommendations regarding selected dietary components on CVD prevention

Recommended	Ineffective or harmful	Uncertain effect
EPA and DHA from oily fish or fish-oil supplements	Hormone therapy and selective estrogen receptor modulators (including soy isoflavones)	Vitamin D
Plant stanols and sterols	Antioxidant supplements (including vitamins E, C, and beta-carotene)	α-Linolenic acid
	Folic acid in post-childbearing years (with or without vitamins B6 and B12)	Resveratrol

Dietary Factors with Demonstrated Benefit

Eicosapentaenoic Acid and Docosahexaenoic Acid

Epidemiological studies have revealed that cultures that traditionally consume a lot of fish, including Inuits in Greenland and Alaska and Japanese living in fishing villages, have dramatically lower rates of heart disease than cultures that do not (reviewed in [27]). Very long-chain *n*-3 PUFAs (also known as omega-3 PUFAs) are posited to be responsible for this dietary pattern effect. They are primarily found in oily fish, and EPA and DHA are the two most abundant forms. These *n*-3 PUFAs have been shown in diverse studies to have many cardioprotective effects, including decreases in plasma triglycerides [28], resting heart rate [29], and blood pressure [30]. They appear to be antiarrhythmic and thus may be particularly beneficial for preventing sudden cardiac death (SCD) (reviewed in [31]).

In 1989, the Diet and Reinfarction Trial (DART) examining myocardial reinfarction in men revealed that of three major dietary recommendations (reduction in fat, increase in fatty fish, and increase in cereal fiber), only fatty fish reduced mortality [32]. Ten years later, results of the large Gruppo Italiano per lo Studio della Sopravvivenza nell'Infarto Miocardio (GISSI)-Prevenzione study demonstrated that dietary supplementation with *n*-3 PUFAs in capsule form (but not vitamin E) in recent myocardial infarction (MI) survivors reduced fatal cardiac events, nonfatal MI, and stroke by 10–15 % after 3.5 years [33]. There was up to a 45 % reduction in SCD. A subsequent publication stratifying the results by follow-up time showed that protection against SCD was evident as early as 4 months [34]. However, only 15 % of the GISSI participants were female and it was not placebo-controlled. The Japan EPA Lipid Intervention Study (JELIS), involving over 18,000 hypercholesterolemic Japanese patients with or without previously diagnosed coronary artery disease (CAD), explored the effect of administration of highly purified EPA in combination with statin therapy compared to statins alone in a 5-year follow-up [35]. Major coronary events were reduced by 19 % in the EPA group, but there was no difference in SCD, unlike the GISSI trial. However, there are several caveats. There was also no true placebo group in this trial, 70 % of the participants were women even though Japanese women have a much lower incidence of CAD than Japanese men, and the Japanese population consumes on average five times more

fish than other countries (suggesting that perhaps the effects on SCD are saturable). This was also a study of just EPA rather than EPA and DHA.

There are relatively few studies focused on women and the effects of EPA and DHA. The handful of randomized controlled trials that are devoted to women typically have few participants and are of short duration [36–39]. One small-scale study found that a fish-oil concentrate lowered serum triacylglycerides by 26 % in postmenopausal women after 28 days of treatment [39]. The NHS found protective effects of high fish consumption (≥5 times per week), particularly for CHD deaths, after 16 years of tracking women who were healthy at baseline [18]. The protective effects in preventing CHD were particularly strong for diabetic women (RR = 0.36 for women who consumed fish ≥5 times per week compared to women who seldom did [40]). While striking, large randomized placebo-controlled trials in women with long-term follow-up are needed to support the observations of the prospective trials.

There are several issues awaiting resolution. First, the optimal dosage is still somewhat unclear. Prospective studies mentioned above have noted a dose–response for fish intake in CVD protection. The AHA currently recommends 2 servings of oily fish per week or, secondarily, ~0.5–1 g of EPA and DHA per day from supplements [2, 25]. Some studies have shown triacylglyceride-lowering effects with larger doses, but it is recommended that a physician be consulted for advice on high-dose treatment [41, 42]. The efficacy of whole fish versus supplements is also debated [42]. While both fish consumption and supplementation with fish oil or EPA and DHA have been effective, it remains to be determined whether supplements can fully recapitulate the benefits of whole fish. This may be particularly relevant when whole fish is incorporated into the diet as a substitute for other animal protein sources that may be high in saturated fat. Finally, there is controversy over whether naturally derived or highly purified supplements are preferable [35, 41]. There are concerns about environmental toxins, such as methylmercury, dioxins, and PCBs, in natural sources (both whole fish and perhaps in even higher concentrations in fish-oil capsules). Other concerns include overfishing and access to/affordability of fresh fish. Considering the strong evidence for the benefits of EPA and DHA, answering these questions is important.

Plant Stanols and Sterols

High levels of serum cholesterol, particularly low density lipoprotein (LDL) cholesterol, are correlated with increased risk of CVD. It has been estimated that a 10 % reduction in total serum cholesterol would decrease the incidence of heart disease by >30 % for both men and women [43]. Serum cholesterol levels can be lowered by blocking cholesterol synthesis but also by interfering with cholesterol absorption. Phytosterols are plant-derived sterols structurally related to cholesterol. They can decrease both total and LDL cholesterol by reducing absorption and increasing elimination of cholesterol [44]. Although phytosterols have been added to hypercholesterolemic patients' diets since the 1950s [45], and consumption typically lowers total cholesterol by ~10 %, focus has now shifted to the synthetic derivative sitostanol.

Sitostanol is a more potent cholesterol-lowering compound than sitosterol [46] and can easily be incorporated into foods, mainly in the form of spreads. In a 1-year randomized, double-blind study of 153 mildly hypercholesterolemic men and women, sitostanol reduced total serum cholesterol 10.2 % and LDL cholesterol by 14.1 %. Triglyceride and high density lipoprotein (HDL) cholesterol levels were unaffected [47]. In a small study of postmenopausal MI survivors, 2–3 months treatment with sitostanol ester margarine lowered total cholesterol 13 % and LDL cholesterol 20 % [48]. Sitostanol was also effective at lowering cholesterol even when women were taking statins to inhibit cholesterol synthesis. Although there are many trials incorporating sterols and stanols as part of the intervention protocol, they are mostly small-scale and relatively short-term and focus primarily on blood lipid profiles as endpoints [49]. It would be interesting to see the results of a long-term, adequately powered study on CVD incidence and mortality in women.

Dietary Factors with Possible Adverse Effects

Isoflavones

CVD risk in women increases after menopause, but estrogen can improve cholesterol levels and vascular tone, leading to the hypothesis that HRT could counteract the increased postmenopausal CVD risk. Alarmingly, HRT actually further increases risk [50], although the study design of waiting a number of years postmenopause to initiate HRT has been raised as a confounding factor [51]. Nonetheless, alternative therapies with estrogenic molecules have been pursued to lower the postmenopausal risk of CVD in the hopes of avoiding the negative consequences associated with HRT. Phytoestrogens are nonsteroidal plant-derived compounds with both estrogenic and antiestrogenic properties as well as receptor tyrosine kinase inhibitory effects and antioxidant properties. The phytoestrogens genistein and daidzein belong to the isoflavone family and are very abundant in soy. A low incidence of CVD has been noted in populations that consume a lot of soy-based foods, such as countries in the Pacific Rim. Certain studies have suggested that replacing animal protein with soy protein in the diet can lower blood lipid levels [52]. In 1999, a randomized trial of 156 mildly hypercholesterolemic men and women compared the effect of 9-week consumption of different isoflavone doses (prepared by adding back isoflavones to isoflavone-stripped soy protein) to casein, which is isoflavone-free [53]. At the highest isoflavone levels (62 mg/day), total cholesterol and LDL cholesterol were reduced by 4 % and 6 %, respectively. The effect was ascribed to isoflavones, although they did not compare to isoflavone-stripped soy protein without any added isoflavones or consider isoflavones either in the absence of protein or added instead to casein. Other studies have noted that, while soy protein can be effective in lipid lowering, isolated isoflavones typically are not, nor is isoflavone-stripped soy protein [54, 55]. This is also true in studies focused on postmenopausal women [56–60]. This discrepancy is still not completely understood, although the

possibility remains that the soy protein effect is simply a misinterpretation of the effect of animal protein reduction. It has also been suggested that soy protein and isoflavones may somehow synergize, rendering either impotent without the other. To date, most isoflavone intervention studies have lacked the required power to detect benefits or risks of supplemental isoflavone intake. In addition, effects of estrogenic compounds in postmenopausal women may be sensitive to timing of intervention. In light of this accumulation of research, the AHA currently recommends against isoflavone dietary supplementation due to the unproven effects in CVD prevention and potential adverse effects in other disease settings [26]. They are also somewhat less enthusiastic about the impact of the potential cardiovascular benefits of soy protein than they were in 2000 [61]. The United States Food and Drug Administration currently allows labels of soy-containing foods to state that they may reduce the risk of heart disease, although they were cautious in their 1999 ruling to not specifically include isoflavones in the statement (Federal Register 64 FR 57699).

Antioxidant Supplements

It has been hypothesized that oxidative stress leads to CVD, among other chronic diseases [62]. The rationale is that free radicals can lead to LDL oxidation, lipid peroxidation, and DNA damage, culminating in CVD. However, whether free radicals are a consequence or a cause of CVD remains a subject of debate [63, 64]. Numerous observational and epidemiological studies have noted that diets high in fruits and vegetables are associated with decreased rates of CVD [65]. As fruits and vegetables are rich sources of antioxidants, which can scavenge free radicals, many trials have studied whether increased consumption of antioxidant vitamins (from the diet or in the form of dietary supplements) can prevent primary or secondary CVD. These include, but are not limited to, vitamins C and E and beta-carotene.

Vitamin C is the predominant circulating water-soluble antioxidant in humans. Although it has strong antioxidant properties, it can also act as a pro-oxidant under certain conditions [66]. Two prospective studies in women have reported effects of supplemental vitamin C, although not dietary vitamin C. The NHS found a modest protective effect of supplemental vitamin C in women in the highest quintile for intake as determined by FFQs [22]. However, these women also had healthier lifestyles overall, leading to moderate confounding. The Iowa Women's Health Study tracked women at higher CVD risk due to diabetes for 15 years [67]. A FFQ similar to the NHS was used here, although only one FFQ was administered at baseline. They also found a supplemental vitamin C effect, but, contrary to the NHS, women in the highest quintile compared to the lowest were at increased risk of CVD mortality (RR = 1.84), CAD (RR = 1.91), and stroke (RR = 2.57). Rates of cardiovascular death in nondiabetic subjects were unaffected by vitamin C supplementation. Only one randomized trial, the Women's Antioxidant Cardiovascular Study (WACS), looked at controlled administration of vitamin C supplements (as well as vitamin E

and β-carotene, each individually assigned) for 9.4 years in at-risk populations of women [68]. They found no beneficial or harmful effect of any of these antioxidant supplements on CVD prevention.

Vitamin E is a class of lipid-soluble antioxidants comprised of eight different isomers: four tocotrienols and four tocopherols, including α (the major isoform in human plasma and tissue) and γ (the major isoform in food). It is a major constituent of LDL particles, where it presumably protects lipid and protein components from oxidative damage. Consequently, vitamin E supplementation has been studied in numerous CVD prevention trials. The Women's Health Study was a randomized controlled trial that studied vitamin E supplementation for over 10 years in healthy women ≥45 years old [69]. Overall, there was no significant effect on the combined primary endpoint of major cardiovascular events. However, there was a significant 24 % reduction in the secondary endpoint of cardiovascular death. Moreover, subgroup analysis revealed a 26 % decrease in the primary endpoint among women older than 65. The prospective NHS also suggested a 41 % decrease among long-term vitamin E supplement users [21], although this was self-reported usage. Interestingly, a meta-analysis that pooled prospective studies found that high dietary intake of vitamin E, though not supplemental, was protective for women (24 % reduction) but not for men [70]. The Iowa Women's Health Study also found a protective effect associated with high dietary vitamin E (RR = 0.38) but not supplemental in postmenopausal women [71]. The WACS, as mentioned above, found no protective effect for at-risk women [68]. Many other trials with both sexes participating have found no protective effect of vitamin E, including the GISSI-Prevenzione [33], the Primary Prevention Project [72], and the Heart Outcomes Prevention Evaluation (HOPE) trial [73]. Disturbingly, in some long-term, high-dose (≥400 IU/day) trials, harmful effects have been observed, such as a 40 % increase in hospitalization for heart failure [73] and an increase in all-cause mortality (meta-analysis described in [74]).

It has been suggested that there could be heterogeneity in results due to the source of vitamin E; natural sources include multiple isoforms while synthetic sources are typically just α-tocopherol. As stated above, food sources contain mostly γ-tocopherol. This is important given the emerging evidence that γ-tocopherol may actually be a more powerful antioxidant than α-tocopherol (reviewed in [75]), and α-tocopherol may displace γ-tocopherol in vivo, suggesting that too much α-tocopherol inhibits the actions of γ-tocopherol [76]. In addition, α-tocopherol has even been observed under some circumstances to act as a prooxidant towards human LDL [77]. Overall, vitamin E supplementation may be beneficial in certain populations, but the potential for harm from chronic high exposure warrants caution.

β-Carotene (part of the vitamin A family) has also been proposed as an important plant-derived antioxidant and studied for a potential protective effect in CVD. Unfortunately, randomized controlled trials have found no benefit in either healthy [78] or at-risk women [68]. The β-carotene arm of the Women's Health Study [78] was prematurely terminated due to both the lack of effect in a companion long-term study (12 years) of men [79] and the observed increase in risk among patients

at high risk for lung cancer [80, 81]. High vitamin A levels from dietary intake did not correlate with any change in death from CHD in the Iowa Women's Health Study [71]. Combined use of these and other antioxidants has also not proven beneficial [82, 83] and has even been observed to counteract the blood lipid-lowering effects of statin therapy [82].

Folic Acid and Other B Vitamins

Homocysteine is a cysteine homologue that serves as an intermediate in the biosynthesis of cysteine from methionine. Numerous observational studies have correlated high plasma homocysteine levels with CVD risk, including postmenopausal women with no previous history of CVD [84]. Of note, a 2002 meta-analysis suggested that a lower homocysteine level was more strongly associated with decreased CHD risk in women compared to men [85]. Plasma homocysteine levels have been shown to increase after menopause [86] and can be lowered by HRT [87, 88]. Supplementation with certain B vitamins, including folic acid (B_9), B_6, and B_{12}, can significantly lower homocysteine levels by catalyzing the synthesis of cysteine from homocysteine. Therefore, numerous studies have tested whether B vitamin supplements can effectively decrease CVD.

In 2006, two randomized, placebo-controlled, double-blind studies were published. The HOPE-2 study [89] treated patients with existing vascular disease or diabetes with a combination of folic acid, B_6, and B_{12} for 5 years, while the Norwegian Vitamin (NORVIT) trial [90] treated recent acute MI survivors with the same combination, just folic acid plus B_{12} or B_6 alone for an average of 40 months. While both studies successfully lowered homocysteine levels in the folate groups, neither showed any benefit to recurrent CVD. In fact, the NORVIT results suggested a 22 % increase in cumulative CVD risk and a 30 % increase in nonfatal myocardial infarctions (MIs) with the triple combination therapy. In 2008, the Women's Antioxidant and Folic Acid Cardiovascular Study (WAFACS), an expansion of the WACS trial, published the results of an ongoing trial in diagnosed or high CVD risk American women health professionals [91]. The women in this randomized controlled study also received a similar triple combination therapy and were monitored for CVD morbidity and mortality for over 7 years. The results confirmed previous studies: there was no difference in the combined endpoint from treatment with B vitamins, although homocysteine levels were significantly lowered. No harmful effects were noted in this study. Compared to secondary prevention studies, there is a relative lack of randomized controlled trials that consider B vitamins in the primary prevention of CVD in healthy women. In 1998, the prospective NHS did suggest that high folate and B_6 intake led to an RR of 0.55 compared to women with the lowest intake levels of these vitamins [20]. However, the primary source of folate and B_6 was from multi-vitamins, complicating analysis of this study. It is possible that elevated homocysteine levels are simply an indication of CVD but are not a direct cause, thereby explaining why interventions with B vitamins are not effective in preventing CVD.

Dietary Factors with Inconclusive Effects

Vitamin D

Vitamin D insufficiency has been hypothesized to contribute to the development of CVD [92]. Indeed, epidemiological evidence suggests that low levels of circulating vitamin D are associated with an up to 80 % increase in incident CVD [93]. Over 1/3 of otherwise healthy American young adults and even higher numbers of Europeans may have insufficient levels of vitamin D [94]. More than 60 % of postmenopausal women with osteoporosis may be vitamin D deficient [95]. Vitamin D has widespread effects throughout the body but is generally tied to calcium absorption. Vitamin D receptors are expressed both in cardiomyocytes and in blood vessels. In the context of CVD, vitamin D is speculated to protect from valvular and arterial calcification.

Several women-specific trials have studied the relation of vitamin D to CVD. In the Iowa Women's Health Study, dietary questionnaires that included questions about calcium and vitamin D food sources and supplements were administered to 34,486 postmenopausal women at baseline; cardiovascular death was assessed within the 8-year study period [96]. While high calcium intake was associated with a 30–35 % reduction in CVD death, vitamin D proved ineffective in this large-scale, long-term study. However, the analysis was based on one questionnaire given at the onset, it only looked at death rather than overall CVD incidence, and it did not consider combined effects of calcium and vitamin D. The calcium/vitamin D supplementation arm of the WHI was a randomized, controlled trial that looked at CVD as a secondary outcome in 7 years of follow-up after daily dosing with 400 IU of vitamin D along with calcium [97]. Unfortunately, treatment had no effect on CVD. This study was complicated by background use among some patients of calcium supplements, poor adherence, and HRT, which has been documented to increase CHD incidence [50]. It is also possible that the dose used was inadequate, as optimal vitamin D intakes are still a contested issue. Of course, it is also conceivable that calcium/vitamin D is unrelated to the development of CVD. One other small, short-term trial involving women found no effect of high doses (2,500 IU/day) of vitamin D on endothelial function or arterial stiffness. The ongoing VITAL trial will test high doses of vitamin D (2,000 IU/day) in the primary prevention of cancer and CVD in healthy populations over the course of 5 years [98]. 20,000 middle-aged men and women are currently being recruited. This extensive trial will hopefully yield more insight as to the benefits, if any, of vitamin D supplementation towards CVD prevention.

Resveratrol

In 1992, Renaud described "The French Paradox," which observes that, despite a diet relatively high in saturated fat, the French experience a very low mortality rate from CHD [99]. Alcohol consumption was the only dietary factor that could counteract

the otherwise positive association of saturated fat with CHD mortality. An in vitro study, which demonstrated that the phenolic component of red wine in the absence of ethanol protected against LDL oxidation at 10 μM concentrations, quickly followed in 1993 and demonstrated that this fraction performed significantly better than α-tocopherol [100]. Resveratrol may also inhibit platelet aggregation and eicosanoid synthesis [101]. In vivo, resveratrol has been shown to prevent reperfusion-induced arrhythmias and mortality from ischemia–reperfusion injury in rats [102]. Micromolar plasma concentrations of polyphenols can be achieved in humans by ~2 glasses of red wine per day, suggesting that the in vitro effects may be clinically relevant [103]. In further support of the contribution of resveratrol to the French Paradox, resveratrol administration to mice on a high fat diet extended lifespan [104].

Clinical trials testing purified resveratrol in the prevention of CVD are scarce, but one ongoing trial (NCT01449110) is considering the effect of resveratrol on vascular health in postmenopausal women. Prospective studies in healthy women, including the NHS, have shown that moderate consumption of alcohol in the form of beer, wine, and liquor confers a 60 % protective advantage against coronary disease and also protects against stroke [15]. Very few of the women in the NHS qualified as heavy drinkers, therefore potential harmful effects at excessive levels could not be assessed. Moderate alcohol consumption also reduced risk in diabetic women, who are at high risk for CHD, by up to 52 % [105]. The role of resveratrol in these intriguing results would be significantly strengthened by randomized controlled trials in order to justify the growing popularity of resveratrol supplements.

α-Linolenic Acid

ALA is an intermediate chain *n*-3 fatty acid found in flaxseed, soybean, rapeseed, and canola oils as well as walnuts. It is an essential dietary fatty acid for humans. It is speculated that it has antiarrhythmic properties and thus can protect against SCD. After 18 years of follow-up, the Nurses' Health Study reported a 38–40 % reduction in SCD when comparing the highest levels of ALA intake to the lowest [19]. They did not see such an association for nonfatal CHD. This is consistent with their earlier 10-year follow-up report [106]. As a prospective study, it is impossible to say whether ALA is directly responsible for the observed effect, but the results are intriguing. A companion study of men did not find a significant correlation of ALA with SCD, although there was association with other CHD indicators [107].

Small proportions of ALA (~8 %) can be converted to the marine long-chain *n*-3 fatty acids (primarily EPA but also DHA) in the liver, potentially linking these dietary factors (reviewed in [108]). Although this conversion rate is low, it has been reported to be 15 % higher in women than in men on controlled diets containing equivalent amounts of ALA and no EPA or DHA [109]. Estrogen signaling may account for this difference as oral estrogen administration to transsexual men increased DHA levels 42 % over control [109]. Given the potential sex difference in ALA effectiveness and metabolism but the dearth of randomized controlled trials, particularly for women, more investigation is certainly warranted.

Exercise

The Cardiovascular Effects of Chronic Endurance Exercise

Decades of research on the cardioprotective effects of exercise have led to consensus statements from both the AHA and the Council on Clinical Cardiology recommending regular physical activity for the prevention and modulation of CVD in men and women [24, 110]. Unfortunately, the current knowledge regarding these basic effects and how they modulate CVD risk and progression is mainly based on studies involving men only, despite data suggesting an important sexual dimorphism in this response. We therefore review the systemic and molecular mechanisms that are modulated by exercise with special consideration for those that are specific to women.

Adaptive Responses of the Cardiovascular System to Exercise in Males and Females

The cardiovascular system exhibits remarkable adaptive responses in response to exercise. Importantly, to efficiently circulate oxygenated blood to peripheral tissues, the strength and speed of cardiac contraction must be enhanced through the thickening of cardiac tissue (cardiac hypertrophy). In addition to cardiac morphologic adaptation, a complex feedback system involving the vasculature, nervous system, and metabolic processes in peripheral tissues modifies hemodynamics. Over time, the cardiovascular system becomes "toned" such that decreased sympathetic and increased parasympathetic activity reduces resting heart rate and blood pressure.

Although basic cardiovascular adaptations to exercise have been studied since the 1940s, the sexually dimorphic nature of cardiovascular responses to exercise has only recently been appreciated. Cardiac hypertrophy, for example, is observed in female rats that are swim-trained, whereas the hearts of male rats do not increase in size. As a result, females experience a greater contractile advantage with this form of exercise [111, 112]. Unlike with swimming, however, male rats that are treadmill-trained exhibit a morphological and functional cardiac advantage over females [113]. These data support the conclusion that the hearts of females and males respond differently to various forms of exercise.

Both sexes also exhibit distinct vascular adaptation in response to exercise, stemming from differential endothelial and autonomic activity. Interestingly, women exhibit enhanced basal parasympathetic tone compared to age-matched men, which is thought to account for much of the cardioprotection experienced by younger women, even in the absence of exercise [114, 115]. Recent data on sexually dimorphic responses to exercise emphasize a need for creating unique recommendations for men and women. Because these outcomes are best described for endurance training compared to resistance or strength training, we will focus on the protective and modulatory effects of aerobic exercise.

Exercise Reduces Risk and Progression of Cardiovascular Disease

It is well-established that engaging in regular aerobic exercise lowers the risk of developing CVD; the rate of many forms of CVD in physically active people is significantly lower than in those who are sedentary [110, 116, 117]. Correspondingly, women report exercise as the number one strategy for reducing the risk of developing CVD [9]. However, medical professionals are less likely to advise women than men to engage in exercise [118]. This dichotomy may be due to the relative lack of evidence-based studies in women: the most comprehensive report in recent years, the United States Surgeon General's Report on Physical Activity and Health, considered women in only 2 % of the studies [119]. Additionally, from 1950 to 1995, of 43 clinical studies examining the relationship between exercise and CVD risk, only seven included women [120]. Interestingly, each of the small number of early studies involving women defined exercise as housework, employment-related tasks, or leisure time activities [121–124], a definition thought to be sufficient because about 82 % of physical activity could be attributed to housework [125]. Despite the conservative description of exercise, the notion that physical activity is cardioprotective in women is supported in current research.

The type, intensity, and duration of exercise can have a significant and unique impact on the degree of cardioprotection experienced by men and women [126]. Early studies hypothesized that intense exercise exerts a more dramatic benefit than moderate activity, albeit in men [127]. Because walking is a popular form of exercise among women [128], defining the unique cardiovascular effects of low or high intensity activity was necessary. A prospective study of middle-aged women demonstrated that low intensity exercise is at least as beneficial as vigorous exercise, suggesting that walking is a sufficient method for reducing risk; indeed, walking led to about a 40 % reduction in CVD over the 10-year study [129]. Other recent large studies demonstrated that as little as 2 h of activity per week can promote this benefit [125, 130, 131]. Interestingly, the pace or intensity of exercise in women did not affect risk of developing CVD, an effect that also held true for women who had other risk factors including diabetes and cigarette smoking [130]. The data strongly suggest that reasonable recommendations could be made for sedentary women, a population previously thought to be resistant to engaging in strenuous exercise programs like running [128]. Basic recommendations for women have therefore been updated to include mild to moderate exercise, such as walking 30 min a day, at least 5 days a week for the prevention of CVD [132].

In addition to modulating risk, exercise has been convincingly shown to slow the progression of existing CVD in both men and women [133–136]. Despite these data, less than 20 % of patients with cardiac events participate in cardiac rehabilitation programs, including those that involve exercise regimens [137]. Women are even less likely to participate in exercise rehabilitation after MI [138], likely a result of a lack of evidence-based studies; prior to 1990, meta-analyses on the modulation of CVD by exercise excluded women [139]. In light of dramatic differences in the

manner in which men and women respond to CVD, these studies are required. Women with hypertension or aortic stenosis, for example, exhibit greater contractility, enhanced hypertrophy, and better prognoses compared to men [140, 141]. However, with ischemic attack, men fare better than women [142]. Interestingly, the few recent studies that included women did not include men, so identification of possible sex differences in response to specific forms of exercise has not been achieved. One small study demonstrated women participating in exercise rehabilitation programs experienced greater functional cardiac benefits to exercise than men with existing CVD [143]. However, the women considered had poor risk profiles (higher rates of diabetes and hypertension) compared to men and no distinction was made among the various forms of CVD. Thus, the type of pathologic insult appears to be important in terms of baseline sexual dimorphism and may dramatically affect how the patient responds to an exercise rehabilitation program. Because of a lack of detailed information on the effects of exercise on existing CVD, we focus on cardioprotective effects of exercise in individuals without CVD.

Protective Effects Induced by the Cardiovascular Responses to Exercise

Recent conflicting studies demonstrate effects of exercise ranging from significant positive modulation to no change on many systemic and molecular processes. Discrepancies are likely due to the diversity of types, durations, and intensities of exercise utilized, each of which has unique effects on the cardiovascular system [144]. However, a number of mechanisms have been proposed with convincing supporting data, many of which exhibit sexually dimorphic effects. Here, we review current clinical and basic science research supporting the notion that the cardiovascular systems of women and men respond differently to exercise and explore the mechanisms that are thought to modulate effects of exercise on the cardiovascular systems of women.

Although much of the cardiovascular benefit of exercise is attributable to responses of the autonomic nervous system and vasculature, it should be noted that many processes contribute to the cardiovascular benefit of exercise in both men and women. Sensitivity of various tissues to insulin, levels of serum lipids, and coagulative properties of blood can directly and indirectly influence cardiac health. In the broadest sense, physical activity also alters metabolism and, not surprisingly, decreases body weight and adiposity [145]. Decreased blood pressure [146, 147], reduced serum triglycerides, and increased HDL [148–150] are also consistently observed among individuals who engage in regular exercise programs. As a result, exercise essentially reduces all symptoms of metabolic syndrome [151], thereby indirectly improving cardiac processes associated with this syndrome. Many of these responses have been studied in men and women; results reveal a significant sexual dimorphism in basal function and alterations due to physical activity.

The longer-term systemic effects of exercise include a toned cardiovascular system with a slower heart rate and lower blood pressure, largely mediated by increased parasympathetic and decreased sympathetic activation in men and women [152]. These autonomic mechanisms are dynamic and sexually dimorphic through life; for example, although autonomic function generally declines with age, compared to age-matched men, females exhibit increased baseline parasympathetic activation and decreased sympathetic involvement in heart rate regulation, effects that are correlated with protection against some forms of CVD [153]. Exercise further reduces sympathetic activity in women, which could confer cardioprotection and reduce overall rates of CVD [153, 154]. A small study also indicated that exercise rescues autonomic function in postmenopausal women; 6 months of an exercise program specifically designed for women about 65 years of age significantly improved several measures of autonomic function [155]. Although few studies have examined the autonomic responses to exercise in women, it generally appears that women have stronger involvement of the parasympathetic nervous system, while in men the sympathetic nervous system dominates [154]. The predominance of parasympathetic function in women likely contributes to cardioprotection with pathologic cardiovascular challenge [153].

Molecular Mechanisms of Cardioprotection Induced by Exercise in the Heart

In addition to systemic differences between men and women that may confer cardioprotective effects even without exercise, many studies have demonstrated that females' hearts have a greater resistance to ischemic injury than males, and this may be enhanced by exercise [156, 157]). This effect is mediated by a complex mechanism aimed at reducing oxidative stress and apoptosis.

Oxidative Stress

In cardiac muscle, high rates of ATP use require mitochondrial oxidative phosphorylation and increased production of reactive oxygen species (ROS) including nitric oxide (NO), superoxide, and hydrogen peroxide. This persistent low level of oxidative stress is neutralized by various enzymes in the myocytes and contributes to cardioprotection through the sustained upregulation of genes involved in this process [158, 159]. Levels of urinary excretion of products of lipid peroxidation, a measure of oxidative stress, are higher in men compared to premenopausal women [160] and suggest a larger degree of cardiac oxidative stress in men in the absence of a physical stressor. In fact, females exhibit higher expression of many genes [161]) that combat the detrimental effects of oxidative phosphorylation in the cardiomyocytes [162, 163] and that are associated with cardioprotection against ischemic injury [164, 165]. For example, basal levels of heat shock protein 72 (HSP72)

expression, which is cardioprotective in ischemia and is associated with anti-apoptotic pathways (see below), is twice as high in females compared to males, a difference that is abrogated by ovariectomy [161, 163]. Indeed, mild oxidative stress induced by exercise further increases HSP72 expression in females, an effect that is also dependent upon the presence of estrogen [166].

Apoptosis

In light of basal differences in gene expression in males and females, the differential effects of exercise in males and females is of interest. Activity and expression of the cardiac ATP-sensitive potassium (K_{ATP}) channel, for example, are higher in females, and importantly, this channel is critically responsible for enhanced protection in an ischemic setting compared to males [157] through reduced apoptotic pathway activation [167, 168]. The channels are activated during periods of decreased ATP [169] and are upregulated with exercise. Activity changes the membrane potential of cardiomyocytes, thus reducing the duration of the action potential and contributing to the increased speed of contraction required during exercise [170]. Activity of this channel is required for the cardioprotection induced by exercise through its anti-apoptotic effects [159, 171]. This mechanism significantly contributes to persistent protection against apoptosis in the context of a regular exercise program in females.

The mild ischemia and oxidative stress generated in the myocardium by exercise is thought to stimulate several cardioprotective pathways within cardiomyocytes via induction of anti-apoptotic pathways. Protein kinase C (PKC) [172, 173] and cyclic AMP responsive element binding protein (CREB) are both implicated in this process. Phosphorylation of CREB, for example, leads to positive mitochondrial respiration and increased expression of anti-apoptotic pathways (increased Bcl-2, decreased Bax) in the myocardium of females [163, 174]. Increases in heat shock proteins in response to chronic exercise are also correlated with increases in expression of the anti-apoptotic factor, Bcl-2 [163].

Female Sex Hormones May Be Important Modulators of Cardiovascular Function with Exercise

The beneficial effect of exercise on women's cardiovascular health is more evident in younger women compared to women older than 60 years, whereas in men there is no such age effect [175]. As such, it is logical to consider the effect of female sex hormones on cardiovascular function during exercise. Despite strong evidence that younger women experience a greater benefit than older women, incidence of CVD in postmenopausal women (aged 50–70) is also reduced with exercise [176]; women between 65 and 75 years old who begin an exercise program exhibit rates of CVD mortality that are similar to age-matched women who were active at baseline [177]. Therefore, exercise should not be ignored in recommendations for postmenopausal women.

To explore the mechanisms responsible for this age effect, we review the cardiovascular interactions between estrogen and exercise in women. However, it is important to note that endogenous estrogen production also plays an important role in cardiovascular function of men. For example, a case study involving a man with a mutation in the estrogen receptor-α gene exhibited abnormal endothelial function [178]. The individual lacked the flow-mediated vasodilation consistent with reduced NO release in estrogen receptor knockout mice [179]. Additionally, in men without the enzyme required for estrogen production (aromatase), low serum levels of HDL and increased total cholesterol are observed [180]. It is therefore important to recognize that estrogen is a significant modifier of cardiovascular function in both men and women.

Even in the absence of exercise, female sex hormones may confer protection to premenopausal women through direct and indirect regulation of ROS. Steroid hormones like estrogen can scavenge free radicals through a phenolic hydroxylic ring and can regulate expression of enzymes that mitigate oxidative compounds [181]. As a result, females experience smaller infarct size compared to males, an advantage further increased by exercise. This modulation is thought to be achieved by increased expression of sarcolemmal K_{ATP} channels, whose activity has been shown to mediate protective effects in ischemic models of heart disease [182] and expression is upregulated in the presence of estrogen [183]. Additionally, estrogen has been shown to inhibit lipid peroxidation, a process primarily responsible for ROS production [184], which may account for the lower oxidative stress observed in women. As such, premenopausal and postmenopausal women receiving estrogen supplementation have lower serum lipid peroxidation products [185, 186]). No effect on superoxide dismutase expression, the other major ROS mitigating mechanism, has been observed in women in response to estrogen [187].

Convincing data from both clinical and animal studies demonstrate that estrogen modulates many processes related to cardiovascular adaptation to exercise in women. Estrogen directly regulates exercise-induced normalization of serum lipid and cholesterol profiles [188], reduced calcium handling in cardiomyocytes [189], and reduced response to factors that induce cardiomyocyte contractility [190]. Estrogen upregulates nitric oxide synthase (NOS) activity in the endothelium of the vasculature [191]; aortic NO production is reduced in ovariectomized mice but is restored by estrogen supplementation [192]. Acute administration of estrogen also induces coronary and brachial artery vasodilation in postmenopausal women [193, 194], an effect that abrogates reduced autonomic tone at the level of the vasculature.

Exercise induces similar improvements in endothelial function [195] and enhances synthesis, release and duration of action of NO through arterial wall stress [196–198]. Therefore, both exercise and estrogen exert similar effects on NO release [199]. However, when exercise and chronic estrogen replacement were combined in postmenopausal women (who exhibit impaired vascular flow-mediated vasodilation), no further benefit was observed compared to exercise or estrogen alone [199]. Exercise and estrogen also had similar cardioprotective effects on calcium handling in cardiomyocytes [200]. In fact, exercise has a greater cardiovascular benefit in

postmenopausal compared to premenopausal women, likely due to this redundancy [200]. These data suggest that exercise may be a reasonable alternative to hormone replacement therapies that have recently been shown to have detrimental effects on cardiovascular health in some women [50, 200–202].

Conclusions

For much of the twentieth century, research into the underlying causes of CVD in women has lagged behind studies in men despite the fact that it is not only the leading cause of death in both sexes but also kills a higher percentage of women than men. However, there was a tenfold increase in the number of publications examining the effect of biological sex on CVD (ischemic injury) between 1999 and 2009 [203]. The convincing conclusion is that healthy dietary patterns and exercise are beneficial to cardiovascular health, particularly in postmenopausal women where lifestyle can reverse many of the effects of aging and loss of sex hormones. From this accumulation of data, it is obvious that CVD is a lifestyle disease in the majority of cases. Seen optimistically, women have the potential to prevent the development of CVD, but this will require a concerted individual and societal effort to stop the deterioration of risk factor profiles and to continue raising awareness about the impact of CVD on women's health. Clearly, more research is needed in those areas where women have been underrepresented.

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