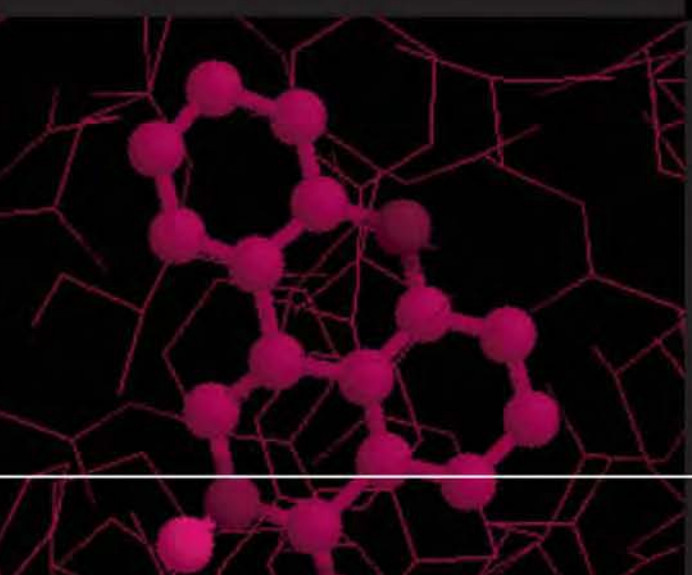
PARP as a Therapeutic Target

Edited by
Jie Zhang



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PARP as a Therapeutic Target

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Cover image: computer modeling illustration of inhibitor GPI 6150 embedded in PARP catalytic domain.
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Dedication

To Jian and Vicki

Foreword

The poly(ADP-ribose) polymerase (PARP) mediated suicide response provides a mechanism by which cells can respond to high levels of DNA damage with activation of a metabolic pathway. This results in rapid cell death before the cell can undergo processes of DNA repair and/or fix DNA damage which might lead to survival of highly mutated and damaged cells.

During the 1970s, research on the eukaryotic, nuclear enzyme PARP demonstrated that the enzyme cleaved its substrate NAD^+ at the *N*-glycosidic bond between the nicotinamide and ribose rings releasing nicotinamide and transferring the ADP-ribose (ADPR) moiety to a covalent linkage with a protein acceptor, frequently the enzyme itself. Subsequent moieties could be added to form a homopolymer of repeating ADP-ribose units with a backbone of alternating ribose to ribose *O*-glycosidic linkages alternating with phosphodiester linkages.[1] A variety of proteins serve as acceptors for attachment of the first ADPR residue.[1] Studies with purified enzymes showed that DNA containing single or double strand breaks is required as an essential enzyme activator and studies with various types of isolated nuclei and permeable cell preparations demonstrated stimulation of enzyme activity by DNA damage.[2-5] A definitive role for the enzyme, with or without polymer synthesis, had not been demonstrated. However, because enzyme activation required DNA strand breaks and because enzyme activity was stimulated by DNA damage, a role in DNA repair was postulated. Many studies focused on the possibility that since PARP was a DNA-binding protein it might be involved in determining chromatin structure.[6] This led to the speculation that poly(ADPR) synthesis caused alteration of chromatin structure so as to facilitate the DNA repair process, perhaps by causing relaxation of nucleosomes to allow access to enzymes of DNA repair.[7]

The concept that PARP was essential to DNA repair was challenged by the observation that treatment of cells with PARP inhibitors in combination with DNA damaging agents resulted in increased unscheduled-DNA repair synthesis.[8,9] However, the interpretation of these experiments was controversial because of the lack of specificity of the inhibitors and the contrasting results obtained in different labs.[10]

Concurrent studies showed cellular levels of nicotinamide adenine dinucleotide (NAD^+), the substrate for PARP, decreased in a dose-dependent fashion when cells were treated with DNA-damaging agents; and PARP inhibitors were shown to prevent or slow down the rate of NAD^+ decrease.[11-14] It was further observed that low doses of DNA damage resulted in reversible decreases in NAD^+ whereas high doses resulted in irreversible depletion.[11-14] We showed also that the decrease in NAD^+ was associated with a proportional decrease in ATP.[15]

The Jacobson's lab developed a high-pressure liquid chromatographic separation and chemical derivatization technique that provided the first analytic approach to quantitate cellular levels of poly(ADP-ribose).[16] Involvement of PARP in DNA

repair was then supported by the demonstration that DNA damage resulted in a burst of poly(ADPR) synthesis. In addition, it was shown that the DNA strand breaks that resulted from processing of UV irradiated DNA stimulated PARP activity but, in contrast, cells derived from patients with *Xeroderma pigmentosum*, which were defective in repair of UV damage, were likewise defective in UV-induced poly(ADPR) synthesis and NAD^+ depletion.[5,17] Thus, it was clear that poly(ADPR) synthesis was somehow involved in the cellular response to DNA damage. However, the role of PARP in repair, fixation, or potentiation of the damage was not clear.

At the end of the 1970s, Nathan J. Berger was working on poly(ADPR) synthesis at the level of whole cells, permeabilized cells, and purified enzymes.[3-5,8] Sosamma J. Berger was working with Oliver Lowry, using enzymatic cycling techniques to determine picomolar quantities of pyridine nucleotides and other metabolites in brain and retinal samples.[18] Combining our techniques, we showed that treating cells with high doses of DNA-damaging agents resulted in irreversible NAD^+ deletion and this process was associated with rapid cell death. The death, measured in minutes to hours, was, in fact, too rapid to be the consequence of genetic damage.

Jim Simms, after completing his Ph.D. with the Jacobsons, joined the Berger lab as a postdoctoral fellow. The lab results quickly showed that the DNA damage induced depletion of NAD^+ were associated with a burst of poly(ADPR) synthesis. More importantly, we showed that PARP inhibitors slowed down PARP synthesis and, consequently, slowed both NAD^+ consumption and ATP depletion. Moreover, the ability of the inhibitors to prevent the fall in NAD^+ and ATP was proportional to their efficacy as PARP inhibitors.[14,15] Thus, the depletion of ATP was secondary to the primary PARP mediated depletion of NAD^+. Additional studies using radioactive thymidine, uridine, and leucine incorporation following DNA damage, showed that the use of PARP inhibitors to prevent NAD^+ and ATP pool depletion allowed for increased DNA, RNA, and protein synthesis. These observations provided an explanation for the apparent increased DNA, RNA, and protein synthesis seen when DNA-damaged cells were treated with PARP inhibitors, i.e., preservation of NAD^+ and ATP pools had a permissive effect on macromolecular synthesis.

These observations resulted in a paradigm shift. Instead of considering PARP as a DNA repair enzyme, we began to think of it as a suicide enzyme activated by high levels of DNA damage to consume NAD^+, deplete ATP, and cause cessation of all energy-dependent processes and consequent cell death. Our studies showed that NAD^+ and ATP could be depleted by two or three orders of magnitude within minutes of high levels of DNA damage. Thus, while PARP might have some role in the DNA repair process, it clearly functioned in response to high levels of DNA damage to consume cellular energy metabolites resulting in the loss of the ability to carry out all energy-dependent processes including DNA, RNA, or protein synthesis and quickly led to cell death.[15,19,20]

Additional studies in our lab confirmed that the decrease in NAD^+ was not simply a shift between oxidized and reduced forms since the NAD^+ depletion was accompanied by a simultaneous depletion of NADH and a slightly delayed depletion of $NADP^+$ and NADPH.[21] There was also depletion of glucose-6-phosphate and other

high energy metabolic intermediates. Shishir Das joined us as a postdoctoral fellow from Larry Loeb's lab and demonstrated that the depletion of energy metabolites was accompanied by a decrease in ATP, UTP, CTP, and GTP as well as by the depletion of dATP, dTTP, dCTP, and dGTP.[22] Thus, the PARP-induced depletion of NAD^+ quickly shut down DNA and RNA synthesis both by restricting energy metabolism and also by depleting the supplies of ribo- and deoxyribonucleotides. The result was that replication and repair synthesis were halted due to lack of substrates as well as the inability to perform energy-dependent processes. Microscopic studies showed the concurrent swelling and lysis of cells and organelles suggesting that all membrane pumps were inactivated by the absence of energy metabolites.

The concept that PARP was a suicide enzyme, activated by high levels of DNA damage, was discussed in a manuscript published in 1982.[14] A subsequent manuscript describing a more detailed metabolic analysis was submitted to the *Journal of Biological Chemistry* and promptly rejected because it was considered obvious that without ATP a cell could not survive. Furthermore, who ever heard of suicide enzymes? This was, of course, before apoptosis and associated suicide enzymes had gained popularity. Depletion of NAD^+ and ATP in response to DNA damage, PARP activation, and the PARP-mediated suicide hypotheses were presented and published in the proceedings of the Princess Takamatsu Symposium on ADP-ribosylation, DNA repair, and Cancer,[1] more formally in a 1983 manuscript[15] and at a symposium of the Radiation Research Society.[20]

In summary, the suicide hypothesis posits that high levels of DNA damage can cause sufficient activation of PARP to rapidly and irreversibly consume its substrate NAD^+. Because of rapid equilibration between NAD^+ and NADH, both pools are depleted. This decrease in pyridine nucleotides interferes with the cells' ability to maintain ATP pools and ATP is rapidly and irreversibly consumed. Other nucleotide and deoxynucleotide pools are depleted, preventing cells from undergoing replication or repair synthesis. Depletion of NAD^+ results also in inhibition of glycolysis which further interferes with ATP generation. The loss of NAD^+ eliminates the precursor for and results in depletion of $NADP^+$ and NADPH levels, cessation of bioreductive synthesis, and the inability to maintain reduced glutathione pools. As a consequence, membrane pumps fail, cells and organelles undergo swelling, and death occurs within several hours of sustaining DNA damage. In many cases, these metabolic consequences of DNA damage can be interfered with by the use of PARP inhibitors.

This suicide hypothesis provides a metabolic mechanism for heavily damaged cells to be eliminated rather than to try to repair their highly damaged DNA. The process is useful since an attempt at repair could lead to survival of cells with a high mutation frequency, especially in the situation where repair might include an error prone pathway. Survival of highly mutated cells, in a multicellular organism could cause a high rate of malignancy or other problems. Therefore, a suicide mechanism for highly damaged cells would benefit the overall organism. In contrast, prokaryotes, which do not possess PARP, would undergo this suicide pathway in response to high levels of DNA damage and would, therefore, have a maximal opportunity for

repairing DNA damage and sustaining mutations that could foster adaptive changes to hostile environments.

While the suicide pathway can be clearly demonstrated under laboratory conditions, it was not clear that it ever occurred under physiologic conditions or even under pathologic conditions associated with any known disease or disorder. It was considered possible that battlefield exposure to high levels of alkylating agents and consequent necrosis of epithelial tissues could be an example of DNA damage with subsequent PARP activation of the suicide response. This suggested that nicotinamide or other PARP inhibitors could have a protective effect or at least slow down the vesicant effect of battlefield alkylating agents.

In 1991 Spragg showed that treatment of vascular endothelial cells with hydrogen peroxide produced sufficient DNA damage to activate the PARP mediated suicide response resulting in cell lysis within hours.[23] This observation suggested that PARP-mediated suicide might have a role in the cytotoxicity induced by reactive oxygen species, and that metabolic modulation, including PARP inhibition, might prevent or ameliorate and consequent cytotoxicity.[24] We suggested that direct intervention to prevent NAD^+ pool depletion was a promising approach to interfere with the consequences of DNA damage since it could be applied regardless of the agents that initiate the DNA damage.[24]

A major resurgence or interest in PARP-mediated suicide occurred in 1994 with the demonstration that it played an important role in mediating nitric oxide neurotoxicity as induced by ischemia reperfusion and by neurotoxins such as glutamate.[25] The central role of PARP in mediating neuronal damage in stroke was supported by demonstrating the protective effect of a PARP inhibitor in a rat stroke model.[26] Around the same time, two separate groups showed that PARP knockout mice were resistant to neuronal death elicited by stroke.[27,28] Thus, studies of PARP inactivation by gene deletion and pharmacologic inhibition confirmed the role of PARP as a mediator of a pathophysiologic process and validated PARP as a viable target for potential therapeutic intervention of ischemia reperfusion injuries.

In addition to its role in pathogenesis of stroke, PARP-mediated suicide is now considered a potential mediator in a variety of neurodegenerative diseases as well as in ischemia reperfusion-mediated damage to the myocardium, kidney, and retina. As discussed later in this book, PARP-mediated suicide appears also to be involved in inflammatory cell-induced tissue destruction, such as airway epithelium and in shock; it is also postulated to be involved in the islet cell loss that occurs in the pathogenesis of diabetes[29] as well as in diabetic cardiovascular disease.

As noted above, the demonstration that PARP-mediated suicide plays a critical biochemical role in pathogenesis of a variety of clinical disorders suggests that the enzyme and the pathway may be important therapeutic targets. The most obvious therapeutic approach is to design pharmacologic agents to inhibit PARP and prevent or at least slow down, the consequent NAD^+ depletion, thereby preventing or retarding the suicide response. Another approach to preserving NAD^+ pools is to improve nicotinamide nutritional status, especially in individuals likely to be subject to PARP-mediated suicide disorders. Similarly, since NAD^+ levels vary in different tissues but generally remain constant within a given tissue, it is clear that pyridine

nucleotide levels are controlled by specific regularity processes and manipulating these processes might help maintain NAD^+ levels even in the presence of high-level PARP activation. Other metabolic interventions may also be directed at preserving NAD^+ and ATP pools.[24] Finally, a genomic approach to help upregulate the enzymes of NAD^+ synthesis and or down-regulate PARP levels might help prevent these conditions. Selective activation of PARP-mediated suicide, perhaps using tumor targeted viruses capable of causing massive DNA damage in selected cells or more selective chemotherapeutic agents, remains an important challenge for cancer therapy.

As a result of the ongoing pursuit of mechanism-based explanations for clinical phenomenon, the interference and/or modulation of PARP-mediated suicide presents new and unique opportunities for prevention and therapy of several important clinical disorders.

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Preface

Drug targets are hard to come by. In the history of drug discovery, the pharmaceutical industry has only worked on a total of about 500 targets. Most drugs, whether extracted from natural products, produced with biotechnology, or ingeniously synthesized by chemists, exert therapeutic effects through a limited number of biological pathways. The prominent drug targets that generate enormous social and economic benefits tend to belong to key regulatory components for essential pathways, e.g., hormones, receptors, channels, transporters, nucleic acids, and enzymes. The industry is primarily driven by its keen interest in identifying novel targets for the next blockbuster drug to treat unmet medical needs. In spite of great expectation bestowed on the human genome sequence project to reveal new targets for drug development, there appears to be ~30,000 + genes, fewer than what was initially anticipated, in the 3 billion base pairs of human genome. This would roughly yield 3000 drug target candidates, if one applies a rule of thumb that about 10% of genes might be related to diseases. Sifting through the genome to identify and validate drug targets by single nucleotide polymorphism (SNP) analysis or proteomics study would require much more time than that for the genome sequencing itself. As for now, the scarcity of new drug targets, and serendipity mode to discover more, perhaps explains the enthusiasm aroused when PARP family proteins, especially PARP-1, emerged as candidate drug targets.

PARP-1 is a fascinating enzyme. This nuclear protein must have afforded eukaryotic organisms evolutionary advantage to be so highly conserved from *Dictyostelium* to human. More than 3 decades of PARP-1 research have accumulated data to support that it may play important roles in safeguarding genome integrity, regulating transcription, facilitating DNA repair, mediating cell death, and participating in DNA replication, DNA recombination, and DNA integration. Although the details of how PARP-1 functions are still being elucidated, its potential as a promising drug target has triggered tremendous interest. Numerous animal studies demonstrate that PARP activation is involved in a broad spectrum of diseases, ranging from cancer to ischemia to inflammation. Studies using PARP-1 gene deletion or PARP inhibitors have resulted in corroborating evidence to validate PARP-1 as a novel target to be intervened for potential therapeutic benefits.

The pharmaceutical industry is streamlining the drug discovery process. More emphasis is put on integrating knowledge for a target from both basic science research and applied pharmacological studies at an early stage of preclinical development to define a strategy to maximize the chance of clinical success. New drug development is a lengthy, risky, and expensive process. A new chemical entity now typically takes a decade to move through clinical trials. The odds for a preclinical candidate molecule to jump over all the hurdles to reach market launch is around 10%. The average cost for the endeavor amounts to hundreds of millions of U.S. dollars and is still increasing. To remain competitive, drug companies face a challenge

to slash the cost and shorten the period of drug development. Recent advance in high throughput screening and structure-based drug design have released enormous power to aid the process. To better harness such power, much of decision making has been shifted to a pre- or early clinical period. The decision process would be facilitated by knowledge that bridges the gap between the basic and pharmacological studies of how the specific drug target acts. As one such novel target, PARP-1 would conform to the rule that better understanding of how it works could lead to a better design of its inhibitors for therapeutic purpose.

For this book, I invited many leading researchers, who have made pivotal contributions shaping the current trend of delineating the role of PARP in various disease conditions, to share their views of recent significant developments and future directions of PARP research. We have attempted to assess the state-of-the-art at the cutting edge of the PARP field. The primary focus is on linking the basic PARP pathway to its roles in pathogenesis. I hope the book will provide interested readers, from basic science or clinical pharmacology researchers to entrepreneurs or managers, with perspectives of multiple potentials for the clinical utilities of PARP inhibitors. If the tone in some chapters sounds provocative or speculative, it is only meant to stimulate critical thinking to push further the boundary of our knowledge on PARP, which we hope would soon translate into clinical benefits.

I thank Dr. Mannfred A. Hollinger for arranging the timely publication of this monograph by CRC Press in the series of *Pharmacology and Toxicology: Basic and Clinical Aspects*. I am much obliged to all the authors for their contributions and candid opinions on the latest development in the rapidly moving field. Finally, I wish to acknowledge my colleagues in the Guilford PARP team, whom I have a privilege to work with. I am very grateful for their hard work and dedication in moving this project forward.

Jie Zhang
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The Editor

Jie Zhang earned his Ph.D. from the biochemistry, cellular, and molecular biology training program at The Johns Hopkins University School of Medicine in May 1991. He then did a postdoctoral stint examining the effect of nitric oxide on the nervous system in the department of neuroscience at Johns Hopkins. While there, he made the seminal discovery that activation of poly(ADP-ribose) polymerase (PARP)-mediated neuronal death resulted from glutamate neurotoxicity. His work identified PARP as a drug target for treating cerebral ischemia, neural degeneration, and other neurological disorders.

In 1994, Dr. Zhang was recruited by Guilford Pharmaceuticals, Inc. to spearhead the PARP project aimed at exploring potential therapeutic benefits of PARP inhibition. Toward that end, he made key contributions to building the PARP team, establishing the PARP high-throughput screening, identifying and optimizing leads with chemists, and coordinating in-house preclinical testing and outside collaborations. As a result, the company has identified multiple families of novel PARP inhibitors and demonstrated their efficacy in various animal models of diseases, which support a broad spectrum of possible clinical indications for PARP inhibitors. Currently, he is a principal scientist leading the PARP biology group to test small molecule PARP inhibitors for future clinical development.

Dr. Zhang maintains a strong interest in basic research and has started several drug discovery initiatives at Guilford. He is on the editorial board of *Emerging Drugs*, and has published 18 original scientific papers in peer-reviewed journals and written 18 review articles and book chapters. He is an inventor of dozens of issued and pending patents related to PARP technology.

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Krishna Juluri, Hyo Chol Ha, and Solomon H. Snyder

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1.1 INTRODUCTION

Poly(ADP-ribose) polymerase-1 (PARP-1) was first identified as a nuclear enzyme involved in DNA repair,[1,2] and as such was of principal interest to studies of the cell cycle and alterations that can lead to cancer. PARP-1 catalyzes the initiation, elongation, and branching of ADP-ribose polymers, derived from the substrate nicotinamide adenine dinucleotide (NAD^+) on to glutamate and aspartate residues of acceptor proteins.[3] Evidence that PARP-1 overactivation following cell stress can kill cells by energy depletion[4] led to the notion that PARP inhibition might be cytoprotective. Because neurons in the central nervous system generally do not divide in adult life, and because energy dynamics in the nervous system tend to be relatively sequestered from events in the rest of the body, studies of PARP-1 in the nervous system have been relatively recent. However, abundant evidence now indicates that PARP-1 overactivation plays a major role in neurotoxicity of various conditions including stroke, and that PARP inhibition may provide a more powerful means of preventing stroke damage than many other pharmacological approaches.[5] Studies involving neural as well as other tissues indicate that cell death by PARP-1 overactivation may be selectively associated with necrotic cell death, thus providing a "programmed" necrosis in parallel to the

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programmed cell death (apoptosis) that has been extensively characterized. PARP-1 also appears to play a unique role in facilitating viral integration into the host genome. Finally, studies in the nervous system and other tissues establish a role for PARP-1 in normal, physiological processes unrelated to pathophysiological cell death and linked in part to nitric oxide (NO) biology.

1.2 PARP-1 AND NO IN NEUROTOXICITY

Our own interest in PARP-1 in the brain stemmed from our studies of NO as a neurotransmitter. NO serves multiple roles including acting as an endothelial-derived relaxing factor following synthesis by endothelial NO synthase (eNOS), mediating the ability of macrophages and other cells to attack tumor cells and bacteria following formation by inducible NOS (iNOS), and functioning as a neurotransmitter formed by neuronal NOS (nNOS).[6] Earliest evidence for neural functions of NO came from findings that stimulation of *N*-methyl-D-aspartate (NMDA) type glutamate receptors that enhanced formation of guanosine 3′:5′-cyclic monophosphate (cGMP) also rapidly activated nNOS[7] and that NOS inhibitors blocked the formation of cGMP.[8] Immunohistochemical studies localized nNOS to select neuronal populations, and NOS inhibitors blocked various forms of neurotransmission, providing evidence that NO is indeed a neurotransmitter.[9]

Although NMDA transmission accounts for the physiological neurotransmitter actions of glutamate, glutamate is released in vast excess during stroke and other forms of neurotoxicity. Thus, overstimulation of NMDA receptors leads to excitotoxicity and cell death. Evidence for this includes the ability of NMDA receptor antagonist drugs to block various forms of neurotoxicity and prevent stroke damage. Just as NOS inhibition blocked physiological NMDA transmission, we found that NOS inhibitors and targeted deletion of nNOS prevented neurotoxicity.[10,11] Stroke damage is also reduced in nNOS mutant mice and following treatment with NOS inhibitors.[12,13] The link between NMDA stimulation and NO formation involves calcium entry through NMDA receptors binding to calmodulin, which is bound to NOS leading to stimulation of catalytic activity.[14-16]

Our interest in PARP as a target of NO stemmed from evidence that cGMP was not likely to be the sole target for NO action. There had been reports that NO enhanced ADP-ribosylation of a 39-kDa protein, which was presumed to be a G protein, although its structure was not identified.[17] To purify such a protein efficiently, we synthesized biotinylated derivatives of NAD^+, the substrate for ADP-ribosyl transferase reactions, and found that one of them retained its ability to participate in such reactions.[18] Utilizing an avidin column, we purified the NO-enhanced ADP-ribosylated protein from brain tissue and discovered that it was not a G protein at all, but was glyceraldehyde-3-phosphate dehydrogenase (GAPDH), the well-known glycolytic enzyme.[19] These findings were also obtained independently by other groups[20,21] and some investigators have suggested that the entire NAD^+ molecule may be transferred.[22,23] It now appears likely that this modification of GAPDH is secondary to its *S*-nitrosylation, which is a physiological signal for NO generated from

nNOS.[24] ADP-ribosylation of GAPDH occurs at cysteine-150, which is critical for its catalytic activity.[19] The function of ADP-ribosylation of GAPDH is still unclear; however, it has been shown that GAPDH can translocate to the nucleus during cell death in neuronal and non-neuronal systems,[25] and that blocking the synthesis of GAPDH is cytoprotective.[26] Accordingly, ADP-ribosylation of GAPDH may play some role in the cell death process.

Our studies of PARP began with efforts to find other NO-stimulated modifications of proteins utilizing NAD^+. Employing the same techniques that led to the identification of GAPDH as a target, we found that NO stimulated ADP-ribosylation of a 110-kDa protein, which we showed to be PARP-1.[27] This led us to wonder whether PARP-1 activation might play a role in neuronal cell death following NMDA receptor activation and overproduction of NO. How might NO bring about activation of PARP-1, which is classically associated with DNA damage? NO is itself a free radical but is not extraordinarily reactive. However, NO can react with superoxide anion to form peroxynitrite, itself a potent oxidant, which can also decompose into the toxic hydroxyl free radical.[28] Activation of NMDA-type glutamate receptors increases generation of superoxide anion in mitochondria, which can augment formation of peroxynitrite.[29] Abundant evidence indicates that both peroxynitrite and hydroxyl free radicals oxidize numerous tissue constituents including DNA.[30-32]

By monitoring NOS activity in intact neural tissue, we obtained direct evidence that NO-mediated neurotoxicity involves the formation of peroxynitrite rather than NO acting by itself. NOS activity was monitored by staining for citrulline, which is formed stoichiometrically with NO, and also by staining for nitrotyrosine, which reflects the covalent modification of proteins by peroxynitrite rather than NO.[33] We found that citrulline staining is prominent in the peri-infarct areas of strokes, which are only partially ischemic, with the tissue still being alive, while nitrotyrosine staining is a good marker for fully infarcted tissue zones.

We obtained direct evidence that NMDA neurotoxicity in cerebral cortical cultures involves PARP-1 overactivation, by experiments in which we showed that PARP inhibitors block this toxicity with relative potencies closely paralleling their potencies in inhibiting the PARP-1 enzyme.[27] In an independent study, Cosi et al.[34] observed that glutamate enhances PARP-1 activity of cerebellar granule cells and that PARP inhibitors prevent glutamate toxicity in these preparations. More persuasive evidence for a role of PARP-1 in neurotoxicity came from studies employing mice with targeted deletion of PARP-1 ($PARP\text{-}1^{-/-}$). In cortical cultures we found that NMDA toxicity was virtually abolished in the mutant preparations and was reduced 60 to 70% even in heterozygote $PARP\text{-}1^{+/-}$ cultures.[35]

Neuroprotective effects were evident in intact mice with strokes elicited by occluding the middle cerebral artery. The $PARP\text{-}1^{-/-}$ animals showed an almost 80% reduction in infarct volume while stroke damage was reduced about 65% in the heterozygotes.[35] Independently, Endres et al.[36] observed about a 40% reduction of stroke damage in $PARP\text{-}1^{-/-}$ animals. Discrepancies between the two studies might derive from the background strains employed as well as variations in lesion size.

Many different pharmacological approaches have been employed to diminish neural damage following stroke with drugs including antagonists of NMDA and

AMPA-type glutamate receptors, calcium antagonists, numerous sedatives, NOS inhibitors, and a variety of other drugs. The protection against neural damage in PARP-1 mutant animals is more impressive than with any other treatment.[35] The potent PARP inhibitor 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(*2H*)-isoquinolinone (DPQ) also provides substantial protection against stroke damage whether given before or after middle cerebral artery occlusion.[37] PARP inhibition also protects in animal models of Parkinson's disease caused by the dopamine neuronal toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) as well as retinal ischemia associated with increased intra-ocular pressure, a model of glaucoma.[38,39]

The finding that PARP inhibition provides better neuroprotection than other approaches may be explained by the notion that PARP-1 overactivation is a final common death pathway for disorders as diverse as stroke, Alzheimer's, Huntington's, and Parkinson's diseases. PARP activation presumably stems from DNA damage, so greater protection by PARP inhibition than by other treatments implies that a variety of cytotoxic insults involve DNA damage as a final common mediator. Stroke damage and various other cellular stresses exert their major toxicity through the formation of reactive oxygen species (ROS) such as superoxide anions, formed independently of glutamate and NO, so one would not expect glutamate antagonists or NOS inhibitors to provide complete protection against ischemic damage. Superoxide anion formation, leading to the generation of peroxynitrite and hydroxyl free radicals, can damage cellular components including DNA. DNA damage activates PARP-1, thus explaining in part the impressive neuroprotective effects of PARP inhibition. Still, there are other types of cellular insults caused by ROS such as lipid peroxidation of cellular membranes, mitochondrial damage by calcium and other substances such as MPTP, as well as oxidative damage to numerous macromolecules. It is likely that all of these steps are important and are relevant targets for therapeutic intervention. Since DNA damage leading to PARP-1 activation is a final step in multiple mechanisms of cellular insults, it is logical that PARP inhibition works particularly well in protecting against several types of injury. DNA damage activates PARP-1 almost instantly. PARP-1 is an extremely abundant and active nuclear enzyme whose activity is amplified by its catalysis, consuming 50 to 200 molecules of NAD^+ leading to rapid depletion of NAD^+ and ATP. Thus, while other molecular mechanisms clearly have the capacity to kill cells, the PARP-associated energy depletion may be a rate-limiting lethal insult in multiple cell death pathways that is a particularly well suited target for therapeutic intervention (Figure 1.1).

1.3 PARP-1 MEDIATION OF CELLULAR NECROSIS

Programmed cell death, also called apoptosis, is a major mechanism for the well-coordinated loss of cells during the development of the nervous system[40] and for cell death in a variety of other conditions. Multiple molecular mechanisms of apoptosis have been advanced. Particularly attention has focused upon the family of proteases called caspases (cysteinyl aspartate-specific proteinases).[41] Kaufmann and colleagues[42,43] first established that proteolytic cleavage of PARP-1 is a key event in

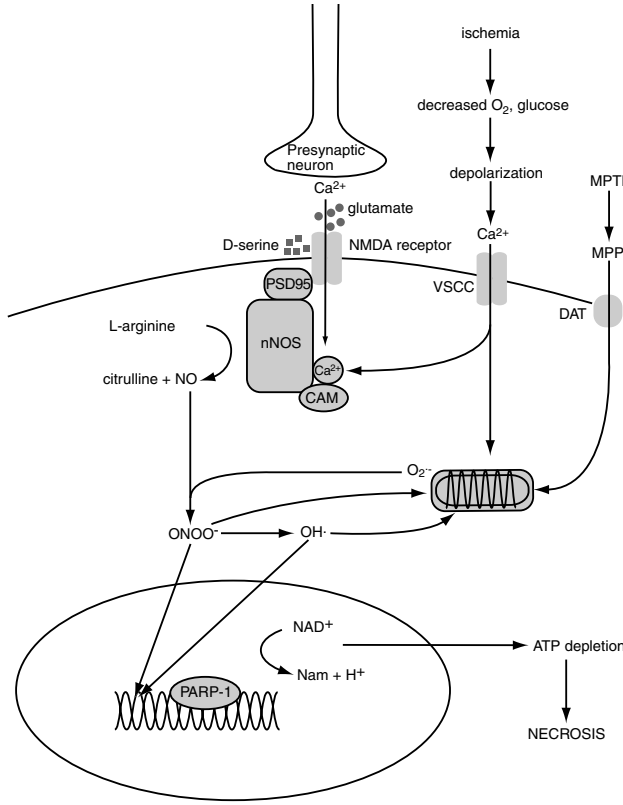

FIGURE 1.1 PARP-1 is a final mediator of several mechanisms of neuronal cell death and is thus an excellent target for therapeutic intervention. NMDA-mediated neurotoxicity occurs through the action of glutamate and D-serine on NMDA receptors. Upon activation, the NMDA channel allows the passage of calcium ions. Neuronal NOS (nNOS) is localized to the NMDA receptor through an interaction with PSD95. Calmodulin (CAM) binds to the entering calcium and activates nNOS, resulting in the production of citrulline and NO from L-arginine. NO reacts with the superoxide anion ($O_2^{-\bullet}$) to form peroxyinitrite ($ONOO^-$), which is itself extremely toxic to cellular molecules including DNA and can also decompose to the toxic hydroxyl free radical ($OH^{\bullet}$). Peroxynitrite can also inhibit mitochondrial respiration, resulting in the generation of superoxide anion. During periods of ischemia, the cell undergoes deprivation of oxygen and glucose, which results in depolarization of the cell, opening voltage sensitive calcium channels (VSCC). This calcium can be used in the NO generation pathway or can damage mitochondria, as well as release more glutamate. In the MPTP model of Parkinson's disease, MPTP is metabolized to MPP^+, which enters the cell through the dopamine transporter (DAT). MPP^+ damages mitochondria, resulting in the generation of superoxide anion. (DAT is not expressed in nNOS-containing neurons; however, for simplification of this figure, it is drawn as such. NO from nNOS-containing neurons diffuses into neurons expressing DAT and reacts with $O_2^{-\bullet}$ as described.) All of these mechanisms result in the eventual damage of DNA, leading to the activation of PARP-1, which utilizes NAD^+ to generate ADP-ribose polymers, releasing nicotinamide (Nam) and H^+. Overactivation of PARP-1 leads to NAD^+ depletion, which in turn results in ATP depletion and cellular death through necrosis.

TABLE 1.1
PARP-1 Deletion or Inhibition Protects Against Necrosis but not Apoptosis

Insults	Method of PARP-1 inhibition	Result	Refs
Necrosis associated: PARP-1 deletion or inhibition confers protection			
In vivo models			
MCAo	Gene deletion, PJ34	Protection against cerebral IR injury by decreasing infarct volume	35,36,69
STZ	Gene deletion	Inhibition of NAD^+ depletion, necrosis in pancreatic beta-cells, and diabetes	50,70,71
Zymosan	Gene deletion	Reduction of multiple organ failure (intestine, lung, and liver), reduction of local inflammation, and suppression of neutrophil recruitment	72
LAD-IR	Gene deletion	Protection against myocardial IR injury by inhibiting the expression of P-selectin and ICAM and suppression of neutrophil recruitment	73
TNBS	Gene deletion	Suppression of neutrophil recruitment and inhibition of ICAM upregulation	74
Acetaminophen hepatotoxicity	NAA, benzamide, thymidine	Protection against analgesic-induced liver damage	75
Chronic colitis	3-AB	Attenuation of inflammation, normalized colonic permeability	76
In vitro models			
NMDA, SNP, SIN-1, OGD	Gene deletion	Inhibition of necrosis in cerebral cortical culture	35
$ONOO^-$, H_2O_2	Gene deletion, 3-AB, hypoxanthine, inosine, adenosine	Maintenance of mitochondrial transmembrane potential and inhibition of necrosis in thymocytes.Inhibiton of necrosis in LLC-PK1 cells.	77-79
LPS/IFN	Hypoxanthine, inosine, adenosine	Protection from inhibition of mitochondrial respiration, inhibition of nitrite production	79

X/XO nitroprusside	Gene deletion	Inhibition of NAD^+ depletion and necrosis in pancreatic islet cells	80
Apoptosis associated: PARP-1 knockout or inhibition does not confer protection			
In vivo models		Developmental apoptosis proceeds normally in PARP-$1^{-/-}$ mice as the morphology and function of the major organs appear to be intact	81
In vitro models			
Low K^+, STA, MPP^+, $ONOO^-$, COL, etoposide	Gene deletion	Apoptosis in cerebellum	44
DEX, ceramide, ionomycin/CD95	Gene deletion	Apoptosis in thymocytes	44
ActD/TNF, MNU	Gene deletion	Apoptosis in hepatocytes	44
CPT-11, - IL-3	Gene deletion	Apoptosis in bone marrow	82
CD95/CHX, MMS	Gene deletion	Apoptosis in fibroblasts	82
MNU	Gene deletion	Apoptosis in splenocytes	45

MCAo, middle cerebral artery occlusion; IR, ischemia and reperfusion; STZ, streptozocin; LAD-IR, ischemia and reperfusion of the left anterior descending coronary artery; ICAM, intercellular adhesion molecule; TNBS, trinitrobenzenesulfonic acid; NAA, nicotinic acid amide; 3-AB, 3-aminobenzamide; NMDA, N-methyl-D-aspartate; SNP, sodium nitroprusside; SIN-1, 3-morpholino-sydnonimine hydrochloride; OGD, oxygen-glucose deprivation; ONOO-, peroxynitrite; H_2O_2, hydrogen peroxide; LPS, lipopolysaccharide; IFN, interferon; X/XO, hypoxanthine/xanthine oxidase; STA, staurosporine; MPP^+, 1-methyl-4-phenylpyridinium; COL, colchicine; DEX, dexamethasone; ActD, actinomycin D; TNF, tumor necrosis factor; MNU, methylnitrosourea; CPT-11, a semisynthetic camptothecin derivative; CHX, cycolheximide; MMS, N-methylmethanesulfonate.

Adapted from Ha, H. C. and Snyder, S. H., *Neurobiol. Dis.* 7 (4), 225-39, 2000. With permission.

apoptosis. Its importance appears related to the requirement of apoptosis for substantial energy resources. Activation of PARP-1 by DNA damage would deplete energy stores and so might prevent energy-dependent apoptotic processes. According to this line of thinking, PARP-1 cleavage would preserve the energetic substrates required for the apoptotic process.

In contrast to the great abundance of research working out specific molecular mechanisms of apoptosis, necrosis had long been thought to reflect a less-controlled collapse of multiple cellular functions. The requirement of PARP-1 cleavage for apoptosis suggests that the cell death caused by excess PARP-1 activation might conceivably involve necrosis. Studies of cell death in PARP-$1^{-/-}$ tissues have led to conflicting results, with some studies showing PARP-$1^{-/-}$ cells or animals to display normal sensitivity to apoptotic agents,[44] whereas others have shown them to undergo more rapid apoptosis.[45] We wondered whether these disparate findings might reflect a selective role of PARP-1 in one or another form of cell death, apoptosis vs. necrosis. To examine this question, we decided to compare apoptosis and necrosis in a single cell type utilizing fibroblasts from PARP-$1^{-/-}$ animals.[46] We employed DNA damaging agents such as 1-methyl-3-nitro-1-nitroguanidine (MNNG) and hydrogen peroxide, which are well known to cause necrosis when used at appropriate concentrations. In addition we employed Fas, also known as α-CD95, a membrane protein that selectively triggers apoptotic cell death following activation by anti-Fas antibodies in conjunction with the protein synthesis inhibitor cycloheximide. MNNG and hydrogen peroxide depleted NAD^+ and ATP from fibroblasts and killed them by a necrotic mechanism. Fas activation, on the other hand, had no influence on ATP and NAD^+ levels while killing the cells by apoptosis. Strikingly, we found that PARP-$1^{-/-}$ cells were protected from the necrotic cell death caused by MNNG and hydrogen peroxide while confirming no protection for the apoptosis elicited by Fas activation.

We then reviewed the literature containing conflicting reports on the relationship of PARP-1 deletion and cell death (Table 1.1). We separated the studies into those in which cell death involved apoptosis and those in which necrosis predominated. Clearly, necrotic cell death is selectively prevented by PARP-1 depletion, whereas apoptosis is not influenced. Examples of cell death that involve necrosis and which are protected by PARP-1 deletion include NMDA mediated neurotoxicity,[47] myocardial ischemia,[48] inflammatory death,[49] as well as streptozotocin-induced diabetes.[50] On the other hand, apoptotic death caused by TNF-α influences on hepatocytes or thymocyte death caused by ceramide, CD95, ionomycin, or dexamethasone are not protected by PARP-1 deletion (see Table 1.1).

It is interesting that PARP-1 plays a role in both necrosis and apoptosis but in opposing directions. Other evidence supporting this conclusion includes observations that ATP depletion transforms apoptosis into necrotic cell death with intracellular ATP levels determining the mode of cell death.[46] Lee and Shacter[51] observed reversible changes between apoptosis and necrosis. They induced apoptosis in Burkitt lymphoma cells by etoposide, a topoisomerase II inhibitor, or the calcium ionophore A23187 and transformed apoptosis into necrosis with associated ATP depletion following treatment with hydrogen peroxide. They then showed that PARP inhibitors prevented the ATP depletion caused by hydrogen peroxide and converted the cell

death process from necrosis back to apoptosis. Taken together, these findings suggest that necrosis is not simply a passive disintegration of cellular activities but, like apoptosis, is an active process selectively associated with PARP-1 activation (Figure 1.2).

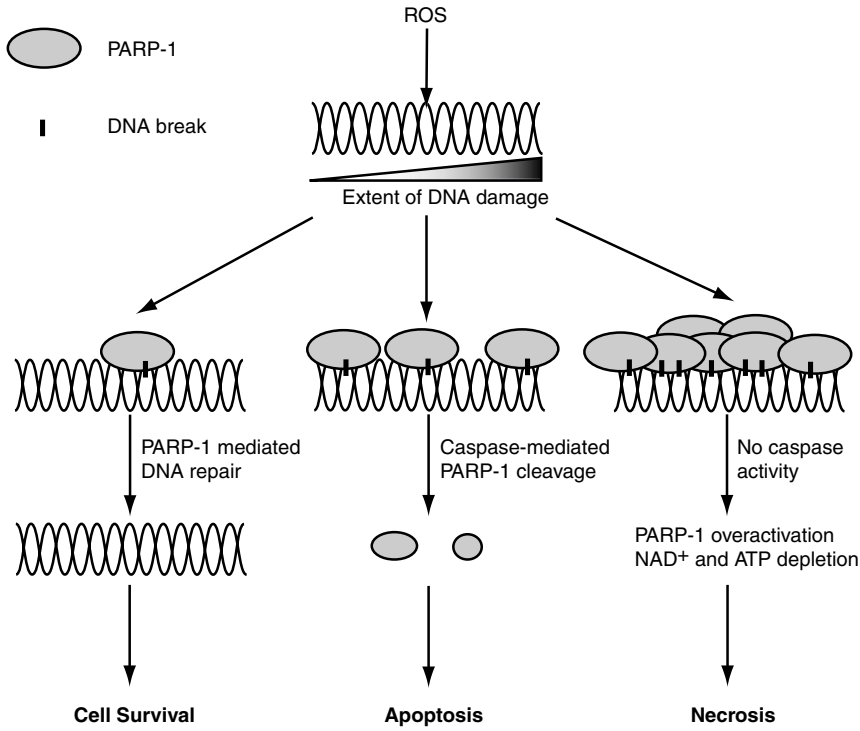

FIGURE 1.2 PARP-1 activity plays a key role in determining the fate of the cell following oxidative injury. Since PARP-1 is activated by DNA damage, there is a positive correlation between the level of DNA damage and PARP-1 activity. At low levels of DNA damage, PARP-1 binds to strand breaks and is activated, mediating DNA repair through processes such as the break excision repair pathway. At higher levels of DNA damage from which the cell cannot recover, PARP-1 is again activated. As the cell begins down the programmed cell death pathway, caspases are activated, which cleave and inactivate PARP-1, and the cell dies through apoptosis. When massive DNA damage occurs, PARP-1 is active, depleting the cell of NAD^+, preventing the regeneration of ATP, thus resulting in the depletion of the cellular ATP pool. As the apoptotic pathway is ATP dependent, programmed cell death cannot occur, and caspase-mediated inactivation of PARP-1 cannot occur, further depleting the cell of NAD^+ and ATP, and resulting in necrotic cell death.

1.4 REQUIREMENT OF PARP-1 FOR EFFICIENT HIV-1 INTEGRATION IN CONTRAST TO THE EXPLOSIVE CELL DEATH ASSOCIATED WITH ISCHEMIA

Human immunodeficiency virus-1 (HIV-1) infection is insidious, involving the integration of the viral DNA into the host genome. Mechanisms for this integration are well established in cells of the immune system.[52-54] HIV-1 infection of the brain is prominent in many patients, but the exact mechanisms for central nervous system damage are not clear, and the extent to which HIV-1 can infect neurons directly is controversial. The overall pattern whereby HIV-1 is integrated into the host genome is reasonably well established. In infected cells viral reverse transcriptase synthesizes double-stranded DNA from HIV-1 RNA. The resulting HIV-1 DNA is part of a large preintegration complex that enters the nucleus and is integrated into the host chromosome. The viral integrase enzyme associates with ends of the linear double-stranded viral DNA and catalyzes integration. At each end of the DNA molecule, the integrase removes two terminal 3′ nucleotides, exposing recessed 3′ hydroxyl groups. The integrase then catalyzes a nucleophilic attack of the recessed 3′ hydroxyl groups on the phosphodiester bonds in each target cellular DNA strand. Each strand of the viral DNA then joins cellular DNA, leaving a 4 to 6 base gap and a 2-base mismatch at the end. The subsequent gap repair provides 4 to 6 base duplications of the target DNA at each host–virus DNA junction leading to formation of an integrated pro-virus. Host cell DNA repair machinery presumably accomplishes this gap repair. Following integration, the pro-viral DNA becomes part of the host genome, thus leading to productive infection.

The ability of several PARP inhibitors, as well as antisense and dominant-negative constructs, to diminish retroviral infection in some studies suggested a role for PARP-1 in this process.[55] However, some studies failed to find such effects.[56] The benzamide PARP inhibitors employed in these studies are weak, acting in the millimolar range, and exert their own toxic effects,[57-59] precluding strong conclusions about a role of PARP-1 in viral integration. Overexpression of antisense and dominant-negative constructs can also elicit nonspecific influences.[60,61] To obtain more definitive evidence, we evaluated HIV-1 infection in PARP-1$^{-/-}$ fibroblasts.

Since HIV-1 selectively infects human rather than mouse cells, we employed a pseudotyped replication-defective HIV-1 vector with HIV-1 particles utilizing a vesicular stomatitis virus envelope glycoprotein (VSV-G). This pseudotyped virus has a broad host range enabling us to utilize mouse cells. Whereas more than 90% of wild-type fibroblasts are infected, PARP-1$^{-/-}$ fibroblasts are almost completely protected from infection. The prevention of infection was evident at multiple time points from 48 to 120 h indicating that PARP-1 deletion did not merely delay infection.[62]

Prevention of infection might involve interference with viral entry into cells, reverse transcription, viral integration into the host genome, or transcription of HIV-1 genome products. Utilizing a specific assay for viral integration, we demonstrated an abolition of HIV-1 integration in the PARP-1$^{-/-}$ fibroblasts. By contrast, we found no abnormality in viral entry into cells in the mutant fibroblasts or reverse transcription of the HIV-1 genome.

How might PARP-1 regulate HIV-1 integration? PARP-1 contains two zinc fingers that attach to the ends of DNA strand breaks. We presume that PARP-1 binds to the 4 to 6 base gap produced by the integrase and that this interaction activates PARP-1. Activated PARP-1 synthesizes poly(ADP-ribose), which modifies histones, leading to chromatin relaxation or decondensation. PARP-1 itself is also ADP-ribosylated leading to its dissociation from the gap. We suspect that these processes help repair the gap by facilitating the access of repair enzymes leading to completion of the integration process. The resolution would yield 4 to 6 base duplications of the target DNA at each end of the host–virus DNA junctions resulting in an integrated provirus.

We do not know whether PARP-1 also influences HIV-1 transcription. We were not able to examine this possibility directly because the loss of integration in the mutant fibroblasts precluded analysis of transcriptional events.

A role of PARP-1 in HIV integration may have therapeutic implications. Specifically, potent and selective PARP inhibitors might be useful in preventing HIV-1 infection of cells.

1.5 BASAL ACTIVATION OF PARP BY GLUTAMATE-NO NEUROTRANSMISSION

Because of its grave consequences for cellular function, DNA damage has been assumed to be negligible under basal conditions. Similarly, PARP-1, which is selectively activated by DNA strand breaks, is also thought to take place only following tissue damage. However, various physiological events could conceivably lead to DNA strand breaks, such as activation of calcium/magnesium-dependent endonucleases,[63] transcription, or DNA replication. Most techniques employed to monitor DNA damage are not exquisitely sensitive. Moreover, they are applied to crude tissue extracts so that strand breaks in discrete areas of tissues, especially the brain, would be unnoticed. The same considerations apply to techniques for detecting PARP activation. To explore the possibility of basal levels of DNA strand breaks and of PARP activation in discrete brain regions, we developed a novel autoradiographic approach to monitoring PARP activity.[50] Specifically, we assessed the conversion of [^{33}P]NAD$^+$ to poly(ADP-ribose) (PAR). In this technique tissue sections are incubated with [^{33}P]NAD$^+$ and extensively washed to remove radiolabel not covalently bound to protein. Novobiocin, a specific inhibitor of mono(ADP-ribosyl)ation eliminates background mono(ADP-ribosyl)ation. This technique, which we designate PARIS — poly(ADP-ribosyl)ation *in situ* — provides autoradiographic visualization of poly(ADP-ribosyl)ation in fresh-frozen tissues by monitoring the conversion of the radiolabeled NAD$^+$ to labeled PAR. We monitored DNA strand breaks by *in situ* end labeling (ISEL).

We observed discrete concentrations of PARIS radiolabel in the brain and pituitary and pineal glands (Figure 1.3). High levels also were apparent in cerebellar granule cells, hippocampal dentate gyrus, the olfactory bulb, as well as ependymal and subependymal ventricular cells. The PARIS signal was exclusively in neurons.

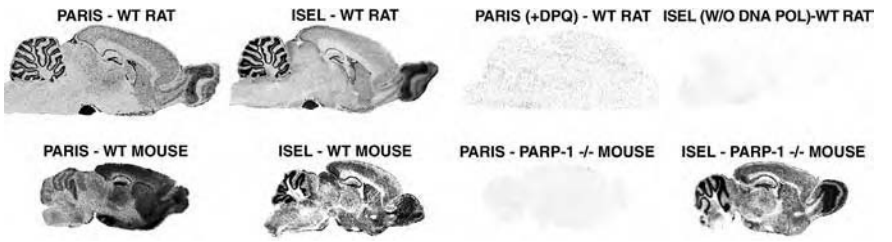

FIGURE 3.3 PARIS assay and ISEL show that basal PARP activity and DNA strand breaks are co-localized in rat and mouse brains. Strongest PARIS labeling occurs in cerebellar cortex (more so in rat than mouse), dentate gyrus of the hippocampus, olfactory bulb, ventricular ependymal cells, cerebral cortex, and striatum, and is sensitive to 10μ*M* DPQ. PARP-$1^{-/-}$ mouse brain shows significantly decreased PARIS signal but retains identical ISEL label. (From Pieper, A. A. et al, *Proc. Natl. Acad. Sci. U.S.A.*, 97(4), 1845-1850, 2000. With permission.)

Strikingly, ISEL localization was identical to that of PARIS, indicating that basal PARP activity in the brain reflects DNA damage.[64] Localizations of PARIS differed from that of PARP-1 protein, indicating that the PARIS signal did not simply reflect the distribution of the enzyme, but instead PARP-1 activation due to DNA damage. Besides the basal activity of PARP and DNA damage, under conditions of cerebral ischemia, DNA damage, and PARP activity occurred in parallel with similar increases and localizations following artery ligation.

We wondered what might determine the basal activation of PARP. Glutamate transmission through NMDA receptors with nNOS activation leads to pathophysiological stimulation of PARP. Might this be the case for basal activity? Treatment with the NMDA receptor antagonist MK801 reduced PARIS and ISEL signals substantially, establishing a role for NMDA transmission. PARIS and ISEL were also diminished following treatment with 7-nitroindazole, a selective nNOS inhibitor, as well as in the brains of $nNOS^{-/-}$ mice.

Basal DNA damage and PARP activity were not restricted to the brain. ISEL assays revealed substantial basal DNA damage with levels in the bladder 50 times higher than those in the pancreas. Highest ISEL signals were evident in tissues with a substantial cellular turnover such as the thymus, bladder, small intestine, colon, and kidney with no difference between wild-type and PARP-$1^{-/-}$ animals. PARIS levels were substantial in a number of peripheral tissues, with highest values in the testes, about 30 times higher than the lowest levels found in the liver. We observed co-localization of basal PARIS and ISEL in a variety of tissues including the retina, thymus, skeletal muscle, testes, liver, and lung. In some tissues we did not observe a correlation between PARIS and ISEL levels with substantial discrepancies evident in the kidney, stomach, and small intestine.

Confirmation that the PARIS signal involves PARP comes with the almost complete depletion of PARIS from several tissues, such as the brain, pancreas, and testes,

of PARP-$1^{-/-}$ animals (Table 1.2). However, in other organs gene deletion leads to lesser decreases and in some cases no decrease at all. Skeletal muscle, whose PARIS activity is the second highest detected in the body, shows no loss of PARIS in the mutant mice with negligible decreases also evident in the eye and spleen. PARIS signal declines only 35 to 70% in kidney, thymus, heart, and lung of PARP-$1^{-/-}$ animals. On the other hand, PARIS activity is largely depleted in the liver, colon, small intestine, brain, testes, pancreas, bladder, and stomach of PARP-$1^{-/-}$ animals. Several forms of PARP other than PARP-1, derived from distinct genes, have been recently described.[65-68] Presumably, these other forms of PARP account for residual enzyme activity in the mutant mice.

There are a number of implications of the basal regulation of DNA damage and PARP activity by glutamate-NMDA-NO neurotransmission. Abundant research indicates that NMDA transmission influences nuclear events. PARP activation may provide one route for such effects. Long-term nuclear alterations are thought to be particularly important in the NMDA transmission that involves plastic changes in learning and memory, mediated particularly by the hippocampus. The fact that the hippocampus is a notable "hot spot" for basal PARP activity fits with a role for PARP in mediating these plastic changes.

Overactivation of PARP-1 mediates cell death by depleting NAD^+, leading to ATP depletion in tissues. We wondered whether basal NMDA receptor signaling

TABLE 1.2
Residual Basal Poly(ADP-Ribosyl)ation in PARP-$1^{-/-}$ Tissues

PARP-$1^{-/-}$ Tissue	Percentage of WT Basal PARIS Signal
Brain	6.9 ± 2.1
Pancreas	2.9 ± 3.0
Testis	4.8 ± 0.9
Liver	9.9 ± 3.3
Colon	14.0 ± 10.5
Small intestine	12.5 ± 6.1
Stomach	19.8 ± 0.4
Bladder	19.2 ± 9.3
Thymus	31.5 ± 11.7
Lung	40.9 ± 15.0
Heart	64.0 ± 2.6
Kidney	63.3 ± 11.0
Spleen	82.1 ± 20.7
Skeletal muscle	94.2 ± 7.3
Eye	105.8 ± 12.9

Percentages of WT PARIS signal are means ± SEM for the average of five assays from five different animals. WT values varied from 4% to 7%. *Source*: Pieper, A. A. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 97 (4), 1845, 2000. With permission.

might alter physiological energy dynamics. In cerebral cortical neuronal culture we found that inhibitors of NMDA transmission, such as MK801 and aminophosphonovalerate, elicit 40 to 80% increases in NAD^+ levels.[64] Similarly, NOS inhibitors such as 7-nitroindozole and L-nitroarginine cause 30 to 80% increases in NAD^+ levels.[64] The superoxide anion and peroxynitrite scavenger manganese(III) meso-tetrakis(4-benzoic acid) porphyrin (MnTBAP) also increases NAD^+ levels by 50%.[64]

How might neurotransmission regulate energy dynamics? We suggest that NMDA transmission lowers NAD^+ levels physiologically, limiting the ability of neurons to initiate other activities that would threaten energy reserves of the cell.

1.6 CONCLUSIONS

PARP-1 appears to be a major player in neuronal activity in the brain exhibiting both physiological, basal activity and overactivity during cytotoxic stresses. In both circumstances one of the major activators of PARP-1 is glutamate acting through NMDA receptors and NO. The profound protection from stroke damage in PARP-$1^{-/-}$ animals implies that multiple molecular mechanisms of cytotoxicity elicit their toxic actions primarily through DNA damage and PARP-1 activation. At a cellular level, PARP-1 primarily mediates necrosis rather than apoptosis and is crucial for HIV-1 integration into the host genome.

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2 Role of Poly(ADP-Ribose) Polymerase-1 in Neurologic Disorders

Marc F. Poitras, Valina L. Dawson, and Ted M. Dawson

CONTENTS

2.1 CELL DEATH

Cell death is a normal physiological process that plays an essential role from the beginning of embryogenesis to adulthood.[1,2] Cell death is instrumental in morphogenesis, homeostasis, and differentiation. It also plays a major role in the pathogenesis of tissue damage in diseases.[3-5] In the developing central nervous system, cell death plays an important role in growth and differentiation. Although mature neurons are among the most long-lived cell types in mammals, immature neurons die in large numbers during development, shaping and sculpting the developing brain.[6] Neuronal cell death in developing brain is thought to be responsible for matching neuronal populations to their target areas, a process believed to be controlled by a limiting supply of target-derived growth factors[7] and by afferent synaptic activity.[8,9] After maturation, neurons become postmitotic and lose their ability to divide. Neuronal cell death induced by pathological or traumatic insults has a deleterious effect on the central nervous system because of the inability of the tissue to repair or replace the damaged neurons. In human brain, neuronal degeneration can occur after acute insults, such as stroke and trauma, as well as during progressive adult-onset diseases such as Parkinson's disease and Alzheimer's disease. Excitotoxicity plays an important role in many neuropathological conditions including stroke, Parkinson's disease, and

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Alzheimer's disease.[10,11] Effective approaches to prevent or limit neuronal cell death in these diseases remain elusive due largely to an incomplete understanding of the neuronal death pathways *in vivo*. Despite the fact that some biochemical features of neuronal cell death have been elucidated, the key players still remain to be identified. Recently, poly(ADP-ribose) polymerase-1 (PARP-1) has been identified as a very important mediator of neuronal cell death.[12-14]

Cell death was originally classified as two distinct types called apoptosis and necrosis according to their respective morphological and biochemical features (Figure 2.1).[1,15-17] As more-detailed examinations of neuronal cell death are being performed, the distinctions between apoptosis and necrosis are becoming blurred. Furthermore, recent observations suggest that cell death may exist as a continuum with apoptosis and necrosis at opposite ends.[18-20]

Apoptosis could be executed in a programmed fashion as observed during development or be initiated under pathophysiological conditions. Apoptosis is an active cell death process characterized by chromatin condensation with extensive DNA fragmentation and nuclear pyknosis often accompanied by karyorrhexis.[1,16] During apoptosis, the cytoplasm condenses and the cell shrinks while the cellular organelles

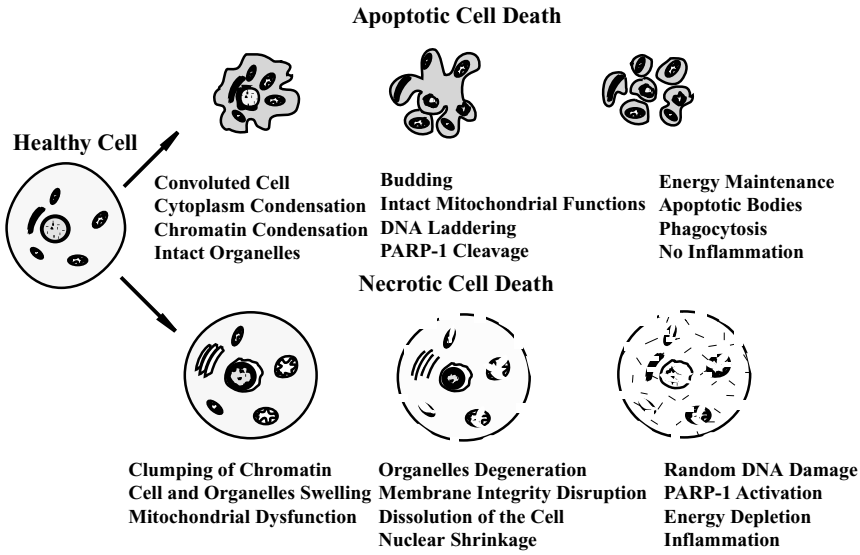

FIGURE 2.1 PARP-1 in apoptotic and necrotic cell death. Apoptotic and necrotic death are two types of cell death characterized by distinct morphological and biochemical features. Apoptotic and necrotic death are probably at opposite ends of a cell death continuum. Despite their distinct features, apoptotic and necrotic cell death may be controlled by the same subset of executor proteins. PARP-1 may be one of the cellular switches that can determine whether a cell will complete the apoptotic program or will die by necrotic cell death. In necrotic cell death, excess DNA damage leads to PARP-1 activation, massive NAD consumption, and cellular energy depletion. In apoptotic cell death, PARP-1 is cleaved by caspases and inactivated, preventing energy depletion and allowing the completion of the apoptotic program.

and the mitochondrial functions remain intact. The cell then becomes convoluted, as fluid is actively removed, and undergoes a process termed *budding*.[16] During this process, the nucleus and the organelles become fragmented and dissociate with bits of condensed cytoplasm forming cellular debris called *apoptotic bodies*. The membrane integrity of the apoptotic bodies is maintained until the cellular debris is removed by phagocytosis, preventing the release of proinflammatory factors and the initiation of an acute inflammatory response.[16,21,22] Most of the morphological changes that were initially described by Kerr et al.[1] are caused by a set of cysteine proteases, known as caspases, that are activated specifically in apoptotic cells.[23] Along with other important downstream proteins such as mitochondrial proteins and endonucleases, caspases as central executioners bring about most of the visible changes that characterize apoptotic cell death.[24] Caspases selectively cleave a restricted set of target proteins leading to the well-organized destruction of the cell. PARP-1 is one of the earliest proteins that are cleaved during the initial phase of apoptosis when the majority of the proteins are still intact.[25] PARP-1 cleavage is such a common event during apoptosis that it becomes a biochemical hallmark for the identification of a cell undergoing apoptosis. It is still unclear why PARP-1 is cleaved during apoptosis. Studies have shown that wild-type (WT) and PARP-1 knockout (KO) cells were equally sensitive to various apoptotic agents, suggesting that PARP-1 would not be required for the execution of apoptosis.[26-29] PARP KO cells exposed to alkylating agents were shown to display either an accelerated apoptotic death[29,30] or a complete resistance to necrotic cell death depending on the intensity of the treatment.[31] A caspase uncleavable mutant of PARP-1 introduced in PARP-1 KO fibroblasts delays apoptotic cell death following exposure to anti-CD95 or an alkylating agent.[29] PARP-1 KO fibroblasts stably expressing another PARP-1 caspase uncleavable mutant were shown to display an accelerated cell death following exposure to tumor necrosis factor-alpha (TNF-α)[32,33] and staurosporine.[33] This accelerated cell death was attributable to both the induction of necrosis and increased apoptosis.[32] NAD and ATP depletion was also observed following exposure of the cells to TNF-α. Under these conditions, the PARP inhibitors 3-aminobenzamide prevented NAD depletion and concomitantly inhibited necrosis but accelerated apoptosis. Furthermore, other studies have shown that inhibition of caspase-3-mediated PARP-1 cleavage by a human anti-PARP-1 autoantibody decreases actinomycin D–induced HL60 cell death.[34] Recent studies have also shown that the C-terminal caspase cleavage fragment of PARP-1 produced during apoptosis can inhibit PARP-1 activity (thus preventing energy depletion) by interfering with its homodimerization,[35] allowing the completion of the apoptotic program. All together, these results suggest that PARP-1 is a key regulator, controlling the cellular switch that determines whether a cell will die by completing the apoptotic program or by energy depletion leading to necrosis.

As mentioned before, necrotic cell death is probably at the other end of a cell death continuum as opposed to apoptosis. In contrast to the well-organized cell destruction that occurs during apoptosis, when a severe insult rapidly disrupts cellular homeostasis, cell death can occur through necrosis with morphology depicting in part the initial damage or the dysfunction.[16] During necrotic cell death, the causal events are likely to occur before the characteristic morphological changes are

observed.[36] In necrosis, both the nucleus and cytoplasm show ultrastructural changes, with the main features including clumping of chromatin, swelling and degeneration of organelles, destruction of membrane integrity, immediate inhibition of mitochondrial function, and dissolution of the cell.[20] The nuclear pyknosis with irregularly shaped condensed chromatin clumps contrasts with the formation of few uniformly and regularly shaped chromatin aggregates observed in pyknotic nuclear during apoptosis. Furthermore, during necrosis, the nuclei of dying cells do not bud to form discrete membrane-bound fragments but rather condense.[20] These distinct morphological features of the nuclear structure are now considered as hallmark features to discriminate between apoptosis and necrosis. Another distinctive characteristic of cellular necrosis is that its rate of progression depends on the severity of the initial insult. In contrast, apoptosis is an all-or-none phenomenon that does not follow a clear dose–response principle. In the nervous system, excitotoxicity and ischemic injury have most of the major hallmarks of necrosis. In these models, deletion of PARP-1 or inhibition of PARP activity results in neuronal survival.

2.2 EXCITOTOXICITY

In early 1970, Olney and colleagues[37-40] clearly demonstrated in rodents and primates the neurotoxic effects of the excitatory amino acid glutamate, establishing the bases for the emerging concept of excitotoxicity. Excitotoxicity is now associated with a variety of neurodegenerative diseases.[10,20,41-44] The concept of excitotoxicity describes the phenomenon by which the presence of pathological concentrations of glutamate overactivate the different subtypes of ionotropic and metabotropic glutamate receptors, leading to neurotoxic effects. The ionotropic glutamate receptors subtypes *N*-methyl-D-aspartate (NMDA) and non-NMDA act as ligand-gated Ca^{2+} and Na^{+} channels, respectively, whereas the metabotropic receptors are 7 transmembrane domain G protein–coupled receptors that activate the inositol phosphates cascade and inhibit the cAMP cascade.[11,45,46] Prolonged overactivation of these receptors induces a massive and sustained increase in intracellular Ca^{2+} concentrations, resulting in a downstream activation of Ca^{2+}-dependent processes including protein kinases, protein phosphatases, proteases, phospholipases, and endonucleases.[47-50] Glutamate neurotoxicity is mainly mediated through NMDA receptor activation. Mitochondria accumulate much of the Ca^{2+} entering the neurons via activated NMDA receptors. Mitochondrial Ca^{2+} overload rapidly alters mitochondrial function, shutting down energy production and increasing formation of oxygen radicals.[50,51] Ca^{2+} influx through NMDA receptors also results in subsequent activation of neuronal nitric oxide synthase (nNOS) and production of NO[52,53] (Figure 2.2). The role played by NO as a physiological messenger molecule in the central nervous system is now well established.[54,55] However, if NO is generated under conditions where superoxide anions are also present, it can form the very potent oxidant peroxynitrite.[56] NMDA receptor overactivation often stimulates superoxide anion production by damaged mitochondria, setting the stage for peroxynitrite generation.[45,57] The strong oxidant activity of peroxynitrite causes tyrosine nitrosylation of proteins, as well as lipids and DNA oxidation.[57,58]

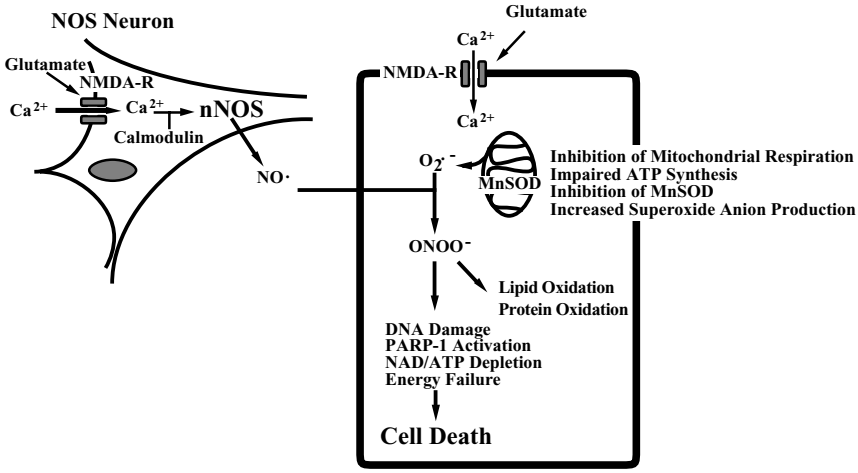

FIGURE 2.2 PARP-1-mediated excitotoxicity. Prolonged stimulation of NMDA receptors by excess glutamate induces a massive and sustained increase in intracellular Ca^{2+} concentrations resulting in downstream activation of Ca^{2+}-dependent processes including nNOS-mediated NO production. The cellular Ca^{2+} overload also induces mitochondrial dysfunction altering energy production and increasing superoxide anion $O_2^{\bullet -}$) generation. The combination of NO and superoxide produces the potent oxidant peroxynitrite ($ONOO^-$). Peroxynitrite inactivates mitochondrial enzymes, further increasing superoxide anion generation and decreasing energy production. Peroxynitrite also induces massive DNA damage, leading to PARP-1 overactivation and NAD depletion. In an effort to restore NAD levels, ATP is also depleted. Under conditions where dysfunctional mitochondria cannot maintain ATP generation, PARP-1 activation rapidly leads to cell death by NAD and ATP depletion.

Peroxynitrite causes DNA strand breaks leading to PARP-1 activation, protein ADP-ribosylation, and massive NAD consumption.[59-61] For every mole of NAD consumed by PARP-1, 4 mol of ATP are required to restore or maintain cellular NAD levels, demonstrating the high energy requirements of PARP-1 activation.[62-64] Peroxynitrite also inactivate mitochondrial proteins such as succinate dehydrogenase, NADH dehydrogenase, mitochondrial ATPase, and manganese superoxide dismutase, resulting in a further increase of superoxide production and decrease in energy generation.[65-69] As mentioned before, damaged mitochondria cannot maintain energy production during excitotoxicity. Under these conditions, PARP-1 activation would exacerbate cellular energy depletion. Transient activation of PARP-1 during excitotoxic insults was demonstrated both *in vitro*[70] and *in vivo*.[71,72] NMDA- and NO-mediated neurotoxicity in cortical cultures is blocked by PARP inhibitors in proportion to their potency in inhibiting PARP-1.[14] PARP inhibitors also reduce glutamate toxicity in cultured cerebellar granule cells[70] and attenuate NMDA-mediated toxicity in the lung.[73] One should still be aware of the possible contribution of the newly

identified members of the PARP family in studies using PARP inhibitors.[74,75] However, we have showed that NMDA-induced excitotoxicity is virtually abolished in cortical cultures obtained from PARP-1 KO mice and reduced by 72% in cultures from PARP-1 heterozygote mice, confirming the results obtained with PARP inhibitors.[12] Furthermore, the lesions produced after the direct intrastriatal injection of NMDA are almost undetectable in PARP-1 KO mice.[71] Under these conditions, the lesion volume remains ~70% smaller in the PARP-1 KO mice compared to WT animals 3 weeks after the injection, suggesting that PARP-1 inhibition could provide long-lasting neuroprotection.[71] Reintroduction of PARP-1 into PARP-1 KO mice via recombinant replication-deficient Sindbis virus restores NMDA-induced neurotoxicity, ruling out the possibility that the neuroprotection observed in PARP-1 KO mice is due to compensatory mechanisms.[71] As mentioned before, a transient increase in ADP-ribose polymer was observed after excitotoxic insults, suggesting the activation of a poly(ADP-ribose) glycohydrolase (PARG) shortly after PARP-1 activation. Since PARP-1 inhibits itself by automodification, PARG activity may be instrumental in cell death by preventing PARP-1 inactivation. Ying et al.[76,77] have recently shown that PARG inhibitors markedly decrease excitotoxic and oxidative neuronal and astrocyte death supporting a role for PARG in PARP-1 mediated neurotoxicity. These results clearly indicate that PARP-1 activation plays a critical role in excitotoxic neuronal cell death.

2.3 ISCHEMIC INJURY

With an incidence of 250 to 400 in 100,000 and a mortality rate of 30%, ischemic stroke is one of the major causes of disability and death in industrialized countries.[78] Brain damage following a stroke results from a transient or permanent interruption of the cerebral blood flow, usually caused by the occlusion of an artery by an embolus or a local thrombosis. The reduction of oxygen and nutrients (mainly glucose) supply initiates a cascade of events that lead to neuronal cell death (Figure 2.3). This cascade includes excitotoxicity, peri-infarct depolarization, inflammation, and apoptosis. Following the onset of ischemia, impairment of oxygen and glucose supply rapidly shuts down cellular energy production. With energy depletion, membrane resting potential is lost and neuronal and glial cells depolarize. Consequently, voltage-dependent Ca^{2+} channels become activated leading to a massive release of neurotransmitters including glutamate.[79,80] Impairment of energy-dependent neurotransmitter reuptake further increases extracellular glutamate accumulation. Activation of glutamate receptors is a major factor involved in neuronal damage following ischemic insults. Glutamate excitotoxic effects are largely mediated via the activation of NMDA receptors and the subsequent activation of nNOS and generation of NO.[81] The NMDA receptor antagonist MK-801 demonstrates important neuroprotection between 30 and 50% of the infarct volume in animal models of experimental stroke.[82,83] In the same experimental models, similar neuroprotection was observed in nNOS KO mice[84] and in WT animals treated with nNOS inhibitors.[85-88] Because excitotoxicity is thought to be largely mediated by NO production, peroxynitrite formation, DNA damage, and PARP-1 activation, a role for PARP-1 was investigated.

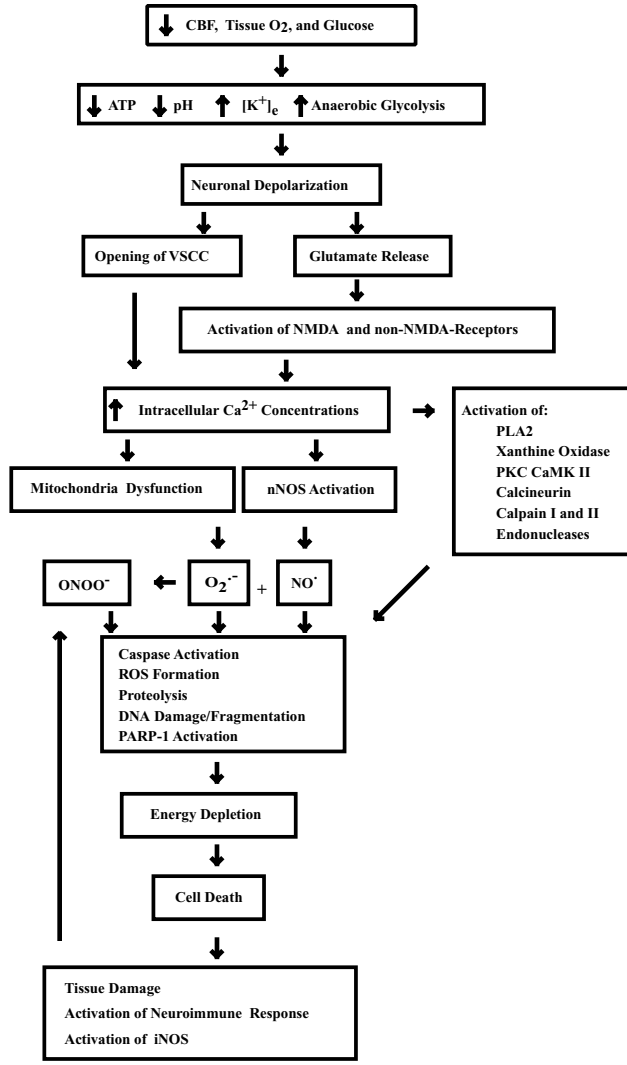

FIGURE 2.3 PARP-1 activation following ischemic insults. Transient or permanent interruption of the cerebral blood flow induces a rapid impairment of O_2 and glucose supply leading to a decrease in intracellular pH and ATP levels and a disruption of ionic gradients. Neuronal depolarization rapidly follows, inducing a massive release of glutamate and other neurotransmitters. Overactivation of NMDA and non-NMDA receptors by glutamate induces a sustained elevation in intracellular Ca^{2+} concentrations, resulting in downstream activation of a cascade of biochemical events that includes nNOS-mediated NO production and superoxide anion generation. The combination of NO and superoxide anion produces the potent oxidant peroxynitrite. Peroxynitrite induces massive DNA damage leading to PARP-1 activation, NAD and ATP depletion, and ultimately to cell death. CBF = cerebral blood flow; NMDA-R = *N*-methyl-D-aspartate receptor; $ONOO^-$ = peroxynitrite; O_2^- = superoxide anion; nNOS = neuronal nitric oxide synthase; PLA_2 = phospholipase A_2; PKC = protein kinase C; ROS = reactive oxygen species.

Ischemic insult simulated by 60 min oxygen and glucose deprivation routinely induces 60 to 80% cell death in neuronal cortical culture. Under these conditions, neuronal cell death was completely abolished in WT cultures treated with PARP inhibitors and in PARP-1 KO cultures.[12] Furthermore, in an experimental animal model of stroke, the infarct volume produced by the occlusion of the middle cerebral artery (MCAO) was reduced by up to 80% in PARP-1 KO mice as compared to WT controls.[12,13] Recent studies have demonstrated an important increase of PARP-1 mRNA[89] and protein levels[90] after ischemic insults. Increased PARP-1 activation, as evaluated by immunohistochemical staining of poly(ADP-ribose), was also detected in the ipsilateral, not contralateral hemisphere in animals and humans following ischemic insults.[12,91-95] ADP-ribose polymers were undetectable in PARP-1 KO mice confirming the genotype and phenotype of these animals.[12]

Interestingly, the poly(ADP-ribose) polymer formation observed after reperfusion was reduced in nNOS KO mice, supporting a role for NO in PARP-1 activation and subsequent neurotoxicity.[91] Similar neuroprotection, not as profound as observed in PARP-1 KO mice, was also obtained with PARP inhibitors following experimental stroke.[91,95-99] The discrepancy between the neuroprotection observed in PARP-1 KO mice vs. WT animals treated with PARP inhibitors may be attributed to the dosing paradigm, the bioavailability of the inhibitors, their selectivity, their specificity, their potency, and their mechanism of action. For example, 3-aminobenzamide, a first-generation PARP inhibitor, was shown also to inhibit protein, RNA, and DNA synthesis, DNA repair, as well as the activity of other members of the PARP family.[100-108] Despite the fact that PARP inhibitors have some PARP-1-unrelated effects, the main conclusions obtained with PARP-1 KO mice well support those obtained in studies using PARP inhibitors. PARP inhibitors have also been shown to attenuate NMDA-induced glutamate release and overall neurotransmitters dysregulation. These results suggest that PARP activation may be related in part to the amplification of excitotoxicity, possibly by cellular energy depletion and additional transmitter release or reduced reuptake.[97] PARP inhibition would then be beneficial at the earliest stage of the ischemic insult by preventing excessive glutamate release.

There is increasing evidence that inflammation contributes to ischemic brain injury.[109,110] Secondary damage to the brain tissue following ischemic insult results in part from neutrophils infiltration at the site of injury, iNOS-mediated NO production, as well as from the production of neurotoxic mediators by activated inflammatory cells.[111,112] Neutrophil infiltration is mediated by upregulation of adhesion receptors and molecules. Endothelial expression of P-lectin and intercellular adhesion molecule 1 (ICAM-1) is markedly reduced following myocardial ischemia and reperfusion in PARP-1 KO mice.[113] Neutrophil infiltration is also reduced in PARP-1 KO mice and in WT animals treated with PARP inhibitor in experimental models of inflammation[61,114] and in myocardial ischemia and reperfusion.[115] Furthermore, Jijon et al.[116] demonstrated that PARP inhibitors can reduce TNF-α and interferon-γ secretion, inducible nitric oxide synthase expression, and nitrotyrosine levels, and therefore significantly reduce inflammation. It is conceivable that the inhibition of PARP-1 or the deletion of PARP-1 would also protect the central nervous system in a similar way by reducing neutrophil infiltration and the subsequent inflammatory response. Genetic deletion of PARP-1 and

pharmacological inhibition of PARP-1 demonstrate the critical role played by PARP-1 in the pathogenesis of ischemic injury from the initiation of the excitotoxic cascade to the development of the late inflammatory response.

The most important conclusion that can be drawn from these studies is that deletion or inhibition of PARP-1 provides the best protection observed so far against ischemic insults in animal models of stroke. One would wonder why such a striking neuroprotection is only achieved by shutting down PARP-1 activity and not by interfering with other targets such as NMDA receptors and nNOS that are upstream of PARP-1 activation. NMDA receptor activation and nNOS-derived NO production are among several other pathways that can lead to DNA damage and PARP-1 activation. A possible answer would then be that DNA damage and PARP-1 overactivation constitute a final common end point of most of the several biochemical pathways involved in ischemic injury. It is clear that PARP-1 activation plays a major role in neuronal cell death following an ischemic insult. However, it is still not clear whether PARP-1 activation-mediated neuronal cell death is caused uniquely by cellular energy depletion or whether it involves downstream effectors that would be activated by ADP-ribosylation or energy depletion.

2.4 PARKINSON'S DISEASE

Parkinson's disease (PD) is a common adult-onset neurodegenerative disorder that affects 1% of the population over 65 years old. PD is clinically characterized by bradykinesia and hypokinesia, resting tremor, rigidity, gait disorder, and postural reflex impairment.[117,118] The neuropathology of PD is characterized by severe neurodegeneration of dopaminergic neurons of the substania nigra pars compacta and their projections to the striatum, and by the presence of Lewy bodies in some of the surviving cells. The loss of substantia nigral neurons resulting in striatal dopamine depletion underlies most of the clinical features of PD. It is still unclear why the dopaminergic neurons of the substantia nigra die. Although the early stage of PD can be treated by drugs that can restore dopamine levels, these treatments, which have their own debilitating side effects, do not stop the progression of the disease and eventually fail to control the symptoms.

The discovery of a by-product of heroin synthesis, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which can induce the selective loss of dopaminergic neurons, reduction of striatal dopamine content, and cause virtually all the motor features of PD in human and nonhuman primates, provided a new experimental model for parkinsonism.[119-122] In the central nervous system, MPTP is oxidized to MPP^+ by monoamine oxidase B (MAO-B).[123,124] The selective sensitivity of dopaminergic neurons to MPTP resides in the fact that these cells express a high affinity dopamine transporter that can uptake MPP^+.[125] MPP^+ is then actively transported into the mitochondria where it inhibits complex I, resulting in mitochondrial respiratory chain dysfunction, increased superoxide production, and decreased ATP synthesis (Figure 2.4).[126-130]

Studies have shown that CuZn-superoxide dismutase (SOD) KO[131] and Mn-SOD heterozygote mice[132] display enhanced vulnerability to MPTP whereas transgenic

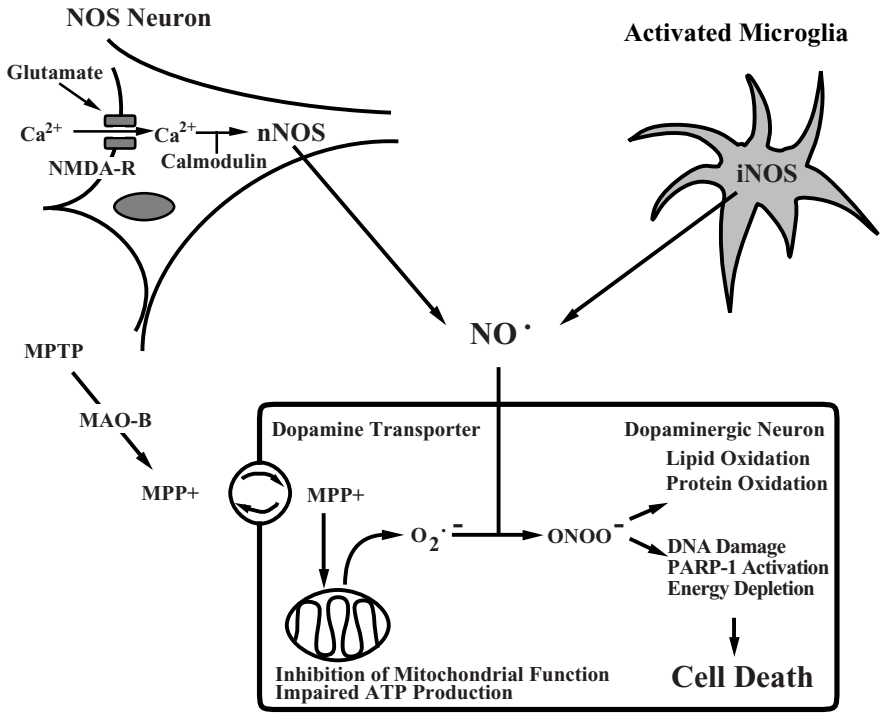

FIGURE 2.4 PARP-1 activation in the MPTP model of Parkinson's disease. In the brain, MPTP is oxidized to MPP^+ by MAO-B and selectively taken up by the high-affinity dopamine transporter expressed in dopaminergic neurons. MPP^+ then accumulates into the mitochondria where it induces mitochondrial respiratory chain dysfunction, increasing superoxide production and decreasing ATP synthesis. Superoxide anion combines with the locally produced NO and generates the strong oxidant peroxynitrite. Peroxynitrite induces DNA damage, leading to PARP-1 activation, NAD and ATP depletion, and eventually to necrotic cell death. Moribund cells and cellular debris then trigger a local inflammatory response that leads to microglial cell activation, iNOS expression, and subsequent NO production, which is induced in activated microglia, contributing to the ongoing oxidative stress, exacerbating neuronal cell injury.

mice with increased expression of either CuZn-SOD[133] or Mn-SOD[134] display lower sensitivity to MPTP, confirming the important role played by superoxide anions in MPTP-mediated neurotoxicity. Superoxide anions are rather poor reactive radicals that are not very toxic by themselves. However, when combined with NO, superoxide anions can form the very strong oxidant peroxynitrite. Many studies have now directly or indirectly demonstrated increased protein nitrosylation induced by MPTP, suggesting peroxynitrite generation following MPTP exposure.[135-138] Although the dopaminergic neurons from the substantia nigra do not express nNOS, they can still be affected

by nNOS-mediated NO production because their terminals are physically associated with nNOS-containing nerve terminals. The contribution of NO in MPTP toxicity was strongly suggested through studies showing the neuroprotection provided by relatively selective nNOS inhibitors.[139-144] Although some effects of the nNOS inhibitor 7-NI could also be attributed to MAO-B inhibition,[140] similar neuroprotection was observed in nNOS KO mice confirming the role played by nNOS in MPTP-mediated toxicity.[139,145] Microgliosis is an important neuropathological feature in PD and is also prominent in the MPTP mice.[146-148] Gliosis was also observed in humans with MPTP-induced parkinsonism.[149] During microgliosis, iNOS expression and NO production are increased in activated microglia, suggesting a possible role for iNOS-derived NO in MPTP pathogenesis. Consistent with this notion, significant neuroprotection was observed in iNOS KO mice exposed to MPTP.[147,148] As mentioned before, peroxynitrite generated from excess superoxide anion and NO production will rapidly induce DNA damage, eventually leading to PARP-1 activation.

A role for PARP-1 activation in MPTP-mediated toxicity was proposed by Cosi et al., who first demonstrated the ability of PARP inhibitors to prevent regional specific dopamine, NAD, and ATP depletion induced by MPTP.[150-152] Here, again, the nonspecific effects of PARP inhibitors preclude anyone from making definitive conclusions when PARP inhibitors are used *in vivo*. However, consistent with these previous observations, Mandir et al.[153] demonstrated a significant increase in PARP-1-mediated protein ADP-ribosylation after MPTP exposure.[153] Immunohistochemical experiments performed with a specific anti-ADP-ribose polymer antibody revealed that protein ADP-ribosylation was confined to the MPTP-sensitive TH-positive neurons of substantia nigra pars compacta of WT mice. Failure to observe any ADP-ribose polymer formation in nuclei of PARP-1 KO mice and nNOS KO mice confirms the involvement of PARP-1 activation in MPTP-mediated neurotoxicity and further support the proposed role of NO and peroxynitrite in MPTP mediated PARP-1 activation.[153] Additionally, the TH-positive and Nissl-stained neurons of substantia nigra pars compacta were completely spared in PARP-1 KO mice exposed to MPTP, demonstrating the remarkable resistance of PARP-1 KO mice to MPTP toxicity. Interestingly, MPTP-induced PARP-1 activation produces a very unusual ADP-ribosylated protein profile, suggesting a possible role for these new PARP-1 targets in MPTP pathogenesis.[153] These results strongly support a role for PARP-1 activation in MPTP-induced parkinsonism and suggest that PARP-1 inhibition could have significant protective benefit against PD, by preventing the progression of the neurodegenerative process and the development of the characteristic clinical manifestations.

2.5 CONCLUSIONS

PARP-1 activation has been shown to be instrumental in neuronal cell death induced in several models of neuronal injury. One common feature of these models is the generation of reactive oxygen species (ROS) and free radicals, such as superoxide anions and NO, and the production of very potent oxidants such as peroxynitrite that can induce DNA damage. Another common feature of these experimental models is

the alteration of mitochondrial function, and the subsequent inhibition of the mitochondria to maintain ATP production under conditions where large amounts of NAD and ATP are required by PARP-1 activation. PARP-1 deletion or PARP-1 inhibition demonstrates significant neuroprotection in all these experimental models by preventing the feed-forward action of NAD consumption on mitochondrial function and ROS generation, and by limiting the secondary neuronal injury induced by the inflammatory response that follows the initial necrotic neuronal death. Inhibition of PARP-1 with more specific and more selective inhibitors could provide considerable benefit for the prevention of the deleterious effects of neuronal injuries after ischemic stroke or during the progression of PD, as well as other neurologic disorders.

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3 Involvement of PARP-1 and Poly(ADP-Ribosyl)ation in the Maintenance of Genomic Stability

Cynthia M. Simbulan-Rosenthal, Dean S. Rosenthal, Bassem H. Haddad, Danith Ly, Jie Zhang, and Mark E. Smulson

CONTENTS

3.1 INTRODUCTION

Poly(ADP-ribosyl)ation of various DNA-binding proteins constitutes one of the earliest nuclear events triggered by DNA strand breakage associated with DNA repair, DNA replication, and cell death.[1-5] This post-translational modification utilizes nuclear nicotinamide adenine dinucleotide (NAD) as a substrate and is catalyzed by an expanding poly(ADP-ribose) polymerase (PARP) gene family, which now includes PARP-1, PARP-2, and PARP-3.[6,7] PARP-1 (E. C. 2.4.2.30) is activated by binding to double- or single-strand DNA breaks via its two zinc fingers[8,9] and undergoes extensive autopoly(ADP-ribosyl)ation in a central automodification domain through an ester linkage between poly(ADP-ribose) (PAR) homopolymers and glutamic acid

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residues in this domain.[8,10] PARP-2, PARP-3, and other PARP homologues account for the residual PARP activity in PARP-1-deficient cells.[6,7,11] Each PARP-1 molecule has 18 to 28 automodification sites;[12] and PAR chains of up to 200 residues are covalently bound mainly to PARP-1 and to other nuclear acceptor proteins. Poly(ADP-ribosyl)ation of nuclear proteins in response to DNA strand breakage is transient in intact cells and is restricted mostly to the potential targets located adjacent to DNA breaks.[13] The half-life of PAR is only 1 to 2 min as a result of its rapid degradation by PAR glycohydrolase.[14] The nuclear protein substrates of PARP-1 include histones, DNA topo I and II,[15,16] DNA pol α and δ, proliferating cell nuclear antigen (PCNA), and approximately 15 components of the DNA synthesome,[16] Ca^{2+}-Mg^{2+}-dependent endonucleases,[17] as well as an increasing number of transcription factors such as p53,[18,19] TFIIF, TEF-1, TBP, YY1, SP-1, and CREB.[20,21] Poly(ADP-ribosyl)ation of DNA pol α and δ [22,23] and topo I and II[15,24,25] modulates their activities; in most instances, such modification inhibits enzyme activity, presumably as a result of a marked decrease in affinity of the proteins for DNA caused by electrostatic repulsion between the negatively charged DNA and PAR.

Inhibition of PARP-1 activity with chemical inhibitors (reviewed in Althaus) or by dominant-negative mutants,[26-30] as well as PARP depletion by antisense RNA expression[4,5,16,23,31-33] has shown that the enzyme plays pleiotropic roles in various nuclear processes including DNA repair, recombination, differentiation, DNA replication, stress response, and cell death, as well as maintenance of genomic stability. Extensive automodification of PARP-1 allows it to cycle on and off DNA ends during DNA repair *in vitro*.[34-37] PARP-1 and poly(ADP-ribosyl)ation are implicated in base excision repair (BER).[29,30,32,38,39] In addition, other functions have been ascribed for PARP independent of its catalytic activity. The enzyme interacts with components of the multiprotein DNA replication complex[16] as well as the BER complex (i.e., XRCC1, DNA pol β, and DNA ligase III).[38,40] The binding of PARP-1 to DNA pol α occurs *in vitro*[41] and *in vivo* during the S and G_2 phases of the cell cycle,[4,42] and alters DNA pol α activity in the absence of DNA breaks or NAD.[41] PARP-1 has also been shown to play a role as a coactivator of gene transcription by interacting with specific transcription factors.[43-46] Further, poly(ADP-ribosyl)ation or functional interaction of PARP-1 with other DNA damage sensors such as DNA-PK[47,48] and p53[18,49] is implicated in DNA damage signaling cascades.

The pleiotropic roles of PARP-1 have also been increasingly examined by gene disruption in PARP-1 knockout mice. Several PARP-1 knockout mice, established by disrupting the PARP-1 gene at exon 2,[50] exon 4,[51] and exon 1[52] by homologous recombination, express no immunodetectable PARP-1 protein nor exhibit any significant poly(ADP-ribosyl)ation. The observations that PARP-1-deficient mice are viable, fertile, and appear normal implies functional redundancy of this protein, as supported by the discovery of novel PARP homologues.[6,7] Thus, although PAR-synthesizing activity has been detected in PARP-$1^{-/-}$ cells, attributed to PARP homologues, this activity is only 5 to 10% of the poly(ADP-ribosyl)ation activity of wild-type cells, and it may not fully compensate for PARP-1 depletion.[7,11] Despite variations in the physiological phenotypes of PARP-1 knockout animals, these mice exhibit increased genomic instability as shown by elevated levels of sister chromatid exchange (SCE)

in response to genotoxic agents.[50,51] Thymocytes from these animals show a delayed recovery after exposure to γ-radiation,[50] splenocytes undergo abnormal apoptosis, and primary fibroblasts exhibit proliferation deficiencies and defects in DNA repair upon exposure to DNA-damaging agents.[51,53] This chapter focuses on the putative roles of PARP-1 and poly(ADP-ribosyl)ation in the maintenance of genomic integrity.

3.2 PARP-1 AND GENOMIC STABILITY

Inhibition or depletion of PARP-1 by chemical inhibitors[54-56] or by expression of dominant-negative mutants[28,30] or antisense RNA[31,32] promotes genomic instability, as revealed by increased DNA strand breakage, DNA recombination, gene amplification, micronuclei formation, and SCE in cells exposed to genotoxic agents. PARP-deficient cell lines are hypersensitive to such agents, also exhibiting increased basal and DNA damaged-induced SCE,[57] further implicating PARP-1 as a guardian of the genome that facilitates DNA repair and suppresses DNA recombination. SCE, a widely used sensitive indicator of genetic damage, has been implicated as a key mechanism for the induction of genomic instability, given that misalignment of recombining sister chromatids leads to unequal SCEs that can then mediate various genetic alterations, such as gene deletions, chromosomal translocations, or gene amplification.[58] Consistent with extensive evidence showing that abrogation of PARP-1 activity potentiates DNA alkylation-induced SCE, conditional overexpression of PARP-1 in stably transfected hamster cells causes cellular accumulation of PAR and strongly suppresses MNNG-induced SCE, suggesting that PARP-1 activity plays an important role in the regulation of SCE formation.[59] PARP overexpression and amplification of human chromosome 1q41-q44, which contains the PARP gene, also correlates with low genetic instability in human breast carcinomas.[60]

PARP depletion by gene disruption in exon 2 PARP-$1^{-/-}$ cells results in an elevated frequency of spontaneous SCE and micronuclei formation in response to treatment with genotoxic agents,[50,61] providing further support for a role of PARP-1 in the maintenance of genomic integrity. Exon 4 PARP-1 knockout mice exhibit extreme sensitivity to γ-irradiation and methylnitrosourea and also show increased genomic instability as revealed by a high level of SCE.[51] Immortalized cells derived from these mice exhibit a reduced growth rate, G_2–M accumulation, and chromosomal instability on exposure to DNA-alkylating agents, presumably as a result of a defect in BER[53] or, alternatively, due to greatly reduced poly(ADP-ribose) formation during base excision repair in these cells.[39]

3.3 PARP-1 AND CHROMOSOMAL STABILITY

The development of tetraploidy or aneuploidy, another marker of genomic instability, is typical of many tumors and is associated with progression to malignancy or metastasis.[62] Tetraploidy results when cells exit from mitosis without either chromosome segregation or cytokinesis; tetraploid cells are genetically unstable and become aneuploid at subsequent mitoses.[63] By flow cytometric analysis, immortalized[64] as

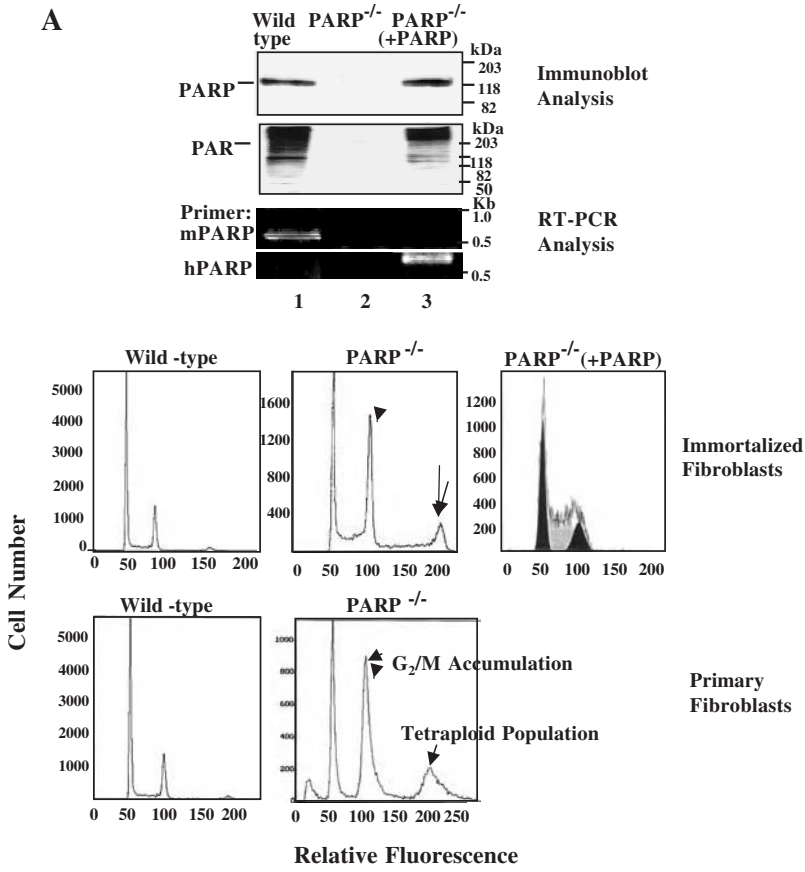
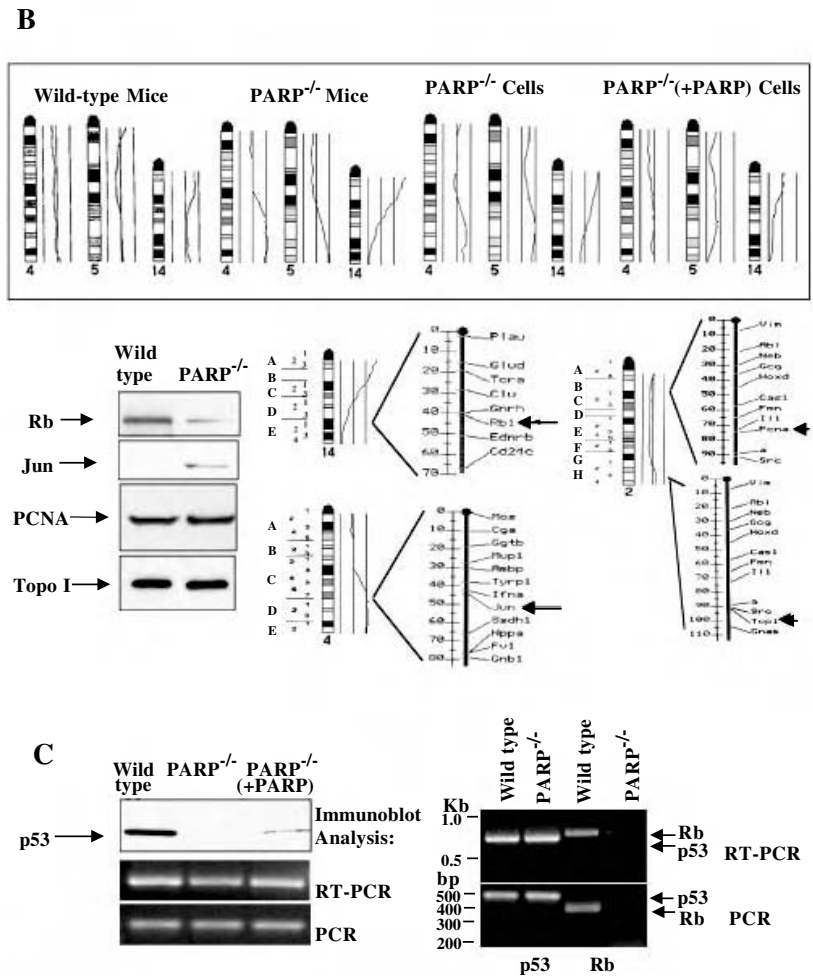


FIGURE 3.1 PARP expression, flow cytometric analysis, and CGH profiles of chromosomes 4, 5, and 14 among wild-type mice, PARP$^{-/-}$ mice, immortalized PARP$^{-/-}$ fibroblasts, and PARP$^{-/-}$(+PARP) fibroblasts. (A) Cell extracts of wild-type, PARP$^{-/-}$, and PARP$^{-/-}$(+PARP) fibroblasts were subjected to immunoblot analysis with anti-PARP and anti-PAR. RT-PCR was performed with specific human (hPARP) and mouse (mPARP) PARP primers. Nuclei from immortalized fibroblasts, harvested 18 h after release from serum deprivation, were prepared and stained with propidium iodide for flow cytometric analysis. In the case of the primary fibroblasts, asynchronously dividing cells were grown to ~60% confluency for 3 days, after which nuclei were prepared and stained as above. In addition to the two major peaks of nuclei at G_0-G_1 and G_2–M apparent in the DNA histograms of wild-type and PARP$^{-/-}$(+PARP) cells, DNA histograms of PARP$^{-/-}$ cells exhibit G_2–M accumulation (arrowheads) and a third peak corresponding to the G_2–M peak of an unstable tetraploid cell population (arrows). (B) Average CGH ratio profiles were computed for all chromosomes and used for the mapping of changes in copy number, with only the results for chromosomes 4, 5, and 14 shown. The three vertical lines to the right of the chromosome ideograms represent values of 0.75, 1, and 1.25 (left to right, respectively) for the fluorescence ratio between the test DNA and the normal control DNA. The ratio profile (curve) was computed as a mean value of at least eight metaphase spreads, and a ratio of ≥1.25 was regarded as a gain and a ratio of ≤0.75



as a loss. PARP$^{-/-}$(+PARP) fibroblasts did not show the gains at 4C5-ter, 5F-ter, or 14A1-C2 that were apparent in both PARP$^{-/-}$mice and immortalized PARP$^{-/-}$cells, although they retained the partial loss at 14D3-ter. Immunoblot analysis was performed with antibodies to Rb, c-Jun, PCNA, and Topo I of tissue extracts from wild-type and PARP$^{-/-}$mice. CGH profiles of chromosome 14 of the PARP$^{-/-}$ mice showing the loss of 14D3-ter, and the location of *Rb-1* on 14D3; chromosome 4 of PARP$^{-/-}$mice showing the partial gain of 4C5-ter and the location of *Jun* on mouse chromosome 4C5; and a balanced CGH profile of chromosome 2 of PARP$^{-/-}$ mice and the location of *Pcna* and *TopI* genes on 2B-C and 2H are shown. The positions of Rb (110 -kDa), Jun (39 kDa), PCNA (36- kDa) and topo I (100 kDa) are indicated. (C) Cell extracts of wild-type, PARP$^{-/-}$ and PARP$^{-/-}$(+PARP) fibroblasts were subjected to immunoblot analysis with anti-p53 (PAb421). RT-PCR and PCR was performed with specific primers for p53 mRNA and gene (left panel); positions of p53 and p53 cDNA are indicated. RT-PCR and PCR analysis of wild-type and PARP$^{-/-}$ mice liver using p53 and Rb-specific primers (right panel). The positions of p53 and Rb cDNA and of DNA size standards (in kilobases and base pairs) are indicated.

well as primary fibroblasts[65] derived from exon 2 PARP-1$^{-/-}$ mice were shown to contain a genomically unstable tetraploid population (Figure 3.1A). The tetraploid population was not observed in wild type cells. Further, this tetraploid cell population was no longer apparent in PARP-1$^{-/-}$ cells stably transfected with PARP-1 cDNA [PARP$^{-/-}$(+PARP)], suggesting that the reintroduction of PARP-1 into PARP-1$^{-/-}$cells may have stabilized the genome and resulted in selection against this genomically unstable population. Similarly, exon 4 PARP-1$^{-/-}$ primary fibroblasts exhibit an increased frequency of polyploidy relative to wild-type or PARP-1$^{+/-}$ cells.[66]

Further characterization of the genetic alterations associated with PARP-1 deficiency was performed by comparative genomic hybridization (CGH) analysis, a cytogenetic technique that detects unbalanced chromosomal gains and losses in test DNA as a measure of genetic instability.[67,68] With a limit of detection of 5 to 10 Mb,[69] CGH analysis revealed partial gains in chromosomes 4, 5, and 14 and a partial loss of chromosome 14 in PARP$^{-/-}$ mice or immortalized PARP$^{-/-}$ fibroblasts[64] (Figure 3.1B). Reintroduction of PARP cDNA into PARP$^{-/-}$ cells appeared to confer stability because these chromosomal gains were no longer detected in these cells, further supporting an essential role for PARP in the maintenance of genomic stability. Analysis of some key genes that map to regions of chromosomal gain or loss in PARP$^{-/-}$ mice revealed that expression of the tumor suppressor gene Rb and the oncogene *Jun* were altered in PARP$^{-/-}$ cells[64] (Figure 3.1B). The loss of 14D3-ter that encompasses *Rb-1* and presumably numerous other genes from the genome of PARP$^{-/-}$ mice was associated with a marked reduction in Rb protein, transcript, and gene copy number in these animals. Increased expression of the oncogene *Jun* in the PARP$^{-/-}$ mice was also correlated with a gain in 4C5-ter that harbors the *Jun* oncogene, whereas there was no difference in expression of the *Pcna* and *Top1* genes, which reside within regions unaffected by chromosomal gain or loss. These results suggest that the gain or loss of large chromosomal regions, such as that encompassing *Rb-1* and numerous other genes, is due to PARP-1 deletion and concomitant genomic instability in the PARP-1$^{-/-}$ mice.

Additional severe chromosomal aberrations have been detected in PARP-1 knockout mice and primary cells, which include increased frequencies of aneuploidy,[70,71] chromosomal end-to-end fusions, and spontaneous chromosome/chromatid breaks and fragments,[66,70,71] which may be attributable to telomere dysfunction. Telomeres are essential for chromosomal stability, thus, telomerase knockout mice exhibit critical telomere shortening, correlated with increased genetic instability, shortened life span, and increased incidence of spontaneous malignancies.[72] Exon 2 PARP-1$^{-/-}$ mice display increased telomere shortening compared with wild-type littermates,[70] suggesting that PARP-1 may contribute to regulation of telomere length. However, exon 4 PARP$^{-/-}$ mice and primary cells exhibit normal telomere length and chromosomal end-capping despite increased chromosomal instability.[66] Thus, given that telomerase activity is unaltered in PARP-deficient cells,[66,70,71] the role of PARP-1 in telomere length regulation remains to be clarified.

3.4 PARP-1 AT CENTROSOMES AND ACTIVE CENTROMERES

The identification of chromosomal gains or losses in PARP-deficient mice as revealed by CGH or by the presence of polyploidy or aneuploidy[64,66,70,71] suggests that impairment of mechanisms controlling the fidelity of proper chromosome segregation during mitosis contribute to the development of genomic instability in these mice. Centrosomes are cellular organizers of the spindle apparatus involved in establishing mitotic spindle bipolarity, assembly of spindle microtubules, and are critical for accurate chromosome segregation during mitosis. To ensure fidelity of chromosome segregation during mitosis, centrosomes must be duplicated only once at the G_1/S boundary of each cell cycle.[73] Deregulation of centrosome duplication results in abnormal centrosome amplification, which has been correlated with genetic instability in mouse embryonic fibroblasts lacking wild-type p53[74] or BRCA1 tumor suppressor proteins,[75] as well as in malignant human tumors.[76,77] The presence of multiple active centrosomes in these cells, therefore, causes chromosome segregation errors that underlie the development of polyploidy/aneuploidy and, consequently, malignant transformation.[78] The observations that p53 and BRCA1 are both localized to centrosomes during mitosis,[79,80] and that normal centrosome duplication can be restored in $p53^{-/-}$ cells by reintroduction of wild-type p53, indicate that p53 plays a direct role in the regulation of centrosome duplication.[81]

Interestingly, immunocytochemical and biochemical fractionation studies recently revealed that, similar to p53 and BRCA1, PARP-1 also localizes to the centrosomes during mitosis.[82] In addition, the telomeric PARP, tankyrase, another member of the PARP family capable of poly(ADP-ribosyl)ation, is also associated with nuclear pore complexes and centrosomes in a cell cycle–dependent manner.[83] PARP-1 also localizes to active centromeres, another essential structure for proper segregation of chromosomes in mitosis and meiosis, during S/G_2 phase of the cell cycle, and it has been proposed that PARP-1 may be a negative regulator of the activity of protein acceptors at the centromere, such as toposomerase II and HMG-1.[84] These results suggest that PARP-1 or poly(ADP-ribosyl)ation associated with the centrosome or the centromere may be involved in maintenance of chromosomal stability by participating in mechanisms controlling the fidelity of proper chromosome segregation during mitosis. Whether PARP-1 plays a direct or indirect role (via its functional interaction with p53) in regulating centrosome duplication to ensure accurate chromosome segregation to daughter cells remains to be elucidated.

3.5 PARP-1 AND p53 STABILIZATION

One mechanism by which PARP may confer genetic stability is via its putative role in p53 induction, accumulation, and stabilization. p53 monitors genomic integrity and reduces the occurrence of mutations either by mediating cell cycle arrest in G_1 or at G_2–M or by inducing apoptosis in cells that have accumulated substantial DNA damage.[85,86] As discussed above, p53 is also involved in the maintenance of diploidy as a component of the spindle checkpoint[87] and by regulating centrosome duplication.[74,81] p53 was detected in lysates of wild-type cells, but not in $PARP^{-/-}$ cell extracts,

by immunoblot analysis with antibodies to p53 (PAb421) (Figure 3.1C). Stable transfection with PARP cDNA partially restores p53 expression in the PARP$^{-/-}$(+PARP) cells, suggesting that PARP may be directly involved in p53 stabilization and accumulation. Given that the loss of p53 may allow the survival of cells with severe DNA damage, thus promoting tetraploidy,[88] down-regulation of p53 expression in PARP$^{-/-}$ cells may contribute, at least in part, to the genomic instability and the development of tetraploidy in these cells. As discussed earlier, lack of p53 in PARP$^{-/-}$cells may promote further genomic alterations via different mechanisms, including abnormal centrosome amplification, which is associated with lack of wild-type p53 and also generates numerical chromosome aberrations.[89] Cells that are incapable of poly(ADP-ribosyl)ation because of unavailability of NAD[90] and primary fibroblasts from PARP$^{-/-}$mice[91] also show reduced basal levels of p53 and defective p53 induction in response to DNA damage. Interestingly, the loss of the tetraploid population in the PARP$^{-/-}$(+PARP) cells correlates with the partial restoration of p53 expression in these cells (Figure 3.1C).

p53 is transiently poly(ADP-ribosyl)ated by PARP-1 during early apoptosis, which is accompanied by a marked increase in the intracellular abundance of p53.[19] The expression of p53 is induced by a variety of proapoptotic stimuli and is required for apoptosis in many cell types;[92] transcriptional activation of target genes and interaction with other proteins are thought to contribute to p53-dependent apoptosis. It has been shown that p53 is poly(ADP-ribosyl)ated by partially purified PARP-1 *in vitro*, and that binding of p53 to a specific DNA consensus sequence prevents its covalent modification.[93] The location of a PAR attachment site adjacent to a proteolytic cleavage site in p53 also suggests that PAR may protect p53 from proteolysis,[94] because similar protection has been noted after binding of monoclonal antibodies adjacent to this region.[95] The lack of regularly spliced wild-type p53 in PARP$^{-/-}$ cells has also been attributed to decreased protein stability, not lower levels of p53 mRNA.[96] Consistently, reverse transcription polymerase chain reaction (RT-PCR) and PCR analysis of RNA and DNA from immortalized wild type and PARP$^{-/-}$ cells revealed that reduced expression of p53 in the PARP$^{-/-}$ cells was not attributable to lower levels of p53 transcripts or to a decrease in p53 gene copy number (see Figure 3.1C). Modification of p53 by PARP-1 may therefore play a role in p53 accumulation and stabilization,[19,94,97] and explain the apparent lack of p53 in PARP$^{-/-}$ cells.

Although increased expression of the p53 homologue p73 may compensate for the lack of wild-type p53 in immortalized PARP$^{-/-}$ cells,[96] (p73, when overexpressed, can activate p53-responsive genes and induce apoptosis), it is unable to detect DNA lesions and, thus, is not induced by DNA damage.[98] The significant poly(ADP-ribosyl)ation of p53 early in apoptosis[19] suggests that this post-translational modification could play a role in p53 up-regulation by protecting the protein from proteolytic degradation. Both PARP-1 activity and p53 accumulation are induced by DNA damage, and both proteins have been implicated as sensors of such damage. A functional association of PARP-1 and p53 has recently been suggested by coimmunoprecipitation of each protein *in vitro* by antibodies to the other.[18,49] Both the increased sensitivity of PARP-1$^{-/-}$ mice and cells to DNA-damaging agents and their genetic instability are therefore consistent with their deficiencies in PARP-1 and p53. Some of the

consequences of PARP deficiency in PARP$^{-/-}$ mice may, thus, be attributed, at least in part, to indirect effects resulting from changes in other DNA damage checkpoint proteins, such as p53. Recent experiments with PARP-1 and p53 double-mutant cells further revealed that PARP-1 deficiency can render p53-deficient mice susceptible to various tumor types, including high frequencies of carcinomas, in addition to lymphomas and sarcomas; this increased tumorigenesis associated with PARP-1 deficiency was further correlated with the loss of the wild type p53 allele, as revealed by a high rate of loss of heterozygosity (LOH) at the p53 locus in these tumors.[71] These results further confirm a functional interaction of PARP-1 and p53 in the maintenance of genomic integrity and in suppression of tumorigenesis.

3.6 PARP-1 AND CELL DEATH

In addition to its role in DNA repair and chromosomal stability, PARP-1 may also confer genomic stability via a potential role in the elimination of cells that have accumulated an unacceptable level of DNA damage. Because PARP-1 is activated by DNA fragmentation, the role for PARP in cell death via NAD and ATP depletion has been proposed previously.[2] This idea has been supported by recent studies showing that PARP$^{-/-}$ mice are resistant to murine models of a number of human diseases, including focal cerebral ischemia,[99,100] traumatic brain injury,[101] myocardial ischemia,[102,103] streptozotocin-induced diabetes,[52,104,105] MPTP-induced Parkinsonism,[106] endotoxic shock[107,108] and peroxynitrite- and dextran sulfate-induced inflammation,[109,110] suggesting that PARP activation and consequent NAD and ATP depletion, triggered by oxidative or nitric oxide-induced stress, play a role in the pathophysiology of these diseases.

A transient poly(ADP-ribosyl)ation of nuclear proteins also occurs early during apoptosis, prior to commitment to cell death, in various cell lines and with different inducers of apoptosis, and this event is followed by cleavage and inactivation of PARP-1 (Figure 3.2).[5,111,112] Prevention of this early PARP-1 activation by expression of PARP antisense RNA or by PARP-1 gene knockout blocks progression of apoptosis, thus correlating this early poly(ADP-ribosyl)ation with later events in the cell death cascade.[5] The reintroduction of PARP-1 in independent clones of PARP$^{-/-}$ cells reestablishes the response. PARP may play an active role early in Fas-mediated apoptosis either by partial depletion of NAD and ATP, or via the modification of nuclear proteins involved in apoptosis. These studies are consistent with earlier results using chemical inhibitors, indicating that the activation of PARP is required for apoptosis to occur in some systems.[113-116] It should be pointed out that another study indicated that primary PARP$^{-/-}$ cells underwent similar apoptosis compared to PARP$^{+/+}$ cells.[61] Whether these differing observations are due to the use of different cell types (immortalized vs. primary cells) or reagents (e.g., Fas + cycloheximide in our study vs. Fas + actinomycin D) remains to be determined. However, it has also recently been shown that expression of caspase-3-resistant PARP-1,[117,118] as well as wild-type PARP-1,[118] in exon 2 PARP-1$^{-/-}$ cells results in an earlier onset of the apoptotic response, a finding that is consistent with an active role for PARP and

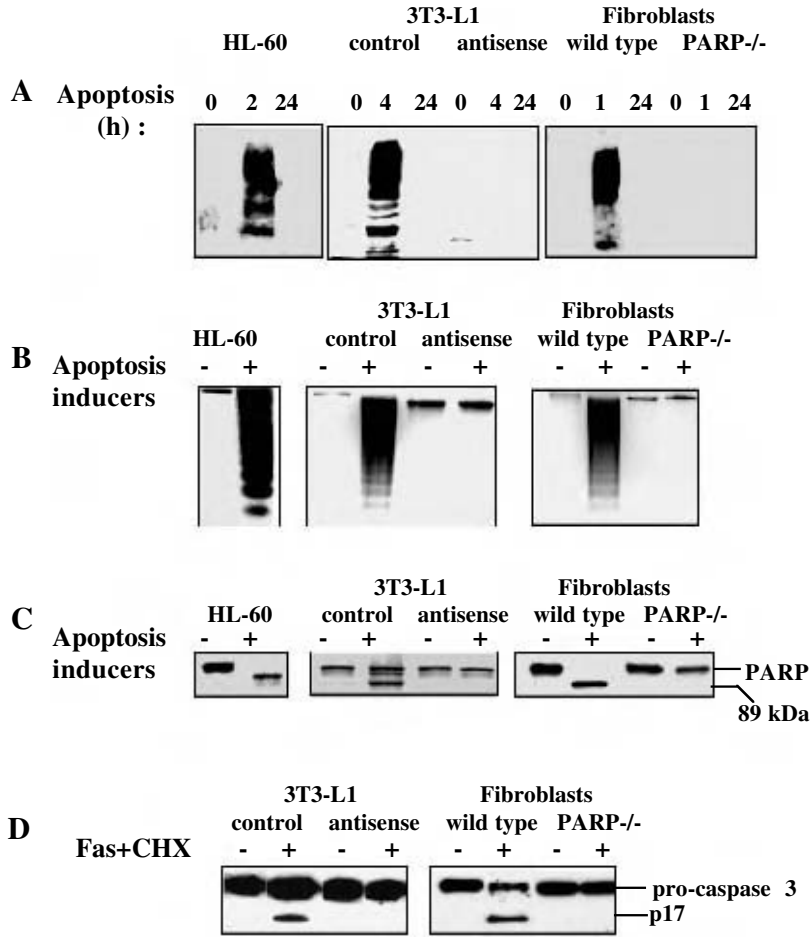


FIGURE 3.2 Time courses of poly(ADP-ribosyl)ation of nuclear proteins (A), DNA fragmentation (B), *in vitro* PARP-cleavage (C), and activation of procaspase 3 (D) during apoptosis in HL-60 cells, 3T3-L1 cells, and immortalized wild-type fibroblasts; effects of PARP depletion by antisense RNA expression and gene disruption. Apoptosis was induced in HL-60 cells by incubation with camptothecin (10 μ*M*); PARP-antisense and control cells were preincubated for 72 h in presence of 1 μ*M* Dex to induce antisense RNA expression and then, together with wild type and PARP$^{-/-}$ fibroblasts, exposed to anti-Fas (100 ng/ml) and cycloheximide (10 μg/ml) for 24 h. Cell extracts were then prepared and subjected to immunoblot analysis with anti-PAR (A) and with mAb to the p17 subunit of caspase-3 (D). Apoptosis was monitored by detection of characteristic internucleosomal DNA ladders by agarose gel electrophoresis and ethidium bromide staining (B). Caspase-3-mediated PARP-cleavage activity in cytosolic extracts was assayed with [^{35}S]PARP as substrate (C). The positions of procaspase 3 and p17, as well as PARP and the 89-kDa cleavage fragment are indicated.

poly(ADP-ribosyl)ation early in apoptosis. In contrast, a presumptive decrease in apoptotic response, as determined by morphological changes, was observed upon expression of uncleavable PARP-1 in cells derived from different PARP knockout animals.[119]

We recently proposed a model, summarized in Figure 3.3, whereby PARP-1 and poly(ADP-ribosyl)ation may play a role in an "amplification loop" toward caspase activation and downstream Fas-mediated apoptotic events.[5,111,112] Stimulation of the Fas receptor has been found to induce the gradual release of mitochondrial factors, such as the apoptosis-inducing factor (AIF), which translocates from the mitochondrial intermembrane space to the nucleus and induces low levels of caspase-independent cleavage of chromatin into large 50-Kb fragments.[120] These large DNA breaks can stimulate PARP activity, rapidly decreasing NAD and ATP levels, which

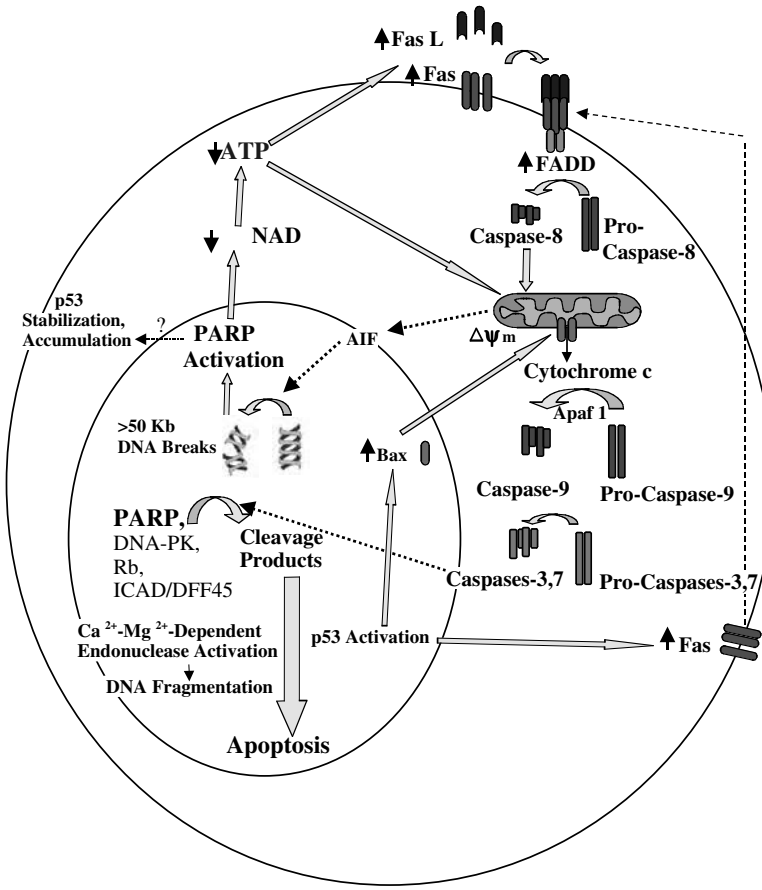


FIGURE 3.3 Model for the involvement of PARP-1 activation and cleavage in an "amplification loop" toward caspase activation and downstream apoptotic events.

contributes to both receptor and mitochondrial pathways of apoptosis. Partial depletion of ATP (~10 to 65% of the control) in turn as been shown to upregulate Fas, Fas ligand, and Fas-associated protein with the death domain (FADD), resulting in induction of caspase-8 and caspase-3 activity.[121] Binding of Fas to Fas ligand recruits FADD via shared protein motifs (death domains), resulting in subsequent activation or amplification of the caspase cascade leading to apoptosis. Further depletion of ATP below a threshold level inhibits later events in apoptosis;[122] thus, subsequent degradation of PARP by caspase-3-like proteases may prevent depletion of NAD and ATP below this critical level as well as release certain nuclear proteins, such as endonucleases[17,123] or p53, from poly(ADP-ribosyl)ation-induced inhibition.[19]

Both PARP activity and p53 accumulation are induced by DNA damage, and both proteins have been implicated in the normal cellular responses to such damage. Whereas PAR synthesis increases within seconds after induction of DNA strand breaks,[124] the amount of wild-type p53, which is usually low because of the short half-life (20 min) of the protein, increases several hours after DNA damage as a result of reduced degradation.[85,125] Colocalization of PARP and p53 in the vicinity of large DNA breaks and their physical association[18,49] suggest that poly(ADP-ribosyl)ation may regulate the DNA binding ability and, consequently, the function of p53. EMSA analysis has shown that PAR noncovalently attached to p53 *in vitro* can block its sequence-specific binding to the p53 consensus sequence, suggesting that poly(ADP-ribosyl)ation of p53 may regulate p53-mediated transcriptional activation of genes important in the cell cycle and apoptosis.[94] Post-translational modification of p53 may alter DNA binding to specific DNA sequences in the promoters of target genes associated with the induction of p53-mediated apoptosis, such as those encoding Bax, IGF-BP3,[126] or Fas.[127] Induction of Bax expression may influence the decision to commit to apoptosis because homodimerization of Bax promotes cell death and heterodimerization of Bax with Bcl-2 has been shown to inhibit the antiapoptotic function of Bcl-2.[126] Wild-type p53 also upregulates Fas expression during chemotherapy-induced apoptosis, and p53-responsive elements were recently identified within the first intron and the promoter of the *Fas* gene.[127] Binding of Fas to Fas ligand recruits the adapter molecule FADD, resulting in subsequent activation or amplification of the caspase cascade leading to apoptosis.

The removal of PAR from p53 during spontaneous apoptosis in human osteosarcoma cells coincides with a marked induction of expression of the p53-responsive genes encoding the pro-apoptotic proteins Bax and Fas at a stage when cells are irreversibly committed to death (Figure 3.4A), suggesting that poly(ADP-ribosyl)ation may play a role in regulating p53 function during the early phases of the cell death cascade.[19] As expected, poly(ADP-ribosyl)ation of p53 with PARP-1 *in vitro* and *in vivo* also inhibits the binding of p53 to its DNA consensus sequence (Figure 3.4B), providing mechanistic insights on how poly(ADP-ribosyl)ation affects p53 functions *in vivo*.[128] Our results with *in vivo* poly(ADP-ribosyl)ation of p53 suggest that p53 may cycle on and off its DNA consensus sequence depending on its level of negative charge based on its poly(ADP-ribosyl)ation state, which may represent a novel mechanism for regulating transcriptional activation of *Bax* and *Fas* by p53 during apoptosis.

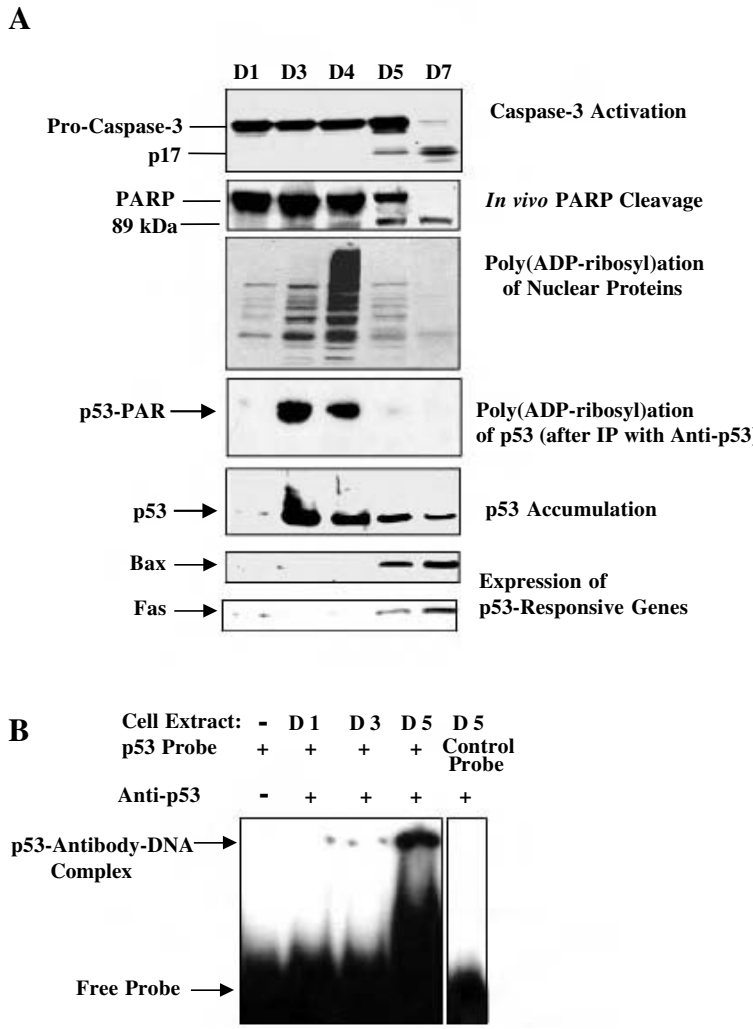


FIGURE 3.4 Time courses of activation of procaspase 3, *in vivo* PARP cleavage, poly(ADP-ribosyl)ation of nuclear proteins, *in vivo* polyADP-ribosyl)ation and accumulation of p53, and expression of p53-responsive genes *Bax* and *Fas* during spontaneous apoptosis in human osteosarcoma cells; effects of poly(ADP-ribosyl)ation of p53 *in vivo* on p53 binding to its DNA consensus sequence *in vitro*. Human osteosarcoma cells were induced to undergo spontaneous apoptosis for 9 days, and, at the indicated times, cytosolic extracts were prepared and subjected to immunoblot analyses with antibodies to caspase-3, PARP, PAR, Bax, and Fas. Equal amounts of protein (100 μg) were also subjected to immunoprecipitation with mAb to p53 and subjected to immunoblot analysis with anti-PAR. The immunoblot was then stripped of antibodies, and reprobed with polyclonal antibodies to p53. The positions of PARP and of its 89-cleavage product, procaspase 3 and its active form p17, p53, Bax, and Fas are indicated. (B) Osteosarcoma cell extracts prepared before (D1), after (D5), and at the peak of poly(ADP-ribosyl)ation (D3) were subjected to gel supershift assays with anti-p53 and the ^{32}P-labeled 30-bp oligonucleotide containing the consensus p53-binding sequence. Control assays were also performed in the absence of cell extract, or with an unrelated control oligonucleotide (NF-κB consensus sequence) to confirm specificity of binding.

3.7 PARP-1 AND REGULATION OF GENE EXPRESSION

To provide further insight into the potential mechanisms by which PARP-1 deficiency affects genomic stability, oligonucleotide microarray analysis was applied to characterize more comprehensively the differences in gene expression between asynchronously dividing primary fibroblasts derived from PARP-$1^{-/-}$ and wild-type mice.[65] Of the 11,000 genes monitored, 91 differentially expressed genes were identified to have altered expression as a result of PARP-1 deficiency. Approximately 40% of these altered genes can be grouped into genes whose products are either involved in the critical regulation of cell cycle progression (12%) or genes involved in maintenance and remodeling of cytoskeletal and extracellular matrix (ECM) (26%). The results of this approach were verified for a subset of genes whose expression appeared to be altered by PARP-1 deficiency with the use of RT-PCR and immunoblot analysis. PARP-1 deficiency results in down-regulation of the expression of genes that contribute to regulation of cell cycle progression or mitosis, DNA replication, or chromosome processing or assembly (Table 3.1). Genes in this category whose expression was decreased by two- to three-fold in PARP-$1^{-/-}$ cells include those for cyclins A, B1, and B2. Expression of these genes is normally upregulated at G_2-M and regulates cell cycle progression by associating with and regulating the activities of cyclin-dependent kinases, which, in turn, phosphorylate and regulate specific target proteins involved in cell cycle progression.[129-131]

Whereas cyclin A is required for DNA synthesis during S through G_2 phases and for mitosis,[129,130] cyclin B1 is a key regulator of mitosis and functions as the regulatory subunit of M phase–promoting factor (MPF).[131] Disruption of cyclin A expression results in cell cycle arrest at G_2,[132] which might explain, at least in part, the accumulation of PARP-$1^{-/-}$ fibroblasts at G_2-M. PARP-1 or poly(ADP-ribosyl)ation is also thought to play a role at or near the S-G_2 transition. Thus, the levels of PARP-1 mRNA,[133] PAR,[134] and PAR-linked acceptor proteins such as histone H1 dimers,[135] increases markedly at the S-G_2 transition. Furthermore, chemical inhibitors of PARP-1 also arrest cells at G_2.[136] G_2–M transition is also prevented by inhibition of cyclin B1 transcription,[137] suggesting that the down-regulation of cyclin B expression apparent in PARP-$1^{-/-}$ cells also contributes to their accumulation at G_2-M. Furthermore, MPF, which comprises cyclin B and the cyclin-dependent kinase CDC2, regulates mitotic initiation by phosphorylating and activating enzymes implicated in chromatin condensation, nuclear membrane breakdown, and mitosis-specific reorganization of microtubules.[131,138,139] Regulation of the intracellular abundance of cyclin B1, the primary regulator of the B-type cyclins,[140] controls mitotic initiation, with a threshold level of cyclin B necessary for mitosis to proceed.[141] Mitotic misregulation as a result of down-regulation of cyclin B1 expression may thus impair chromosome segregation or cytokinesis and contribute to the development of tetraploidy and aneuploidy in PARP-deficient cells.[64-66,70,71]

Expression of $p55^{CDC}$ (CDC20), another vital cell cycle regulator, was also down-regulated in PARP-$1^{-/-}$ cells. $p55^{CDC}$ binds to and activates the anaphase-promoting complex (APC), a multicomponent ubiquitin ligase that mediates the degradation of cyclins, including cyclins A and B, and is essential for chromosome segregation,

TABLE 3.1
Differential Expression Profiles of Cell Cycle, Chromosome Segregation/Assembly, and DNA Synthesis/Repair Genes between Wild-Type and PARP$^{-/-}$ Primary Fibroblasts

Fold △	Gene Name	Function	Effects of Down regulated Expression
Cell Cycle Regulation			
2.1	Cyclin A	Required for DNA synthesis during S through G_2 and for mitosis	G_2–M accumulation
2.7	Cyclin B1	Key regulator of mitosis; regulates mitotic initiation, chromatin condensation, and microtubule reorganization	G_2–M accumulation Chromosome missegregation impaired cytokinesis Tetraploidy and aneuploidy
2	p55^{CDC}	Essential for chromosome segregation, anaphase intitiation, exit from mitosis by mediating Ub-dependent cyclin degradation DNA damage and spindle assembly checkpoints Precise duplication of centrosomes	Dysfunctional aberrant mitotic spindles Chromosome missegregation Tetraploidy and aneuploidy
Chromosome Segregation/Processing/Assembly			
6.8	HMG-2	Promotes assembly of nucleoprotein complexes that facilitate chromatin function	
3.2	pr22 microtubule protein	Regulates microtubule dynamics important for mitotic spindle formation	Dysfunctional aberrant mitotic spindles
2.5	Ubiquitin-conjugating enzyme E2	Mediates selective degradation of key regulatory proteins including cyclins	Impaired timing of late mitotic events
DNA Replication/Repair			
3.1	DNA primase small subunit	Initiates lagging strand DNA synthesis together with DNA polymerase α	Proliferation deficiencies

anaphase initiation, and exit from mitosis.[142,143] The ubiquitin-dependent proteolysis of cyclins is critical for cell cycle progression and promotes its unidirectionality.[144] p55^{CDC} is further implicated in the DNA damage and spindle assembly checkpoints that delay exit from mitosis to prevent chromosome missegregation.[145-147] Furthermore, this protein also ensures the precise duplication of centrosomes, which are important in spindle assembly, by coordinating the timely disengagement of mother and daughter centrioles.[148] Centrosome amplification increases genetic instability in malignant tumors as a result of dysfunctional aberrant mitotic spindles and consequent chromosome missegregation during mitosis.[75,77] Finally, modulation of

the activity of the APC–$p55^{CDC}$ complex contributes to another checkpoint mechanism that blocks sister chromatid separation when chromosomes are misaligned; defects in this mechanism result in aneuploidy in human cells.[149] These various functions of $p55^{CDC}$ suggest that the down-regulation of $p55^{CDC}$ apparent in PARP-$1^{-/-}$ cells may also contribute to the chromosome missegregation that gives rise to tetraploidy and aneuploidy in these cells.

In addition to cell cycle–regulatory genes, the expression of several genes whose products participate in spindle assembly or chromosome segregation or processing was also downregulated two- to seven-fold in PARP-$1^{-/-}$ cells (Table 3.1); these genes include those for histone H2A.1, HMG-2, and pr22. The chromatin-associated nuclear protein HMG-2 induces changes in DNA structure that enhance binding of transcription factors, and promotes the assembly of nucleoprotein complexes that facilitate chromatin function.[150] The pr22 protein regulates microtubule dynamics that are important for formation of the mitotic spindle during mitosis, for cellular motility, and for intracellular transport processes.[151,152] $PARP^{-/-}$ cells also exhibit reduced expression of the ubiquitin–conjugating enzyme E2, a component of the ubiquitin-proteasome pathway that mediates selective degradation of key regulatory proteins including cyclins.[153] Misregulation of this pathway induced by down-regulation of E2 expression may thus also contribute to impaired timing and coordination of late mitotic events in PARP-1-deficient cells.

In summary, PARP-1 deficiency can cause misregulation of the mitotic machinery of dividing cells, potentially leading to genomic alterations, such as formation of unstable tetraploid and aneuploid cells predisposed to chromosome segregation abnormalities, as well as partial chromosomal gains and losses. These mitotic errors may further lead to altered expression of genes that contribute to cancer and aging. Thus, the expression of genes that encode extracellular matrix (ECM) or cytoskeletal proteins implicated in cancer initiation or progression or in normal or premature aging was upregulated in PARP-$1^{-/-}$ cells, including genes that encode for annexin III, caveolin, cortactin, cyclins D1 and D2, tissue inhibitor of metalloproteinase-2 (TIMP-2), and β-amyloid precursor protein (APP) (Table 3.2). Expression of PARP-1 is decreased in association with aging and progeria.[154] Our results suggest that reduced PARP expression may be an early factor that contributes to the pathogenesis of cancer and age-related diseases.

Results of DNA microarray analysis suggest that PARP-1 is involved in the upregulation of the expression of critical cell-cycle regulatory genes, such as those encoding cyclins A and B, and $p55^{CDC}$. Increasing evidence has shown that PARP-1 plays dual roles in transcription, depending on the concentration of its substrate NAD and the presence of DNA strand breaks. In the absence of NAD, PARP promotes activator-dependent transcription by interacting with RNA polymerase II–associated factors;[43] it also binds transcription enhancer factor 1 (TEF1) and the transcription factor AP-2 to increase the transcription of muscle-specific genes and AP-2–mediated transcription, respectively.[44,45] Transient transfection of an E2F-1 gene promoter-luciferase reporter construct into wild-type, $PARP^{-/-}$ and $PARP^{-/-}$(+PARP) cells also increases both E2F-1 promoter activity and E2F-1 expression in wild-type and $PARP^{-/-}$(+PARP) cells after re-entry into S phase, but not in $PARP^{-/-}$ cells, indicating

TABLE 3.2
Differential Expression Profiles of Extracellular Matrix/Cytoskeletal and Other Cell Cycle Genes between Wild-Type and PARP$^{-/-}$ Primary Fibroblasts

Fold △	Gene Name	Function	Effects of Overexpression
ECM/cytoskeletal Proteins			
3.3	Cortactin	Intracellular signaling that mediates reorganization of the actin cytoskeleton	Overexpressed in several human cancers; promotes tumor cell migration/invasion
2.1	Caveolin	Integral membrane scaffolding; protein	Diabetes, Alzheimer's disease Prostate and breast cancer progression
24.8	TIMP 2	Endogenous inhibitor of matrix metalloproteinases	Breast cancer progression
3.2	IGFBP-2	Binding proteins for insulin-like growth factors	Adrenocortical hyperplasia and cancer
20	IGFBP-4		
3.9	APP	β-Amyloid precursor protein	Alzheimer's disease, amyloidosis
2.2	β-2 microglobulin	Similar to APP	Amyloidosis, carpal tunnel syndrome microglobulin
5.3	Protease nexin (PN-1)	ECM protein	Systemic sclerosis, age-related connective tissue diseases due to ECM accumulation, autoimmunity
Cell Cycle Progression			
2.2	Cyclin D1	Regulates G_1 phase of the cell cycle	Overexpressed in a variety of human cancers; an early event in breast and colorectal cancers
3.5	Cyclin G	G_2–M transition	G_2–M arrest and accumulation Overexpressed in human breast and prostate cancer cells
3.3	MDM2	Inhibits p53 and Rb	Amplified in human leukemias

that PARP up-regulates the activity of the E2F-1 gene promoter during early S phase.[155] Interestingly, cyclin A represents one of the E2F-1-responsive genes during S phase re-entry,[156] suggesting that PARP-1 may up-regulate cyclin A expression via its co-activation of E2F-1-mediated transcription during early S phase. PARP-1 also functions as a co-activator of Tax-activated transcription *in vivo* given that transient co-transfection of PARP$^{-/-}$ cells with an HTLV reporter construct and expression vectors for Tax and PARP-1 markedly increases Tax-specific transcription.[46] In contrast, in the presence of NAD, PARP-1-dependent silencing of transcription involves poly(ADP-ribosyl)ation of specific transcription factors, which prevents both

their binding to the respective DNA consensus sequences and the formation of active transcription complexes.[20] Thus, although PARP-1 is not a transcription factor, some of its functions may be mediated by direct or indirect effects on gene expression.

3.8 PARP-1 INHIBITORS: EFFECTS ON GENOMIC STABILITY

PARP-$1^{-/-}$ mice suffer far less tissue injury in murine models of a number of human diseases, including focal cerebral ischemia,[99,100] traumatic brain injury,[101] myocardial ischemia,[102,103] streptozotocin-induced diabetes,[52,104,105] MPTP-induced parkinsonism,[106] endotoxic shock,[107,108] and peroxynitrite- and dextran sulfate-induced inflammation,[109,110] suggesting that PARP-1 activation, triggered by oxidative or nitric oxide–induced stress, plays a role in the pathophysiology of these diseases. Thus, potent and specific inhibitors of PARP-1 are now being developed as novel therapeutic agents in the treatment of these diseases. For acute treatment, PARP-1 inhibitors have been shown to diminish brain damage after cerebral focal ischemia,[100,157-162] protect cardiac myocytes in regional heart ischemia,[102,103,163] reduce renal ischemia-reperfusion injury[164] as well as decrease neuronal injury in a head trauma model.[165] The onset of some diseases, such as the development of hyperglycemia in streptozotocin-induced type I diabetes, is delayed in PARP-$1^{+/-}$ mice,[104] suggesting that even partial inhibition of PARP can provide therapeutic benefits, and treatment with potent PARP-1 inhibitors at the time of diagnosis may, thus, prevent or slow disease progression. Even weak PARP-1 inhibitors, such as nicotinamide, have shown some limited beneficial effects in clinical trials for the treatment of human diabetes.[166,167] Most PARP-1 inhibitors, including the benzamide and isoquinoline families, however, exhibit low potency and specificity; thus, in the search for more specific, potent, small molecule PARP-1 inhibitors, GPI 6150 (1,11b-dihydro-[2*H*]benzopyrano[4,3,2-*de*]isoquinolin-3-one) was recently identified and shown to exhibit remarkable efficacy in reducing tissue damage in animal models of human diseases,[103,161,165,168,169] such as cerebral and myocardial ischemia, traumatic brain injury, arthritis, septic shock, type I diabetes, parkinsonism, and inflammation. GPI 6150, a specific competitive inhibitor of PARP (K_i = 60 n*M*) as revealed by enzyme kinetic analysis, has no effect on other NAD-utilizing enzymes and is among the most potent PARP inhibitors (IC_{50} = 0.15 μ*M*).[170]

As discussed earlier, inhibition or depletion of PARP-1 increases genomic instability in cells exposed to DNA-damaging agents. Whether this genomic instability is attributable to PARP-1 deficiency or lack of PARP-1 activity remains unknown. Inhibition of PARP-1 by chemical inhibitors[54-56] or by expression of dominant-negative mutants[28,30] promotes genomic instability, as revealed by increased DNA strand breakage, DNA recombination, gene amplification, micronuclei formation, and SCE in cells exposed to genotoxic agents. It is of interest particularly to laboratories developing potent PARP-1 inhibitors as therapeutic agents to test whether pharmacological inhibition of PARP, at least during the duration of acute treatment intended for the initial clinical use, could increase genomic instability, as indicated,

for example, by development of tetraploidy. To investigate the effects of PARP-1 inhibition on the induction of tetraploidy, immortalized wild-type and PARP-1$^{-/-}$ fibroblasts were exposed for 3 weeks to 20 μM GPI 6150.[171] GPI 6150 initially decreased cell growth in wild-type cells, but there was no effect on cell growth or viability after 24 h, although GPI 6150 inhibited endogenous PARP activity in wild-type cells by ~91%, to about the residual levels in PARP-1$^{-/-}$ cells. FACS analysis of unsynchronized wild-type cells exposed for 3 weeks to GPI 6150 did not induce the development of tetraploidy, suggesting that, aside from its catalytic function, PARP-1 may play other essential roles in the maintenance of genomic stability.

3.9 CONCLUSIONS

PARP-1, a member of a growing gene family, has been shown to play active roles in earliest nuclear events triggered by DNA strand breakage associated with DNA repair, DNA replication, and cell death, in response to diverse forms of stimuli resulting from normal metabolic processes, as well as environmental factors. Although the mechanisms of its actions are yet to be defined, PARP-1 appears to function via its modification of or association with a number of nuclear proteins involved in repair, transcription, and genomic stability. A number of techniques have helped define the roles of PARP-1, including the use of *in vitro* replication and repair systems, chemical inhibitors, conditional overexpression, dominant-negative and uncleavable mutants, antisense RNA, and knockout animals. Although differing in experimental outcomes, the observation that PARP-1$^{-/-}$ animals are resistant to several diseases associated with oxidative damage has been a consistent finding among different investigators. Although conclusions with regard to the role of PARP-1 in cell death have varied among different groups, it appears that the response may depend upon the level and type of damage, as well as the cell type. In severely damaged cells, PARP-1 activation induces poly(ADP-ribosyl)ation of key nuclear proteins, including p53, and a concomitant lowering of NAD and ATP levels, resulting ultimately in cell death, the form of which (apoptosis vs. necrosis) may depend upon the time of onset of caspase-mediated PARP-1 cleavage. In mildly damaged cells, PARP-1 may signal a DNA repair response. Similarly, the role of PARP in chromosomal and genomic stability is a consistent theme. The putative mechanisms that underlie the increased genomic instability in PARP-deficient mice may include (1) a diminished ability of cells to undergo repair, (2) inability of cells to eliminate cells with a threshold level of DNA damage, (3) impairment of mechanisms that control the fidelity of chromosome segregation, and (4) loss of expression of PARP-regulated genes that themselves regulate genomic stability such as p53, Rb, and cyclins A and B. Ultimately, PARP-1 deficiency impairs mitotic function, thereby resulting in the genomic alterations and chromosomal abnormalities as well as in altered expression of genes that may contribute to genomic instability, cancer, and aging. Elucidation of the cellular functions of the other PARP homologues by disruption of these other genes will be necessary to reveal possible functional redundancies. Further work is clearly

necessary to elucidate other molecular mechanisms by which PARP-1 or poly(ADP-ribosyl)ation plays a role in the maintenance of genomic integrity.

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4 Functional Testing of Human PARP in Proliferation, Endotoxic Shock, and Radiosensitivity: A Genetic Rescue Study

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CONTENTS

4.1 INTRODUCTION

Poly(ADP-ribosyl)ation is an important post-translational modification of nuclear proteins induced by DNA damage. Poly(ADP-ribose) polymerase (PARP, or PARP-1, EC 2.4.2.30) is a constitutive chromatin-associated protein representing the major cellular poly(ADP-ribosyl)ating activity.[1] The enzyme is found in most eukaryotes with the exception of yeast. In response to DNA damage induced by alkylating agents, ionizing radiation, or free radicals, PARP binds rapidly to DNA strand breaks

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and undergoes automodification by forming long branched ADP-ribose polymers using NAD^+ as a substrate.[2] The polymer synthesis seems to occur quantitatively according to the number of DNA strand breaks.[1] Therefore, overactivation of PARP induced by massive DNA damage can cause the depletion of intracellular NAD^+. The negative charge of ADP-ribose polymers causes auto-modified PARP to be subsequently dissociated from DNA ends, allowing the DNA repair process to take place.[2] Poly(ADP-ribose) polymers are short-lived *in vivo* because they are rapidly degraded by poly(ADP-ribose) glycohydrolase (PARG).[3] Studies using specific inhibitors of PARP and a dominant-negative mutant of the enzyme, as well as cell lines devoid of PARP activity, have indicated that PARP and poly(ADP-ribosyl)ation play a multifunctional role in a range of cellular processes such as cell proliferation and replication, stress response, cell toxicity and apoptosis, DNA repair and recombination, as well as the maintenance of chromosomal stability [1,4,5]

Together with chemical inhibitor studies, the loss-of-function and overexpression approaches have been useful to study the biological function of PARP *in vivo*[6-13] These studies have shown that constitutive overexpression of a dominant-negative PARP mutant (DNA-binding domain) renders cells' hypersensitive to alkylating agents and chromosomal instability[7,10] and interferes with tumor formation because of increased apoptosis.[14] Perhaps the most powerful approach was the generation of PARP knockout mice by gene targeting,[15-17] which proved to be a valuable tool in determining biological functions of PARP.

Mouse embryonic fibroblasts (MEFs) isolated from PARP null mice exhibit normal DNA repair efficiency, and PARP-deficient splenocytes carry out apparently normal genetic recombination, such as immunoglobulin class switching and V(D)J recombination.[18] However, primary fibroblasts and splenocytes derived from PARP knockout mice show an elevated level of sister chromatid exchange (SCE),[16,18] suggesting an anti-recombinogenic function for PARP. This notion has been further supported by a genetic study in which the PARP deficiency partially rescues stalled V(D)J recombination in SCID mice.[19] In addition, an increased level of micronuclei formation is also found in PARP null cells following DNA damage. Finally, PARP null mice exhibit hypersensitivity to whole-body γ-irradiation and alkylating agents.[16,18,20] Together, these studies highlight the importance of PARP in recovery from DNA damage and suppression of recombination processes involving DNA ends.

PARP knockout mice are particularly useful in elucidating the role of PARP in several pathological processes involving DNA damage-induced cell death. Several studies have demonstrated that in response to acute DNA damage, PARP deficiency renders certain types of cells highly resistant to death. This is exemplified by the resistance of $PARP^{-/-}$ mice to neuronal damage,[21-23] streptozotocin-induced pancreatic islet cell destruction,[17,24-26] and myocardial ischemia/reperfusion damage.[27] Moreover, studies on PARP null mice have demonstrated a role for PARP in the general inflammatory response.[28-31] Mechanistic studies, aiming to elucidate the function of PARP in cell death, have led to the discovery of the role played by PARP in determining the mode of cell death with regard to physiological death (apoptosis) vs. pathological death (necrosis). Our laboratory has shown that introduction of

caspase-resistant PARP on PARP null background leads to necrosis induction coupled with NAD^+ and ATP depletion[12] suggesting that PARP cleavage by caspases during apoptosis prevents its overactivation and induction of necrosis. This notion is supported by other studies showing that overactivation of PARP leads to necrosis mediated by ATP depletion.[25,32] Therefore, it is concluded that the activation or cleavage of PARP plays an important role in the "decision-making" phase of cell death.[5] Taken together, studies using knockout mice demonstrate a central function of PARP in response to DNA damage and environmental insults. Moreover, these studies indicate that modulation of PARP activity, by either chemical or genetic means, could be potentially interesting for the development of new targets for the therapeutic treatment of acute tissue injury and chronic inflammatory response.

Despite high homology between mouse and human PARP, the functions of the latter during DNA damage and stress responses have not been tested in a multicellular organism. In this chapter, we demonstrate, using newly generated transgenic mice exclusively expressing exogenous PARP, the functional similarity of human PARP to its mouse counterpart in a number of biological processes, namely, cell proliferation, DNA damage response, and endotoxic stress. This genetic rescue approach provides further evidence of the important role of PARP in these processes, strengthening previous data derived from different approaches.

4.2 HUMAN PARP TRANSGENE RESCUES THE PROLIFERATION DEFECT OF PARP$^{-/-}$ FIBROBLASTS

A role for PARP in cell proliferation has been suggested by previous studies using chemical inhibitors of PARP in which PARP expression or activity was found to be associated with cell cycle progression and cell proliferation status.[33-35] Chemical inhibition of PARP by 3-aminobenzamide (3-AB) was shown to repress G_1 arrest and to augment G_2 arrest following DNA damage.[36] Furthermore, PARP inhibitors were shown to decrease the recovery of cells from oxidative stress.[37,38] However, caution has to be applied in the interpretation of studies with PARP inhibitors because of the nonspecific effects of these agents.

Our previous studies have shown that primary MEFs and thymocytes derived from PARP$^{-/-}$ mice have a compromised proliferation capacity and that this defect is enhanced when competing with wild-type cells in culture, both under normal and stress conditions.[15,18] By using an *in vivo* proliferation assay employing morulae aggregation, we have reported a growth disadvantage for PARP$^{-/-}$ cells when competing with wild-type counterparts, which very likely reflects general proliferation defects of PARP-deficient cells or a compromised response to environmental stress.[18] Moreover, we have demonstrated that primary PARP$^{-/-}$ MEFs progressively lose their replicative capacity and enter quiescence three to five passages earlier compared with wild-type or heterozygous MEFs. Interestingly, PARP-deficient cells escaped crises and resumed their proliferation earlier than did PARP proficient cells.[39] However, cell cycle analysis fails to reveal apparent differences in cell cycle progression or in the ability of quiescent PARP$^{-/-}$ fibroblasts to reenter the cell cycle when compared to

wild-type controls.[40] These data seem to be contrary to a study using different PARP knockout mice showing that PARP deficient cells exhibited a cell cycle progression defect as evidenced by accumulation at the G_2–M border following treatment by alkylating agents.[16,41]

Nevertheless, the precise mechanism underlying the proliferation defect in the absence of PARP has not been elucidated. Proliferation defects in PARP null cells may be due to the lack of poly(ADP-ribosyl)ation which may affect DNA replication or chromosome segregation, because chromatin contains a large proportion of poly(ADP-ribosyl)ated proteins and poly(ADP-ribosyl)ation facilitates chromatin relaxation.[42,43] It is also possible that DNA metabolic enzymes might require poly(ADP-ribosyl)ation to function efficiently, since several enzymes involved in DNA replication, such as topoisomerases[44] and DNA polymerases, have been shown to be acceptors of poly(ADP-ribosyl)ation.[1] Because proliferation deficiency in $PARP^{-/-}$ cells was augmented under stress conditions, this suggests that target molecules, such as stress response molecules and perhaps cell cycle regulatory molecules, e.g., p53, may need to be poly(ADP-ribosyl)ated when the cells are challenged by external insults. In this regard, it is interesting to note that PARP not only can poly(ADP-ribosyl)ate p53 protein, but also binds to specific domains of p53 protein and modifies p53 activity by poly(ADP-ribosyl)ation.[45,46] Finally, it is also possible that the impaired proliferation of mutant cells might be due to a higher frequency of misrepaired DNA following imposed stress, and could also be caused by the severe chromosomal aberrations present in $PARP^{-/-}$ cells.[47,48]

To investigate further the role of PARP in cell proliferation and other cellular processes, we have generated transgenic mouse lines expressing human PARP. We have constructed a ubiquitin promoter–driven vector for expressing full-length human PARP (Figure 4.1A). Three transgenic founders were obtained after pronuclear injection of the construct. The progeny of these founders was analyzed by Southern blotting for genotyping (Figure 4.1B). To determine accurately the level of transgene expression, crossings between these transgenic mice and PARP null mice[15] were then designed. Three independent transgenic lines (379-1, 379-4, and 378) were crossed with $PARP^{-/-}$ mice, generating mice containing human PARP transgene in a $PARP^{-/-}$ background. Analysis of transgene expression in several tissues by Western blotting revealed that transgenic line 379-1 exhibited a high level of transgene expression in all tissues examined (Figure 4.1C and Table 4.1). On the contrary, lines 379-4 and 378 showed a nondetectable expression level in most tissues examined (Figure 4.1C, Table 4.1, and data not shown). Therefore, we used the 379-1 transgenic line (henceforth named $PARP^{-/-}$Tg+) in genetic rescue experiments to study the ability of human PARP to substitute the functions of its mouse counterpart in cell proliferation and stress response.

To test growth properties of cells exclusively expressing exogenous PARP, MEFs were isolated from E13.5 sibling fetuses obtained from intercrosses of $PARP^{-/-}$Tg+ and $PARP^{+/-}$ mice. We performed a replating assay on primary $PARP^{+/-}$, $PARP^{-/-}$, and $PARP^{-/-}$Tg+ MEFs to determine growth rates through successive passages. Cell numbers were counted at each passaging to measure their capacity to sustain repeated

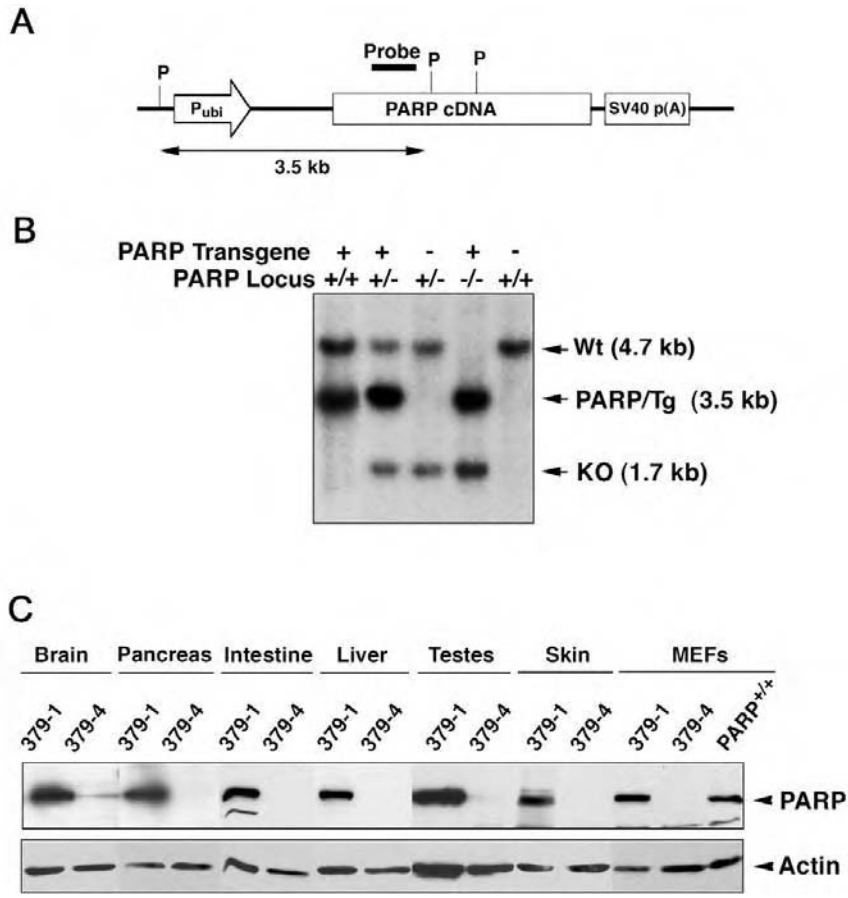

FIGURE 4.1 Generation of transgenic mice expressing human PARP. (A) Schematic view showing structure of the PARP transgene used for microinjection into mouse oocytes [F1(C57BL/6 × CBA)] Transgenic construct was generated by cloning full-length human PARP cDNA[12] and Ubiquitin promoter (P) into pSK(-)Bluescript vector. P = PvuII restriction site. (B) Southern blot analysis of PARP transgene and endogenous PARP locus. Genomic DNA isolated from mouse tails was digested by PvuII and simultaneously hybridized with two probes: transgene-specific probe as indicated in A and endogenous PARP-specific probe as described previously.[15] The 4.7-kb band and 1.7-kb band correspond to the wild-type and the targeted mutant (KO) allele of the endogenous PARP gene, respectively, and the 3.5-kb band corresponds to the transgene. (C) Western blot analysis of PARP transgene expression in transgenic lines 379-1 and 379-4. Protein was extracted from tissues or MEFs originated from mice containing the PARP transgene on a PARP null background ($PARP^{-/-}$Tg+ mice). Equal amounts of proteins (50/μg) were loaded in each lane. Monoclonal anti-PARP antibody (C-2-10; Biomol, Plymouth Meeting, PA) was used to detect PARP expression. Blots were stripped and hybridized to anti-actin antibody (ICN, Costa Mesa, CA) as a loading control.

TABLE 4.1
Expression Analysis of Transgenic Human PARP Protein in Tissues of Mice from Different Transgenic Lines

Transgenic Line	Transgenic Human PARP Expression*									
	Brain	Liver	Spleen	Thymus	Pancreas	Intestine	Kidney	Testes	Skin	Fibroblasts
379-1	++++++	++++	+	+	++++++	+++	+	++++++	++	++
379-4	+	–	–	–	–	–	–	–	–	–
378	+	–	–	–	ND	–	ND	+++++	–	ND

* Expression of transgenic human PARP was determined by Western blot analysis (see Figure 4.1). All tissues analyzed were obtained from mice carrying the transgene on PARP null background ($PARP^{-/-}$Tg+ mice). ++ correspond to expression level of endogenous PARP in MEFs. ND = not determined.

rounds of cell divisions. As shown in Figure 4.2, PARP$^{-/-}$ MEFs exhibited proliferation defects and after three passages lost their replicative ability, consistent with previous reports.[15,18,39] Interestingly, PARP$^{-/-}$Tg+ cells exhibited growth rates identical to PARP$^{+/-}$ cells (Figure 4.2) demonstrating rescue of the proliferation defect in PARP

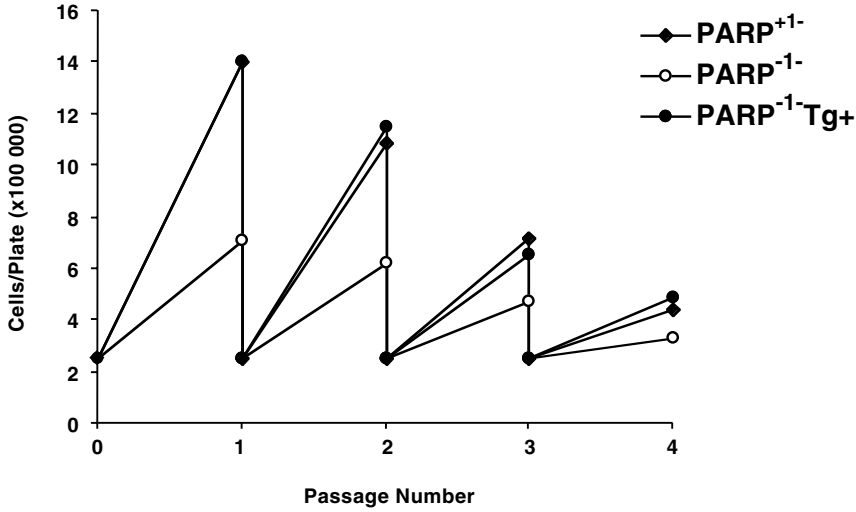

FIGURE 4.2 Proliferation defect of PARP$^{-/-}$ cells is restored by reintroduction of human PARP. Primary MEFs derived from littermates of E13.5 transgenic (PARP$^{-/-}$Tg+), nontransgenic PARP homozygous (PARP$^{-/-}$) or nontransgenic PARP heterozygous (PARP$^{+/-}$) fetuses were seeded at the density of 2.5×10^5 cells/well and passaged every 2 days at the same density. Each plot represents the mean of triplicate samples of MEFs from a single embryo, with cell numbers on the Y-axis against passage numbers in culture on the X-axis.

knockout cells by transgenic human PARP. These results further strengthen the notion of an important role for PARP in the maintenance of normal cell proliferation.

4.3 FUNCTION OF PARP IN ENDOTOXIN-INDUCED SEPTIC SHOCK

We have shown that cells lacking PARP exhibit a severe proliferation defect when experimental stress is applied (see references 15, 18, and 39 and this study), suggesting that PARP plays a role in stress response. It has been reported that PARP-deficient mice are resistant to septic shock induced by lipopolysaccharide (LPS).[30,31] Septic shock is a systemic response to infection by Gram-negative bacteria resulting in high lethality.[49] In experimental animal models, these systemic effects can be reproduced by injection of endotoxin LPS. The mechanisms by which absence of PARP protects

against inflammation are thought to be due to a reduced expression of proinflammatory cytokines.[28,30] In this regard, it was proposed that a decreased NF-κB transactivation ability in PARP$^{-/-}$mice may be responsible for this resistance.[30,50,51]

To define further that the phenotype is indeed due to PARP deficiency, we tested the effect of reintroduction of PARP to PARP null mice on endotoxic shock induced by LPS. Transgenic (PARP$^{-/-}$Tg+) and nontransgenic littermates (PARP$^{-/-}$) as well as control wild-type mice (PARP$^{+/+}$) were injected intraperitoneally with 20 mg/kg body weight of LPS and were followed for survival for a 7-day period. Although the surviving fraction of all three genotypes of mice at the end of the 7-day observation period was similar, at earlier time points nontransgenic (PARP^{-1-}) mice were protected more compared with wild-type mice (e.g., 3 days after treatment 64% of PARP$^{-/-}$ mice survived compared with 17% survival in wild-type mice) (Figure 4.3). Although survival of PARP$^{-/-}$Tg+ mice was similar to that of nontransgenic (PARP$^{-/-}$) mice for the first 2 days after treatment, PARP$^{-/-}$Tg+ showed a decline in surviving fractions compared to PARP$^{-/-}$ mice at days 3 and 4, but still exhibited a higher survival rate than wild-type controls (Figure 4.3). These results suggest that sensitivity of PARP$^{-/-}$ mice to LPS-induced endotoxic shock is

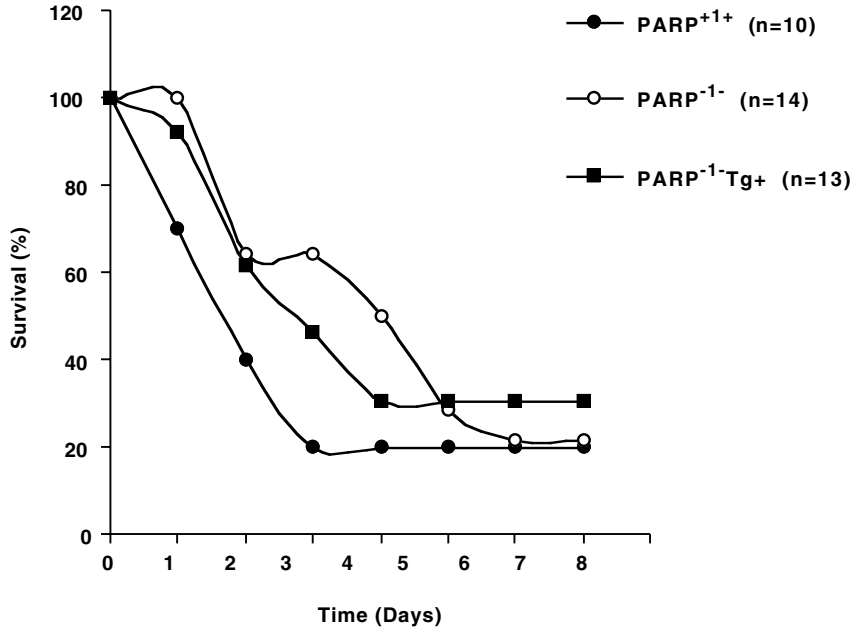

FIGURE 4.3 Introduction of exogenous human PARP partially abolishes resistance to LPS-induced endotoxic shock in mice lacking PARP (129 × C57BL/6 × CBA). Adult mice (6 to 17-weeks old) of indicated genotype were injected intraperitoneally with LPS (20 mg/kg body weight), and survival was monitored over 7 days.

at least partially restored by human PARP transgene. Although our results are in principle consistent with other studies using PARP knockout mice generated from different laboratories,[30,31] there appears to be a difference in the magnitude of resistance to endotoxic shock between those studies and the present study. The lower resistance of $PARP^{-/-}$ mice to endotoxic shock observed in our study compared with others[30,31] may be attributable to the differences in age and genetic background of animals. The fact that human PARP transgene can only partially rescue the response defects of $PARP^{-/-}$ mice to LPS might also be due to insufficient transgene expression levels in particular LPS target cell types.

It is believed that the principal mediator of LPS-induced septic shock is tumor necrosis factor-alpha (TNF-α).[52] Indeed, the major effects of microbial toxins can be directly induced by injection of TNF-α in animal models. Injection of TNF-α or its massive endogenous release leads to severe pathological reactions, such as blood vessel leakage, leukocyte infiltration, and inflammation of various organs, ultimately leading to death.[53,54] Although the full panel of NF-κB target genes that may be involved in PARP-mediated inflammatory response is unknown, reduced synthesis of TNF-α in PARP null mice, resulting from defective NF-κB activation, may explain the protection against LPS-induced septic shock in these mice.[30] However, this proposal was challenged by the findings that PARP null mice are not resistant or sensitive to TNF-α-induced lethality.[55] Therefore, the difference between PARP null mice and wild-type mice in resisting LPS-induced lethality may be due to a mechanism involving molecules other than TNF-α.

Although the underlying mechanisms still await elucidation, the involvement of PARP in the regulation of the NF-κB signaling pathway may be utilized to develop novel therapeutic strategies for septic shock, known as one of the most common causes of mortality in intensive-care units. Pharmacological strategies that enable specific inhibition of PARP-associated NF-κB activation without affecting the functions of PARP in genome integrity and surveillance would be particularly desirable.

4.4 HUMAN PARP PROTECTS PARP KNOCKOUT MICE FROM HIGH DOSES OF γ RADIATION

Following induction of DNA breaks, e.g., by γ-irradiation or alkylating agents, PARP is rapidly activated to catalyze poly(ADP-ribosyl)ation onto itself and various proteins, presumably altering their ability to interact with a variety of proteins involved in sensing and signaling DNA damage, such as DNA-PK, ATM, and p53. Indeed, biochemical and genetic studies have shown that PARP interacts with p53 [45,46,56,57] and Ku80.[58-61] Hypersensitivity to ionizing radiation is a characteristic of PARP knockout mice. After 8 Gy of whole-body γ irradiation at 6 to 8 weeks of age PARP null mice invariably die within 10 to 15 days whereas wild-type mice show 100% survival.[16,18,39] Consistent results were also obtained by Masutani et al.,[20] with a lower dose of γ irradiation (6 Gy). The cause of the acute lethality following irradiation appears to be due to the extensive destruction of gastrointestinal mucosa.[16,20,39] This pathology is

characteristic of radiation-induced death termed as the gastrointestinal syndrome, usually caused by intermediate radiation doses.[62,63]

Sensitivity to ionizing radiation–induced lethality depends on the age of the animals: young animals were found to be more sensitive than older ones.[62] Therefore, we wanted to test whether PARP deficiency results in increased sensitivity to ionizing radiation in older animals, and, if so, whether expression of exogenous human PARP in these animals can rescue radiation toxicity observed in $PARP^{-/-}$ mice. We irradiated transgenic ($PARP^{-/-}$Tg+) and nontransgenic $PARP^{-/-}$ littermates, as well as wild-type ($PARP^{+/+}$) mice with 8 Gy of whole-body γ irradiation and followed their survival. Only 1 of 11 (~10%) irradiated wild-type animals died, whereas 13 of 16 nontransgenic $PARP^{-/-}$ mice (81%) died within 16 weeks postradiation (Figure 4.4), consistent with radiation hypersensitivity of animals lacking PARP.[16,18,20,39] In contrast, only 3 of 17 (18%) $PARP^{-/-}$Tg+ mice had died by 16 weeks postradiation (Figure 4.4). Thus, these results show restoration of normal sensitivity to ionizing radiation in $PARP^{-/-}$Tg+ mice, suggesting that exogenous human PARP can replace the endogenous mouse gene in maintaining its radioprotective functions.

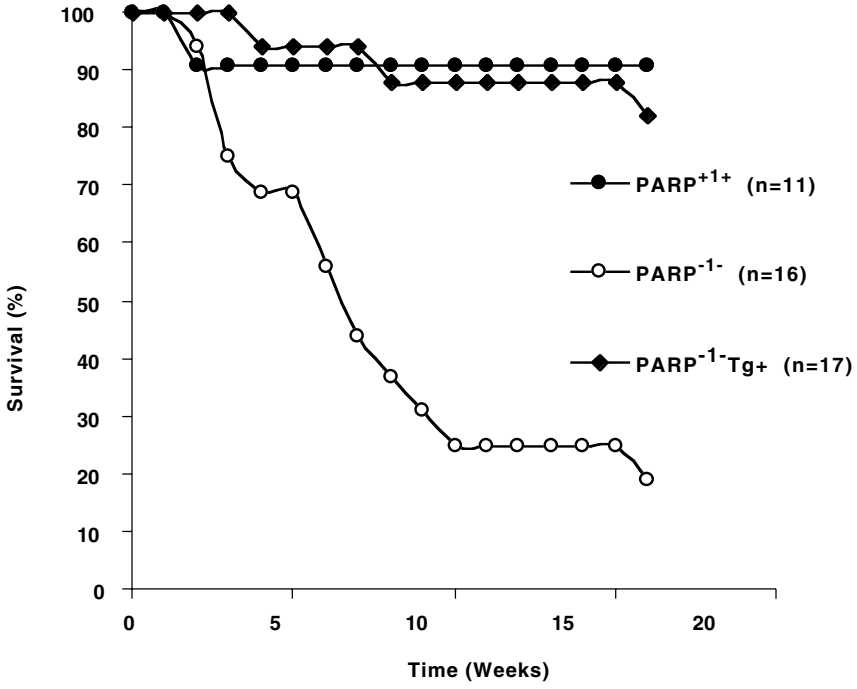

FIGURE 4.4 Ecoptic expression of human PARP transgene rescues radiosensitivity of PARP null mice. Survival curves of $PARP^{-/-}$Tg+, $PARP^{-/-}$, and $PARP^{+/+}$ mice [(129 × C57BL/6 × CBA), aged 17 to 30 weeks] after 8 Gy whole-body γ irradiation.

Together with previous published data, our study indicates that PARP deficiency renders animals hypersensitive to ionizing radiation irrespective of their age. However, there appears to be a significant difference in the kinetics of death between young and old PARP null mice. Whereas 100% of young PARP$^{-/-}$ mice (6 to 8 weeks of age) died within 2 weeks after receiving 8 Gy of radiation,[16,18,39] most older animals used in this study (over 17 weeks) died within 10 weeks postradiation (see Figure 4.4). Different kinetics of death between young and old PARP$^{-/-}$ mice after ionizing radiation may be attributable to damage of different cell types. Early death observed with young PARP null mice is caused by gastrointestinal failure, whereas delayed death in old mice may reflect a hematopoietic syndrome, characterized by bone marrow failure and delayed lethality.[64] This notion was confirmed by a histopathological analysis of lymphoid organs as well as gastrointestinal tract after irradiation. Interestingly, half of PARP$^{-/-}$ mice exhibited severe depletion of lymphoid cells in the spleen, whereas such a depletion was not observed in PARP$^{-/-}$Tg+ and PARP$^{+/+}$ mice (data not shown). Moreover, most irradiated PARP$^{-/-}$ mice exhibited lobular pneumonia (60%) (data not shown), suggesting a compromised immune response. Interestingly, pathological changes in gastrointestinal mucosa were not obvious in all groups of mice at time of autopsy (data not shown). Together with delayed kinetics of death, these histopathological analyses indicate that irradiated older PARP$^{-/-}$ mice die due to the effects on blood-forming organs (hematopoietic syndrome), and not due to gastrointestinal changes, which are usually observed in young mice devoid of PARP. Therefore, this study further strengthens the notion that PARP is a general protector from death in cells after acute DNA damage.

Radiosensitivity caused by PARP deficiency opens interesting perspectives for the use of PARP inhibition (e.g., by chemical inhibitors) for therapeutic purposes. Therefore, PARP inhibition may be synergistic in certain pathological settings in which radiation is therapeutically used to kill cells. For example, the combination of a specific PARP inhibitor and local irradiation may be very beneficial in treating certain tumors in humans.

4.5 REMARKS

We have shown that exogenous human PARP in PARP$^{-/-}$Tg+ mice is functionally active and can replace the mouse endogenous gene in maintaining normal cellular functions such as proliferation and DNA damage/stress response. The generation of transgenic mice exclusively expressing human PARP produced a useful model for testing drugs aiming to inactivate or activate the gene *in vivo*. A better understanding of PARP and poly(ADP-ribosyl)ation homeostasis in response to DNA damage and stress would facilitate the development of therapeutic strategies for the treatment of different human diseases such as inflammatory processes and malignancy. Another process involving PARP that may be amenable to pharmacological modulation is cell death. Since cell biological studies have shown that PARP cleavage has a function in protection from necrotic death generation and characterization of mice exclusively expressing noncleavable mutant PARP will represent a useful model to evaluate the physiological roles of PARP cleavage, particularly in inflammation response.

It is now widely accepted that PARP is a multifunctional protein involved in many important cellular processes. However, a number of questions on the exact mechanisms through which PARP participates in cell proliferation, DNA damage signaling, stress and inflammatory responses remain to be answered and will undoubtedly represent a major challenge to scientists in the field. Characterization of several recently identified PARP-related molecules, i.e., tankyrase,[65] PARP-2,[66-68] PARP-3,[66] and VPARP,[69] is required to delineate functions of poly(ADP-ribosyl)ation *in vivo*. However, since PARP (PARP-1) is the molecule principally responsible for poly(ADP-ribosyl)ation activity, and the only one containing DNA binding domain, it may be predicted that PARP, among other members, will still occupy a central position in the response to DNA damage. Furthermore, elucidation of the biological function of the poly(ADP-ribose) polymer degrading enzyme PARG will represent another important research area. PARG has so far been identified as being responsible for the degradation of poly(ADP-ribose) polymers and is thus believed to be critical for the maintenance of polymer homeostasis. In this regard, disruption of the PARG gene *in vivo* will be instrumental in studying the biological significance of poly(ADP-ribose) homeostasis catalyzed by all "PARP" members, as well as in evaluating inhibitory effects on enzymes for synthesis or degradation of poly(ADP-ribose) polymers.

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5 PARP Inhibitors as Neuroprotective Agents for Brain Injury

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CONTENTS

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5.1 CEREBRAL ISCHEMIA

5.1.1 Ischemic Cascade

During cerebral ischemia and trauma, a complex cascade of events is set in motion that contributes to cellular injury. When cerebral blood flow falls to levels of approximately 20% of control, glucose and oxygen supply become limiting and bioenergetic failure occurs.[1] As high-energy phosphate stores become depleted, the tissue depolarizes, leading to a loss of ion homeostasis. One of the consequences of a depletion of ATP is that the excitatory neurotransmitter glutamate, which is normally taken up from the extracellular space by glutamate transporter proteins (requiring the expenditure of energy), now begins to accumulate in the extracellular space and soon reaches neurotoxic levels.[2] Calcium enters the cell through the activated neuronal *N*-methyl-D-aspartate (NMDA) receptors, as well as through voltage-sensitive calcium channels and via reversal of $2Na^+/Ca^{2+}$ exchange.[3,4] This increase in cytosolic calcium is deleterious to the cell by way of a number of mechanisms including lipolysis, proteolysis, disaggregation of microtubuli, and activation of a number of enzymes, principal among them, nitric oxide synthase.[5,6]

When cytosolic Ca^{2+} increases precipitously during ischemia, two isoforms of nitric oxide synthase, NOS1 (nNOS) and NOS3 (eNOS), bind calmodulin in a Ca^{2+}-dependent manner[7] and nitric oxide (NO) synthesis increases by more than 20-fold.[8] Although NOS2 (iNOS) is not Ca^{2+}-dependent, the induction of this enzyme is stimulated by cytokines released during ischemia.[9] The activation of NOS1 and NOS3 occurs within minutes after the start of ischemia, whereas the induction of NOS2 mRNA takes several hours, but persists over several days. In ischemia, and in particular during recirculation, the production of NO is accompanied by the production of superoxide anions and hydrogen peroxide. These can combine to form hydroxyl free radicals, which are highly reactive and can lead to lipid peroxidation. Of greater importance, however, is that NO combines with superoxide to form peroxynitrite ($ONOO^-$).[10,11] Although itself not a free radical, peroxynitrite is an anion that is extremely toxic to many biological molecules and to DNA. It is extremely reactive with moities that are involved with signal transduction,[12] attacks iron/sulfer centers important for cell respiration,[13] produces lipid peroxidation and causes calcium to be released from the intracellular stores in the mitochondria.[14]

Potentially, one of the more-damaging aspects of oxidative injury involves both single and double strand breaks to DNA. Following transient cerebral ischemia, single-strand breaks are evident within minutes of the start of reperfusion and double-strand breaks are seen as early as 1 h.[15,16] Reactive oxygen species from a number of sources contributes to DNA damage following ischemia, including metabolism of arachidonic acid via the cyclooxygenase and lipoxygenase pathways, oxidation of hypoxanthine by xanthine oxidase, and delocalization of iron.[17] Although NO can, by itself, cause single-strand breaks in DNA,[18] peoxynitrite is much more damaging to DNA,[19] and this DNA damage may play a very significant role in tissue damage following cerebral ischemia.[20]

5.1.2 DNA Damage and PARP Activation

Although DNA fragmentation, consisting of double-strand breaks, is a hallmark of cell death, damaged DNA e.g., single strand breaks and the formation of base adducts, can be detrimental to normal cell function and survival. Small, nonbulky DNA lesions, such as single strand breaks, are normally corrected by base excision repair (BER) mechanisms.[21] Poly(ADP-ribose) polymerase (PARP) (EC 2.4.2.30), a tightly bound chromosomal enzyme located in the nuclei of cells of various organs, including brain,[22,23,24] acts as an endogenous detection system for DNA damage repaired via BER mechanisms.[25] PARP binds to nicks in DNA and acts to catalyze a reaction leading to the cleavage of nicotinamide adenine dinucleotide (NAD) into adenosine 5′-diphosphoribose (ADP-ribose) and nicotinamide, transferring long-branched and negatively charged polymers of ADP-ribose (PAR) to chromatin-associated proteins, including PARP itself.[26,27] This post-translational modification continues until a critical PAR chain length is reached, at which time PARP is released from the DNA and other DNA repair enzymes complete the repair process.[28]

The extent of inherent DNA repair activity depends on the degree of damaged DNA[21] and the stage of the cell cycle,[29] with neurons possessing relatively less DNA repair capacity than normally dividing cells. If the DNA damage is extensive or if normal DNA repair function is impaired, however, cells may mutate or die as a result of permanent genomic damage. Genetic instability may lead to premature neurodegeneration, while inherited diseases with deficient DNA repair systems appear to be associated with neurological deficits.[30] Chronic accumulation of DNA lesions in central nervous system (CNS) neurons occurs with age,[31,32] although the repair capability of aged neurons may not change as compared with young cells.[33] It has been suggested that acute CNS injury, such as stroke or trauma, may accelerate age-related accumulation of genomic damage and lead to deficient control of DNA repair processes.[34]

Although PARP-assisted post-translational modifications of nuclear proteins may be beneficial under some circumstances,[35-37] during periods of energy depletion, such as ischemia or trauma, abnormally high levels of PARP activation can paradoxically be detrimental to the tissue.[38] This "suicide hypothesis" was first described by Berger et al.,[39,40] who reported that cells exposed to DNA-damaging agents exhibited DNA repair activity that decreased when the concentration of the damaging agents became high, suggesting that some important component became limiting in the repair process. Further studies indicated that extensive DNA strand breaks lead to a significant activation of PARP with a resultant energy depletion, since four molecules of ATP are consumed in NAD^+ regeneration.[41] When the cells were treated with PARP inhibitors, the depletion of NAD^+ and ATP (the essential components referred to above) was dramatically attenuated, and it was speculated that when DNA damage is extensive, the activation of PARP turns off the repair mechanism allowing the cell to die rather than survive with highly mutant phenotypes. In ischemia, energy is already severely limited, and the extra energy demands required for regeneration of NAD^+ can hasten tissue damage.

5.1.3 Indirect Evidence that PARP Activation is Detrimental in the CNS

There are several studies linking PARP activation with neurotoxicity.[24,42,43] Zhang et al.[42] used rat cerebral cortical cultures to examine the effect of PARP inhibitors on NMDA- and NO-induced neurotoxicity. They noted that when brain extracts were exposed to NO, a 110-kDa protein, shown to be PARP, was ADP-ribosylated. In addition, they found that benzamide, which is an inhibitor of PARP, significantly decreased cell damage induced by NMDA. When the cell cultures were pretreated with benzamide, cell damage elicited by sodium nitroprusside (SNP) or *S*-nitroso-*N*-aceylpenicillamine (SNAP) was reduced by approximately 50%, indicating that NO itself can activate PARP, leading to neurotoxicity. Application of benzamide up to 1 h after NMDA administration can produce significant rescue of cellular damage. Cultured cerebellar granule cells, when exposed to a toxic dose of glutamate, show a transient increase in poly(ADP-ribose) immunostaining, a persistent increase in enzyme protein immunoreactivity, and an increase in PARP mRNA within 30 min of the insult.[43] Treatment of the cultures with structurally different inhibitors of PARP significantly attenuates the neuronal death suggesting an active role of PARP in the neurotoxicity of glutamate. Exposure of macrophages and smooth muscle cells to peroxynitrite causes DNA strand breaks, activation of PARP, and significant inhibition of cellular respiration.[24] 3-Aminobenzamide treatment, however, prevents the decrease in cellular energy depletion, providing further evidence for a role of PARP activation in peroxynitrite-mediated injury. The substantial evidence linking peroxynitrite with tissue damage in cerebral ischemia[10,20] strongly suggests that PARP activation may be detrimental in ischemia as well.

Similar neuroprotection has been obtained with hippocampal slices.[44] When these slices are exposed to NO, severe neuronal injury results. Both orthodromic and antidromic population spikes are nearly abolished even after 1 h of recovery. Treatment with either nicotinamide at a concentration that produces significant inhibition of poly-(ADP-ribosyl)ation, or with 3-aminobenzamide, dramatically improves recovery, although it does not prevent CA1 population spike loss during the NO application.

These data with cell cultures and brain slices are supported by *in vivo* studies. Mice lacking PARP appear to be highly resistant to damage caused by NMDA injected into the striatum, although they are susceptible to striatal α-amino-3-hydroxy-5-methyl-4-isoxazcle propionate (AMPA) injections.[45] Furthermore, NMDA injections activate PARP whereas AMPA injections do not, and if PARP protein is restored in mice lacking PARP, they regain their susceptibility to NMDA.

Some of the data linking PARP with oxygen radical and nitric oxide toxicity has been obtained in organs other than the brain. Heller et al.[46] found that islet cells exposed to reactive oxygen intermediates (ROI) or NO exhibited a very large decrease in NAD^+ content, which is mostly reversed with treatment with the PARP inhibitor 3-aminobenzamide. Islet cells from mice with an inactivated PARP gene ($PARP^{-/-}$) did not show NAD^+ depletion following ROI or NO exposure, indicating that PARP activation is primarily responsible for the damage to these cells following ROI administration. The role of PARP in non-neuronal tissue is discussed in detail in Chapters 6 through 8.

5.1.4 PARP Inhibition

The hypothesis that activation of PARP may be involved in cellular damage following cerebral ischemia has been directly examined in a number of studies using inhibitors of PARP in models of ischemia. Most of these studies have been undertaken using PARP inhibitors that are relatively nonselective because highly selective inhibitors have not been readily available. The most widely used agents for the inhibition of PARP have been benzamide and its derivatives, primarily 3-aminobenzamide.[47,48] These compounds mimic nicotinamide to competitively inhibit PARP at the NAD^+-binding site. They have low toxicity *in vivo* and are highly selective for PARP compared with mono(ADP-ribose) transferase, although they do inhibit nicotinamide methyltransferase.[49] The ability of 3-aminobenzamide to inhibit PARP has been shown in a number of studies, both *in vitro*[50,51] and *in vivo*.[52] When murine macrophage-like tumor cells or human peripheral lymphocytes are exposed to hydrogen peroxide, they exhibit lysis with a concomitant decrease in NAD and ATP. Treatment with 3-aminobenzamide blocks the energy depletion,[53] influx of extracellular Ca^{2+}, and cell lysis although it does not block DNA damage.[50] The lack of repair of DNA strand breaks is consistent with the role of PARP in mediating this repair. Immunohistochemical staining showed that nuclear PAR decreased within 1 h after treatment with 3-aminobenzamide.[52]

Benzamide and its derivatives, however, can affect cell viability, glucose metabolism, and DNA synthesis,[54] inhibit oxidant-induced DNA single strand breakage due to a direct scavenging effect,[55] act as hydroxyl radical scavengers,[56] and inhibit the expression of inducible NOS.[57] It is necessary to utilize doses within a relatively narrow range when using these compounds to inhibit PARP. Although glucose metabolism is unaffected by 3-aminobenzamide, concentrations of benzamide as low as 3 mM can cause significant decreases in metabolism. The incorporation of methionine and glucose into DNA, both important for *de novo* DNA synthesis, are affected by benzamide at a concentration of 0.5 m*M*, whereas 3-aminobenzamide does not have an effect on DNA synthesis until 3 m*M*. Cosi et al.[58] examined the pharmacokinetics of benzamide in the mouse brain after intraperitoneal (i.p.) administration. They found that following a single injection of 160 mg/kg, the calculated molar concentration of the drug in the striatum was about 0.64 m*M* at 15 to 60 min and 0.09 m*M* at 240 min after injection. Therefore, to keep brain concentrations below the levels at which non-PARP-specific effects occur, *in vivo* administration of benzamide and 3-aminobenzamide should not exceed approximately 200 to 300 mg/kg. The slightly better characteristics of 3-aminobenzamide as a PARP inhibitor have made it more widely used in *in vivo* studies.[59-63] The *in vitro* inhibition of purified PARP by 3-aminobenzamide at 1 m*M* is 88%,[64] so doses of 3-aminobenzamide of 250 mg/kg (low enough that nonspecific pharmacological effects should not be a problem) will lead to PARP inhibition approaching 90%.

Another compound capable of inhibiting PARP is nicotinamide, which is an essential precursor of NAD^+ and thus helps attenuate NAD depletion during ischemia.[65] Nicotinamide is not only a precursor of NAD^+; it is a free radical scavenger and in millimolar quantities it acts as an inhibitor of PARP. The 50%

inhibitory concentration (IC_{50}) of nicotinamide is 210 μ*M*, approximately seven times greater than 3-aminobenzamide.[64] The relative nontoxicity of nicotinamide makes it a potential candidate for stroke therapy.

The lack of specificity and potency of benzamide and its derivatives to inhibit poly(ADP-ribose) polymerase has led to an active search for better inhibitors. Using an *in vitro* assay system Banasik et al.[64] screened 132 compounds for their ability to inhibit ADP-ribosyltransferases. One of the compounds with the greatest inhibitory effect was 1,5-dihydroxyiosquinoline, with an IC_{50} of 0.39 μ*M* — two orders of magnitude lower than benzamide (22 μ*M*) and 3-aminobenzamide (33 μ*M*). A series of PARP inhibitors similar to 1,5-dihydroxyiosquinoline, the dihydroisoquinolinones, was subsequently described,[66] and one compound from this series has been used *in vivo* to examine the role of PARP inhibition in cerebral ischemia.[67,68] This compound, 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone (DPQ), exhibits an IC_{50} of 1 μ*M* (1/200 that of nicotinamide), and competitively inhibits PARP at the NAD-binding site. Recently, two more potent PARP inhibitors have been synthesized. GPI 6150, 1,11b-dihydro-[2*H*]benzopyrano[4,3,2-*de*]-isoquinolin-3-one, has been shown to protect P388D1 cells against hydrogen peroxide cytotoxicity by inhibiting PARP activation with few potential side effects.[69] PJ34, based on a modified phenanthridinone structure, appears to be approximately 10,000 times more potent than 3-aminobenzamide as an inhibitor of PARP as determined by EC_{50} comparisons.[70] The specificity and potency of these new PARP inhibitors should facilitate investigations into the role of PARP in ischemic neural injury.

5.1.5 PARP Activation in Ischemia

The *in vivo* studies that have been undertaken to examine the role of poly(ADP-ribose) polymerase in cerebral ischemia have primarily employed either pharmacological inhibition of PARP or use of transgenic mice in which the PARP gene had been deleted. The conclusion from these studies is that activation of poly(ADP-ribose) polymerase is an important contributor to ischemic cell damage in the brain as well as in other organs.

The *in vitro* data implicating the activation of PARP in cerebral ischemia and the potential for adverse effects of this activation have been discussed above. This section describes the *in vivo* data showing that PARP is indeed activated and the implications of this activation. Among the strongest indications of an activation of PARP is an increase in the PAR polymer during ischemia (Table 5.1). Mice subjected to 2 h of focal ischemia using the intraluminal suture technique exhibit a two- to threefold increase in PAR-positive cells within 5 min of the start of reperfusion.[60] Although poly(ADP-ribose) is still evident 2 h after reperfusion,[71] by 3 h, no significant PAR is seen immunohistochemically,[60] possibly due to its short half-life.[72] As may be expected, PARP-deleted mice do not show any PAR formation following the same ischemic/reperfusion insult.[60,71] Mice treated with 3-aminobenzamide intracerebroventricularly prior to middle cerbral artery (MCA) occlusion and reperfusion exhibit significant attenuation of poly(ADP-ribose) formation.[60] Similar data

have been obtained in the rat using a model in which the distal portion of the MCA is occluded along with temporary common carotid occlusion.[68] At 10 min into reperfusion after 90 min of ischemia, there is an increase in immunoreactivity of poly(ADP-ribose) polymer, with the number of PAR positive cells increasing by a factor of two to three in both layer II-III and layer V of the cortex (Figure 5.1). This increase in polymer formation is totally blunted by the administration of DPQ 30 min after the start of ischemia.

It should be noted that reperfusion is not necessary for the activation of PARP. Faint poly(ADP-ribose) immunostaining is seen in the medial and lateral striatum and in the frontoparietal cortex 1 h into permanent MCA occlusion in the rat.[61] Poly(ADP-ribose) formation peaks in the lateral striatum and the frontoparietal cortex after 2 h of ischemia, with the immunopositivity confined primarily to neurons, and the nuclei staining much more prominently than the cytoplasm. By 4 h, the number of positive cells decreases, and by 16 h of occlusion, poly(ADP-ribose) immunostaining almost completely disappears. Similar to transient ischemia models, immunoreactivity is also markedly inhibited with 3-aminobenzamide.[61] The time course of poly(ADP-ribose) immunostaining in a model of sublethal transient global cerebral ischemia[73] is similar to that seen in focal ischemia. Following 15 min of four-vessel occlusion, PAR formation is seen in the hippocampus one hour after ischemia, but by 24 h, the staining is not different from that seen in sham-operated animals.[62]

The appearance of the poly(ADP-ribose) polymer is also evident following focal ischemia in the neonate. Permanent occlusion of the MCA in the 7-day-old rat along with occlusion of the ipsilateral carotid artery for 1 h produces strong immunoreactivity in the ischemic area after 48 h of reperfusion.[74] Both the cytoplasm and the nuclei are stained, with more pronounced staining in the core of the ischemia than in the peri-infarct zone. Similar to the effect seen in the adult, the number of PAR-positive cells is reduced by 3-aminobenzamide treatment 1 h after MCA occlusion.

Poly(ADP-ribose) polymer formation is only one marker of an activation of PARP. Nagayama et al.[62] measured PARP mRNA using both Northern blot analysis and *in situ* hybridization and found no change either 4 or 24 h into reperfusion following 15 min of global cerebral ischemia. Accordingly, Western blot analysis also failed to see any increase in PARP protein activation. PARP enzymatic activity, however, was increased 4.37-fold and 1.73-fold after 1 and 24 h of reperfusion, respectively, increases that were blocked by administration of 3-aminobenzamide.[62] As implied by the "PARP suicide hypothesis," activation of PARP by DNA strand breaks leads to depletion of NAD^+. By 24 h following 2 h of MCA occlusion, NAD^+ level is depressed to approximately 35% of control.[60] $PARP^{-/-}$ mice, however, show a significantly smaller depression (69% of control), and in mice administered 3-aminobenzamide, NAD^+ is decreased to only 60% of control.

TABLE 5.1
Markers of PARP Activation in Ischemia

Model	Animal	Ischemia / Reperfusion	Drug / Condition	Timing [a]	Dose	Effect	Ref.
PAR Immunohistochemistry							
MCA + LCCA	Neonatal rat	60 min / 48 h	— 3-AB	— + 60 min	— 10 mg	↑ PAR-positive cells PAR positive cells inhibited	Ducrocq et al., 2000
Intraluminal suture	Mouse	120 min / 2 h	Wild-type PARP$^{-/-}$	— —	— —	↑ ↑ PAR-positive cells no PAR-positive cells	Eliasson et al., 1997
Intraluminal suture	Mouse	120 min / 5 min 120 min / 3 or 6 h	— —	— —	— —	2–3 fold ↑ PAR positive cells ≈ nonischemic control	Endres et al., 1997
			3-AB	– 10 min	2-8 mg/Kg (icv)	PAR-positive cells strongly inhibited	
			PARP $^{-/-}$	—	—	no PAR positive cells	
4 vessel occlusion [b]	SD rat	15 min / 1 h 15 min / 24 h	— —	— —	— —	↑ PAR-positive cells ≈ nonischemic control	Nagayama et al., 2000
Distal MCA + BCCA	LE rat	90 min / 10 min	— DPQ	— + 30 min	— 40 mg/kg (ip)	>3 fold ↑ PAR positive ≈ nonischemic control	Takahashi et al., 1999
Intraluminal suture	SD rat	2 – 24 h	—	—	—	Peak ↑ PAR at 2 h, no positive cells by 16 h	Tokime et al., 1998
		2 h	3-AB	– 30 min	1 – 30 mg/kg (i.p.)	PAR positive cells inhibited; maximum at 10 mg/kg	

Table 1 (con't)

Related parameters

Intraluminal suture	Mouse	120 min / 22 h	$PARP^{-/-}$	—	—	Restores NAD+ from 35% of control to 69% of control; no change in amount of DNA fragmentation	Endres et al., 1997
		120 min / 22 h	3-AB	– 10 min	2.8 mg/kg (icv)	Restores NAD+ from 35% of control to 60% of control; no change in amount of DNA fragmentation	
4 vessel occlusion	SD rat	15 min / 1 h	—	—	—	4.37 fold ↑ PARP activity	Nagayama et al., 2000
		15 min / 24 h	—	—	—	1.73-fold ↑ PARP activity	
		15 min / 1 h	3-AB	– 10 min	30 mg/kg	Blocks 4.37-fold ↑ in PARP activity	
		15 min / 24 h	3-AB	– 10 min	30 mg/kg	Blocks 1.73 fold ↑ in PARP activity	
		15 min / 1, 4, 8, 16, 24 h	—	—	—	PARP mRNA expression not altered (*in situ* hybridization); PARP protein level not altered (Western blot)	
		15 min / 1, 24 h	3-AB	– 10 min	30 mg/kg	Increase in DNA base excision repair activity prevented	

MCA = middle cerebral artery; LCCA = Left common carotid arterty; BCCA = bilateral common carotid artery; LE = Long Evans; SD = Sprague Dawley; DPQ = 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone

3-AB = 3-aminobenzamide; PARP = poly(ADP-ribose) polymerase; $PARP^{-/-}$ = PARP null mice; ip = intraperitoneal; icv = intracerebroventricular

a = treatment times with respect to start of ischemia; b = brain temperature controlled at 34°C.

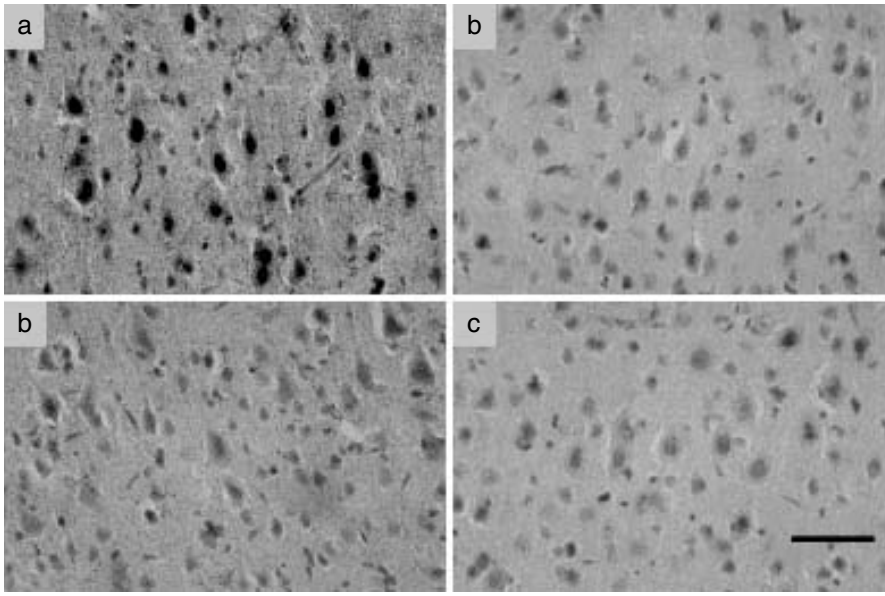

FIGURE 5.1 Immunoreactivity of poly(ADP-ribose) polymer 10 min after reperfusion following 90 min of MCA occlusion in the rat. The number of poly(ADP-ribose)-positive cells and the intensity of nuclear staining was increased in the ischemic tissue (a) compared to the contralateral cortex (b). Animals treated with DPQ (40 mg/kg) 30 min after MCA occlusion exhibited fewer immunopositive cells in the ischemic tissue (c) than untreated animals. The immunoreactivity in the nonischemic side of the treated animals (d) was comparable to that in the untreated animals. The images were obtained from layer V of the frontoparietal cortex (Bregma–1.3). Scale bar = 50 μm. (Modified from Takahashi K. et al., *Brain Res.*, 829, 46, 1999. With permission from Elsevier Science.)

5.1.6 Neuroprotection with PARP Inhibition in Experimental Models of Cerbral Ischemia

5.1.6.1 Pharmacological Studies

5.1.6.1.1 DPQ, GPI 6150, PJ34

Because of its high potency and relative specificity as an inhibitor of PARP,[68] DPQ has been evaluated in a series of focal ischemia studies in the rat.[67] Focal cerebral ischemia was induced by cauterizing the distal portion of the right MCA simultaneous with temporary bilateral common carotid occlusion.[75] Cerebral blood flow falls to approximately 20% of control in the center of the ischemic territory in this model, and remains stable throughout the ischemic period.[67] After 90 min of occlusion, the carotid ligatures were removed and cerebral blood flow returned to at least 50% of the control level. DPQ dissolved in dimethyl sulfoxide (DPQ is not aqueous soluble) was administered to the animals two hours before MCA occlusion and 2 h after

occlusion to maximize inhibition during the early ischemic period. Infarct volume, as determined from 2,3,5-triphenyltetrazolium chloride (TTC) staining 24 h after occlusion, showed a significant decrease in the animals treated with 5 to 20 mg/kg DPQ[67] (Table 5.2). The greatest neuroprotection was seen at the 10-mg/kg dose where infarct volume decreased to 76.4 ± 16.8 mm^3 from 165.2 ± 34.0 mm^3 (a 54% decrease).

When treatment is given only after the start of ischemia (no pretreatment), a similar neuroprotective effect of DPQ is observed.[68] Administration of DPQ at a dose of 10 to 80 mg/kg leads to significant decreases in infarct volume, with the largest effect observed at a dose of 40 mg/kg (a 42% decrease). Treatment with DPQ either prior to the onset of ischemia[67] or following ischemia[68] leads to a U-shaped dose–response curve (Figure 5.2). This suggests dual effects of this compound whereby, at higher doses, the protective effect of PARP inhibition is countered by secondary effects capable of exacerbating neuronal injury acting on another target. This may be analogous to the "non-PARP"-specific effects of 3-aminobenzamide at higher doses.[49,54]

Preliminary data exist on a compound GPI 6150, which is similar to DPQ but with a greater potency as a PARP inhibitor (IC_{50} = 0.1 μ*M*). GPI 6150 was given to rats two hours prior to two hours of MCA occlusion (intraluminal filament technique) as well as two hours after occlusion, and infarct volume was measured following 22 h of reperfusion.[76] The reduction of infarct volume was extremely large (80%), comparable to the neuroprotection seen in $PARP^{-/-}$ mice (see below). Even treatment 1 h after MCA occlusion yielded very significant protection from ischemic injury.

Another potent PARP inhibitor, PJ34, has also been examined in models of focal ischemia in both the mouse and the rat.[77] In the mouse, treatment with PJ34 2 h prior to 1 h of MCA occlusion and 4 h after occlusion leads to a decrease in infarct volume of 48% at 24 h. It should be noted that this decease was measured by the direct technique without correction for brain edema. The decreases in infarct size are even greater in the rat. Treatment 10 min prior to 2 h of MCA occlusion decreases infarct volume by 70% after 2 h of reperfusion and 90% after 22 h of reperfusion. Delaying treatment until 110 min after occlusion (10 min prior to withdrawal of the intraluminal filament) reduced the beneficial effect only slightly — to 70% by 22 h of reperfusion. Following permanent MCA occlusion, a decrease in infarct volume is seen at 4 h, but not at 24 h.[77]

5.1.6.1.2 3-Aminobenzamide

Although 3-aminobenzamide is not as specific or potent as DPQ, there have been several studies examining the effect of 3-aminobenzamide treatment on infarct volume following cerebral ischemia. Using the standard intraluminal occlusion model for producing focal ischemia in the rat, Lo et al.[63] observed a 56% decrease in infarct volume in animals administered 10 mg/kg 3-aminobenzamide intravenously at the start of 90 min of temporary MCA occlusion and 22$^1/_2$ h of reperfusion. The data from models of permanent ischemia, however, are slightly different. If 3-aminobenzamide is administered by intraperitoneal injection (even in doses as high as 100 mg/kg 30 min

TABLE 5.2
Decreases in Infarct Volume as a Result of Treatment with PARP Inhibitors or in PARP Null Mice.

Model	Animal	Ischemia / Reperfusion	Drug / Condition	Timing [a]	Dose	Effect	Ref
Intraluminal suture	SD rat	120 min / 2 h 120 min / 22 h 4 h 24 h	PJ34	– 10 min – 10 or +110 min – 10 min	10 mg/kg (iv)	↓ 70% [b] ↓ 90% or ↓ 70%[b] ↓ 77%[b] NS	Abdelkarim et al., 2001
Intraluminal suture	Mouse	60 min/ 23 h	PJ34	– 2 h and +3 h	2.5 mg/kg (ip)	↓ 48% [b]	Abdelkarim et al., 2001
Intraluminal suture	Wistar rat	24 h	NAm	+ 0.5, 2, 3, or 4 h	500 mg/kg (ip)	↓ 28% (0.5 hr) ↓ 32% (2 h)	Ayoub et al., 1999
Intraluminal suture	Mouse	120 min/ 22 h	3-AB	– 10 min	2.8 mg/kg (icv)	↓ 46% (10 mg/kg)	Endres et al., 1997
MCA + LCCA	Neonatal rat	60 min / 48 h	3-AB	0, 1, 1.5, or 2 h	5, 10, 20 mg/kg	↓56% (10 mg/kg at t = 0) NS (10 mg/kg at t = 1 -2 h) ↓ 41% (20 mg/kg at t = 0) NS (20 mg/kg at t = 1.5, 2 h)	Ducrocq et al., 2000
Intraluminal suture	SD rat	90 min / 22.5 h	3-AB	+ 0 h	10 mg/kg (iv)	↓ 56%	Lo et al., 1999
Intraluminal suture	Wistar rat	120 min / 3 or 7 d	NAm	+ 2 h	500 mg/kg (i.p.)	↓ 46% (3 d) ↓ 35% (7 d)	Mokudai et al., 2000

Table 2 (con't)

4 vessel occlusion[c]	SD rat	15 min / 72 h	3-AB	– 10 min	3, 10, 30 mg/kg	↓ 98% in number surviving cells	Nagayama et al., 2000
Distal MCA + BCCA	LE rat	90 min / 22 h	DPQ	– 2 h / +2 h	5 – 40 mg/kg (i.p.)	↓ 54% (10 mg/kg)	Takahashi et al., 1997
Distal MCA + BCCA	LE rat	90 min / 22 h	DPQ	+ 30 min	10 – 80 mg/kg (i.p.)	↓42% (40 mg/kg)	Takahashi et al., 1999
Intraluminal suture	SD rat	24 h / 24 h	3-AB	– 30 min – 30 min	1 – 100 mg/kg (i.p.) 1 – 30 mg/kg (icv)	No change ↓ 37% (10 mg/kg)	Tokime et al., 1998
PARP null mice							
Intraluminal suture	Mouse	120 min / 22 h	$PARP^{-/-}$ $PARP^{+/-}$	—	—	↓78% ↓56%	Eliasson et al., 1997
Intraluminal suture	Mouse	120 min / 22 h	$PARP^{-/-}$ $PARP^{+/-}$	—	—	↓35% ↓ 31%	Endres et al., 1997

MCA = middle cerebral artery; LCCA = left common carotid artery; BCCA = bilateral common carotid artery; LE = Long Evans; SD = Sprague Dawley; DPQ = 3,4-dihydro-5-[4-(1-piperidinyl)butoxy]-1(2H)-isoquinolinone; 3-AB = 3-aminobenazamide; NAm = nicotinamide; PARP = poly(ADP-ribose) polymerase; $PARP^{-/-}$ = PARP null mice; ip = intraperitoneal; iv = intravenous; icv = intracerebroventricular; NS = not significant.

a = treatment times with respect to start of ischemia;

b = infarct volume by direct measurement, all others by indirect technique;

c = brain temperature controlled at 34°C.

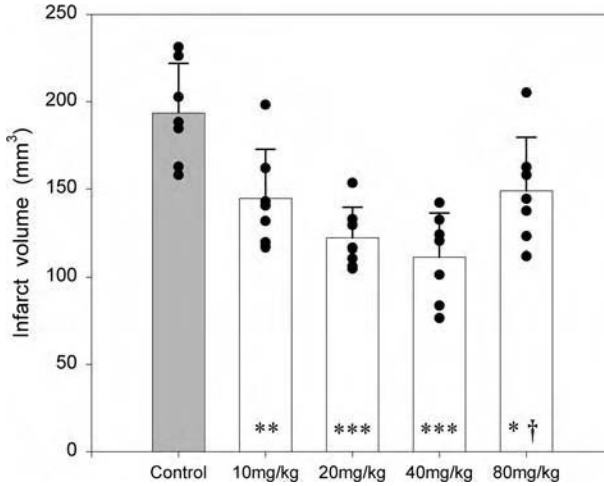

FIGURE 5.2 The effect on infarct volume of *in vivo* treatment with DPQ administered 30 min following the start of 90 min of MCA occlusion in the rat. Infarct volume was determined from 2,3,5-triphenyltetrazolium chloride staining 24 h into reperfusion. Significant differences between the treated and the control groups (* $p < 0.05$, ** $p < 0.02$, *** $p < 0.01$) as well as between the 40 mg/kg and the 80 mg/kg treated groups († $p < 0.05$) are indicated. Bars are means; error bars are standard deviation. (Modified from Takahashi K. et al., *Brain Res*., 829, 46, 1999. With permission from Elsevier Science.)

prior to MCA occlusion), no effect is seen, whereas an intracerebroventricular (ICV) injection decreases infarct volume by 37% with decreases seen in both the cortex and the striatum.[61] Similar results were obtained in the mouse. Intracerebroventricular administration of 3-aminobenzamide 10 min prior to 2 h of MCA occlusion using the intraluminal suture technique reduces infarct volume by 46% after 22 h of reperfusion.[60]

5.1.6.1.3 Nicotinamide

Nicotinamide, which is also a PARP inhibitor, has been examined in both transient and permanent focal cerebral ischemia. When rats were administered nicotinamide (500 mg/kg) either 30 min or 2 h following intraluminal induced permanent MCA occlusion, infarct volume 24 h after the induction of the ischemia was reduced 28 to 34% compared with vehicle-treated animals.[78] Treatment at 3 or 4 h, however, failed to reduce ischemic damage significantly. Similar to the data of Takahashi et al.,[68] the dose–response curve was U-shaped, with 1000-mg/kg treatment failing to produce any beneficial effect. As suggested by the authors, the loss of neuroprotection at the higher dose may be related to the ability of nicotinamide to enhance brain choline levels,[79] which has been implicated in glutamate neurotoxicity.[80] These investigators examined nicotinamide treatment in the same model of focal ischemia in the rat, but in which reperfusion was permitted following 2 h of ischemia.[81] Treatment 2 h after MCA occlusion (at the start of reperfusion) decreased infarct volume 46% 3 days later, and 35% 7 days later.

5.1.6.2 PARP Null Mice

Pharmacological inhibition of PARP has been very useful in investigating mechanisms of damage following ischemia. There are, however, several limitations to the use of inhibitors of PARP in animal studies, including the nonspecificity of the available compounds (see above). In addition, until the pharmacokinetic and pharmacodynamic characteristics of these compounds are known, chronic treatment is difficult. For these reasons, the use of animals with targeted disruption of the gene that encodes PARP has been extremely useful in investigations of the role of PARP in cerebral ischemia. Eliasson et al.[71] subjected $PARP^{-/-}$ mice to 2 h of MCA occlusion and measured infarct volume after 22 h of reperfusion. They found a decrease in infarct volume of 78% compared with wild-type controls, whereas in $PARP^{+/-}$ mice, infarct volume decreased by 56%. This dramatic neuroprotection is greater than that measured by Endres et al.[60] who found a 35% decrease in infarct volume in $PARP^{-/-}$ mice and a 31% decrease in infarct volume in $PARP^{+/-}$ mice using the same ischemia model (Figure 5.3). These differences in neuroprotection may be related to differences in background strains used in the respective studies.[82] In the study of Endres et al.[60] the background strain was mixed (129SV/C57B6), whereas in the study of Eliasson et al.,[71] pure 129SV $PARP^{-/-}$ mice were used. It has been shown previously that the size of infarct following MCA occlusion in the mouse is dependent upon the strain.[83,84] C57B6 mice subjected to 3 hrs of MCA occlusion and 24 h of reperfusion exhibit infarcts that are 33% larger than 129SV mice, in agreement with the larger infarcts observed in the mice used by Endres et al.[60]

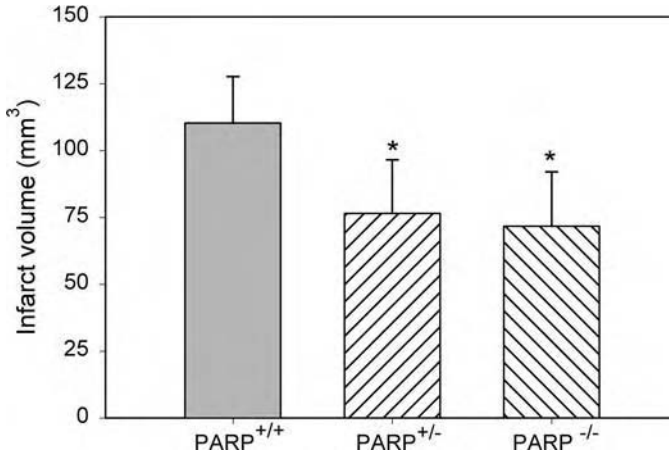

FIGURE 5.3 Infarct volumes in mice after 22 h of reperfusion following 2 h of MCA occlusion. The infarct size, obtained from hematoxylin and eosin-stained sections, was significantly decreased in PARP heterozygotes ($PARP^{+/-}$), and in PARP null mice ($PARP^{-/-}$) compared to wild-type littermates ($PARP^{+/+}$). * $p < 0.01$ compared to the wild-type mice. Data are presented as mean ± standard deviation. (Data from Endres et al., 1997.[60])

5.1.6.3 Behavioral Evaluation

Because of the importance of protecting and restoring cerebral function following stroke and the use of functional parametrics in human stroke therapy trials, many studies of neuroprotection in experimental cerebral ischemia include measurements of function following ischemia and treatment.[85-88] Mokudai et al.[81] made measurements of both sensory and motor disturbances for up to 1 week in rats following 2 h of MCA occlusion by the intraluminal filament technique. They found that animals treated with nicotinamide had significantly improved sensory and motor neurological scores at 3 and 7 days after occlusion, correlating well with the decrease in infarct volume following treatment. Similar data were obtained with 3-aminobenzamide treatment in the mouse 24 h following 2 h of filament-induced MCA occlusion,[60] and with PJ34 treatment in the rat following 2 h of MCA occlusion.[77] In the neonatal rat, 3-aminobenzamide reduces the neurological deficits normally observed 2 to 3 weeks following permanent MCA occlusion combined with temporary common carotid occlusion.[74] This improvement in neurological score correlates, as may be expected, with tissue damage in this model.

5.1.6.4 Mechanisms of Damage

The vast majority of the studies examining the role of PARP in ischemia have been undertaken in temporary ischemia models — models that include reperfusion. It is therefore difficult to determine whether PARP plays a larger role in ischemic or in reperfusion injury. As discussed above, there is a marked increase in PAR polymer within 5 min of the start of reperfusion and, depending upon the ischemia model, the polymer remains elevated for several hours. In the neonatal rat, the PAR polymer is elevated for as long as 2 days after the start of reperfusion.[74] One of the few studies that actually measured PARP activity (as opposed to poly(ADP-ribose) immunoreactivity), in ischemia provides data following only sublethal global cerebral ischemia.[62] In this study, PARP activity was measured along with PARP mRNA and PARP protein level. Although PARP mRNA and PARP protein level were not elevated at any time during reperfusion, PARP activity increased 4.4-fold at 1 h and 1.7-fold at 24 h, suggesting that PARP activation was due to a post-translational mechanism in which PARP binds to the DNA strand breaks.[62] One study to measure PARP activation *during* ischemia found a faint increase in poly(ADP-ribose) immunostaining 1 h into ischemia (permanent intraluminal filament model in the rat), and maximal immunoreactivity at 2 h of ischemia, which decreases in time so that by 16 h of ischemia no immunoreactive cells for poly(ADP-ribose) are observed.[61] In a similar ischemia model in the mouse, no immunoreactive cells were found after 1 h of ischemia[60] in general agreement with the rat study. Taken together, these data suggest that PARP is activated in ischemic tissue 1 to 2 h after the start of ischemia and remains elevated for several hours (data from permanent ischemia studies). At the time of reperfusion there is a sharp increase that persists for a few hours (data from 1 to 2 h ischemia studies).

One way to address the question whether the PARP activation that seems to occur early in the reperfusion period contributes significantly to the tissue damage in ischemia–reperfusion is to treat animals with a PARP inhibitor after the start of reperfusion. Takahashi et al.[59] administered 3-aminobenzamide to rats 15 min after the start of reperfusion in a group of rats comparing them with a comparable group in which the treatment occurred 15 minutes prior to the start of reperfusion. Ischemia was produced by distal occlusion of the MCA combined with bilateral common carotid occlusion. Both the MCA and the CCA were reperfused after 90 min and infarct volumes were measured 22.5 h later. When treatment occurred prior to the start of reperfusion, infarct volume decreased by 33% ($p < 0.01$), whereas treatment after the start of reperfusion was not beneficial; the difference in infarct volume between the untreated and 3-aminobenzamide treated animals was only 2%. To demonstrate that this was not because the pretreated animals received the 3-aminobenzamide sooner with respect to the start of the ischemia, an additional group received only 60 min of ischemia with the 3-aminobenzamide administered 15 min after the end of the ischemia (75 min after the start of ischemia). These animals were thus treated at the same time after the start of ischemia as the 90 min pretreatment group (75 min after the start of ischemia). Again, the pretreatment group exhibited a decrease in infarct volume even in spite of the ischemia being 30 min longer. These studies are consistent with the evidence of a large and rapid activation of PARP activity during the early part of reperfusion,[89] which, unless it is inhibited, can lead to significant tissue damage.

5.1.6.5 Delayed Neuronal Death

The vast majority of the data in the literature seems to support the original "suicide" hypothesis of Berger and associates[39-41,53] in which DNA strand breaks activate PARP and the subsequent consumption of NAD^+ markedly depletes cellular energy levels. According to this hypothesis, PARP inhibition (or deletion) should protect tissue from damage by preventing or attenuating NAD^+ and ATP depletion.[42] Besides cerebral ischemia, this scenario is supported by studies in diabetes,[9,90] septic shock,[92] and myocardial ischemia.[93,94] PARP, however, is involved in DNA repair and maintenance of genomic stability, and plays a role in cell proliferation, differentiation, and transformation.[35,37,95] This would tend to suggest that PARP activation in the face of DNA damage would be beneficial, and not, as discussed above, detrimental to cellular survival. This apparent paradox may be related to the role of PARP in apoptosis. Apoptosis, sometimes referred to as programmed cell death, is an energy-dependent process whereby the cell dies in an organized fashion.[96] Caspase-3, a member of the family of asparate specific cysteine proteases, is enhanced during ischemia,[97,98] cleaving and inactivating PARP.[99] In fact, one of the hallmarks of apoptosis is the cleavage of PARP which seems to be necessary for the proper execution of the apoptotic pathway. $PARP^{-/-}$ mice are extremely sensitive to either alkylating agents or to γ irradiation,[37] and cells from an uncleavable PARP mutant introduced in $PARP^{-/-}$ fibroblasts exhibit a significant delay in apoptosis following CD95 stimulation.[100] Because apoptosis is an energy-dependent event, it has been suggested that PARP cleavage is

important for apoptosis as it prevents PARP activation with the resulting depletion of energy and necrotic death.[101,102] Thus, fibroblasts from PARP$^{-/-}$ mice exposed to DNA-damaging agents that normally elicit necrosis, are protected from ATP depletion and subsequent damage, but are susceptible to apoptotic death.[101] Consistent with these results is the observation that fibroblasts expressing a PARP that is resistant to caspase cleavage undergo an accelerated necrotic death when exposed to tumor necrosis factor-alpha (TNF-α).[102] In addition, there exists an endonuclease that is a substrate for PARP and that is inhibited by poly(ADP-ribosyl)ation.[103] Cleavage of PARP would therefore facilitate DNA fragmentation. These *in vitro* data suggest that PARP inhibition *in vivo* may not only reduce necrotic death, but may also enhance apoptotic death.

We have recently obtained data that pertain to delayed maturation of the ischemic tissue following *in vivo* DPQ treatment, and to the issue of apoptosis vs. necrosis following PARP inhibition in cerebral ischemia.[104] Studies were undertaken in rats subjected to MCA occlusion along with bilateral common carotid artery occlusion. Some animals were treated with DPQ (40 mg/kg) 30 min after MCA occlusion while others received vehicle. After 90 min of occlusion, the carotid ties were released and the animals permitted to reperfuse for a period of 1 day (control and DPQ, n = 7 each), 3 days (DPQ, n = 3), or 2 weeks (control and DPQ, n = 7 each), at which time the brains were removed and infarct volumes measured using TTC staining. In separate animals 1 day after MCA occlusion, three sections from each brain were stained using terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick-end labeling (TUNEL) histochemistry (control and DPQ, n = 3 each).

Similar to our previous report,[68] post-treatment with DPQ reduced infarct volume by 42% 1 day after the start of reperfusion ($p < 0.01$). At 2 weeks, however, the DPQ-treated group showed a significant increase in infarct volume compared to the DPQ 1 day group ($p < 0.01$), with the infarct volume in the DPQ-treated animals no longer significantly smaller than that in the vehicle-treated animals. TUNEL-positive cell density in the ischemic area of the DPQ 1 day group was significantly higher than the control 1 day group ($p < 0.001$). Thus, the inhibition of PARP led to a significant decrease in the infarct size at 24 h, but with delayed maturation of ischemic tissue by 2 weeks, with PARP inhibition reducing necrotic cell death but increasing the apoptotic cell population 1 day after the ischemic insult. We speculate that PARP inhibition in the acute phase of ischemia may divert the mode of cell death from necrosis to apoptosis, which will contribute to the delayed maturation of ischemic damage, possibly through the maintenance of intracellular energy levels. Although these results are consistent with the *in vitro* data of Ha and Snyder[101] and Herceg and Wang,[102] further studies need to be undertaken. TUNEL-positive staining is suggestive of apoptotic cell death but is not specific,[105,106] and additional markers of apoptosis including DNA laddering and electron microscopy should be performed.

It is not clear why DPQ treatment appears to decrease infarct volume 1 day after focal cerebral ischemia but not at 2 weeks. The pharmacokinetics of PARP inhibition with DPQ is not known, so it may be that a single treatment 30 min after MCA occlusion was not sufficient to inhibit PARP for a sufficient length of time during reperfusion to offer long-term neuroprotection. Although most studies show that

poly(ADP-ribose) polymer is not elevated for more than a few hours after ischemia–reperfusion,[60,61] there is an indication that enzyme activity is increased as long as 1 day into reperfusion.[62]

5.2 BRAIN TRAUMA

5.2.1 Similarities between Trauma and Ischemia

Cellular dysfunction and death occurring as a result of traumatic brain injury (TBI) arise from complex biochemical and molecular interactions, and many post-TBI cascades have some overlap with those observed following an ischemic insult. A major difference between trauma and primary ischemia is the insult. A traumatic insult is caused by a physical deformation of the brain due to high acceleration of the head that causes diffuse brain movement or direct head contact with a blunt or penetrating object that causes focal brain deformation.[107] Because of the large heterogeneity among insults, the degree of damage varies greatly among patients. Early events include nonspecific membrane damage, widespread depolarization, rapid shifts in ion concentrations, calcium influx, extracellular excitatory amino acid accumulation, edema, and reduced blood flow.[108] The compromise in cerebral blood flow in the acute period following moderate to severe trauma to the brain can leave the brain susceptible to secondary ischemia,[109-113] which exacerbates energy depletion and oxidative stress. Secondary cell death is the cumulative result of several factors, including energy failure, proteolytic enzyme hyperactivity, excitotoxicity, and free radical damage.

Both experimental and clinical studies of TBI reveal an extensive pattern of cell death, which appear to involve both necrotic and apoptotic pathways.[114-121] This regionally distinct pattern of cell death occurs within hours of the insult and progresses to brain regions remote from the initial injury site over periods of days to months.[122] DNA fragmentation, detected by TUNEL, likely is characteristic of both necrotic and apoptotic cell death and has been detected in similar patterns after both TBI and cerebral ischemia,[115,118,119,123-127] but may correspond to cells that are the most severely injured or those that have already died. The identification of sublethal or prelethal events is important to develop mechanistically based therapeutic strategies. To this end, several events that lead to apoptotic cell death have been measured following TBI, including alterations in regional expression of cell death and cell survival genes[117,128] activation of caspase-3,[118,120] alterations in DNA fragmentation factor (DFF)-like proteins,[129] activation and cleavage of PARP,[130] and alterations in p53.[131] The activation of cellular repair enzymes, such as PARP, may contribute to the rescue of damaged cells, although the capacity for repair in a pathological environment may differ from that which occurs during normal cellular homeostasis.

5.2.2 PARP Activation After Trauma

Although PARP activation is indicative of DNA damage, PARP also undergoes site-specific proteolysis during apoptosis. Since both DNA fragmentation and apoptosis

are known to occur following experimental brain injury,[115,118,119,126,132-134] any potential therapeutic strategy must be considered with respect to the time course of PARP activation and the apoptotic cascade. Following lateral fluid percussion (FP) brain injury in rats, significant endogenous PARP activity has been detected 30 min after injury in the brain (to levels greater than 200% of sham injured), which subsequently returned to baseline levels (Figure 5.4).[130] The levels of PARP activity at this time point may reflect the temporal profile and type of DNA damage that occurs following TBI. Oxidative DNA damage, in which reactive oxygen species (ROS) attack DNA directly, producing single-strand breaks or indirectly causing DNA base modifications, is known to activate PARP.[28,135] ROS-mediated PARP activation may occur through various pathways, including glutamate receptor activation,[136,137] which may also account for PARP activation following TBI, since increases in extracellular excitatory amino acids have been observed acutely following TBI.[138-142] Oxidative DNA damage may also occur in the acute post-traumatic period, as with ischemic insults,[127,143] in light of the observation that hydroxyl radicals have been detected within the first 30 min of experimental brain injury in mice.[144] Furthermore, peroxynitrite ($ONOO^-$) formation is evident within the first 24 h following controlled cortical impact in mice[145] and acute inhibition of $ONOO^-$ in brain-injured mice led to improved motor recovery as early as 1 h postinjury.[146]

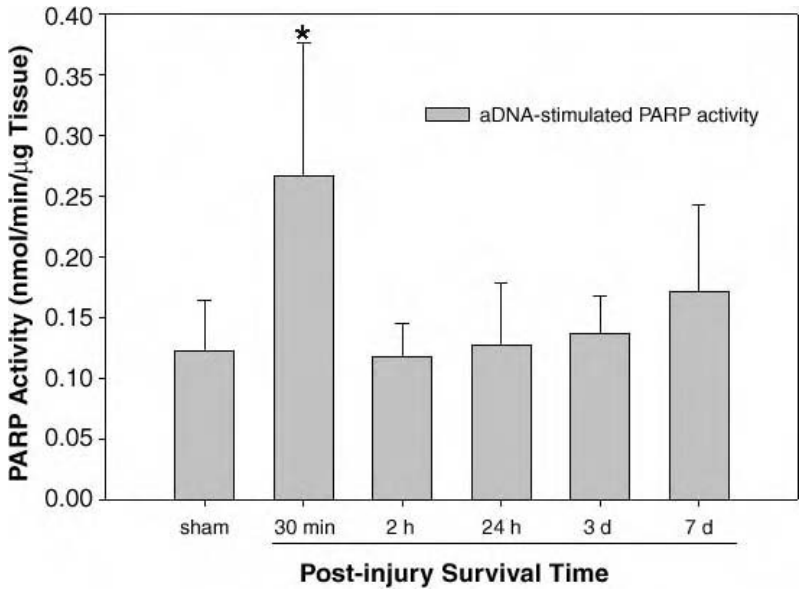

FIGURE 5.4 PARP activity in traumatically injured rat cortex. Endogenous PARP activity expressed as nmol of NAD^+/min/mg tissue for sham and injured cortex at 30 min, 2 h, 24 h, 3 days, and 7 days postinjury in the presence of activated DNA (aDNA) as a substrate. There were no differences in PARP activity for shams generated at different postsurgical times, and therefore they were combined for comparison with injury groups. Bars are means; error bars are standard deviation. * $p < 0.01$, relative to sham. (From LaPlaca, M.C. et al., *J. Neurochem.,* 73, 205, 1999. With permission.)

A different time course of PARP activity was detected when exogenous PARP was combined with genomic DNA from traumatically injured brains,[130] underscoring the importance of choice of methodology. DNA extracted 30 min after the insult did not significantly stimulate exogenous PARP, perhaps because of the requirement of other enzymes not present in the DNA preparation (e.g., damage-specific exonucleases) to induce PARP activity detected in tissue obtained at 30 min postinjury. DNA obtained at 24 h postinjury, however, produced a pronounced stimulatory effect on exogenous PARP. Interestingly, significant DNA fragmentation is not apparent until 12 h postinjury in this model and may be derived from dying or dead cells, in which normal PARP activity is impaired. Differential patterns of DNA damage and PARP activity have been observed in a cellular system of excitotoxicity, which revealed that DNA fragmentation — detected by TUNEL — occurred hours after maximum PARP activity.[147] This is in agreement with preliminary data that demonstrated a lack of TUNEL staining in tissue obtained at 2 h following lateral (FP) brain injury, despite the evidence of oxidative damage as early as 30 min.[148] It is also worthy of note that although it has been shown that PARP repairs single-strand breaks in DNA with greater affinity than double-strand breaks,[149] it is generally recognized that PARP binds (and therefore becomes active) to both single- and double-strand breaks.[150] Furthermore, increased PARP activity should be considered primarily as an indicator of DNA damage and does not necessarily indicate completed repair of DNA.

5.2.3 PARP Degradation After Trauma

Slight fragmentation of PARP has been detected in the injured cortex 3 days following lateral FP injury in rats, with significant cleavage detected 7 days postinjury.[130] Although PARP is activated in the acute posttraumatic period, subsequent PARP activation does not appear to result, possibly due to delayed apoptosis-associated proteolysis. These data suggest that this highly conserved nuclear repair enzyme is initially available for use in DNA repair after traumatic CNS injury (or at least for DNA damage detection), but that a portion of the pool of this enzyme may be subsequently subjected to proteolysis and thus become functionally impaired. The morphological and biochemical changes during the apoptotic cascade are promoted by a variety of members of the caspase family of proteases that are activated by apoptotic stimuli.[151] Activated caspase-3 is known to cleave and inactivate PARP as one of its substrate molecules.[151-153] Caspase-3 is elevated following lateral FP brain injury and may contribute to enzymatic degradation of PARP.[118,133] Caspase-3 may also promote apoptotic cell death via the cleavage of DFF45/ICAD (inhibitor of CAD [caspase-activated DNase]), which activates DFF40/CAD, a key DNA-cleavage enzyme responsible for DNA fragmentation during apoptosis by events unrelated to PARP cleavage.[154-156] Recent studies have demonstrated that the cleavage of DFF45/ICAD and activation of DFF40/CAD occur following traumatic spinal cord injury[157] and lateral fluid percussion brain injury.[129]

5.2.4 Neuroprotection with PARP Inhibition

5.2.4.1 Pharmacological Studies

The inhibition of PARP using pre- and postinjury GPI 6150 administration has been shown to be neuroprotective as documented by a modest, but significant reduction in lesion area 24 h after lateral fluid percussion brain injury in rats of moderate severity as compared with vehicle-treated injured rats ($p < 0.05$) (Figure 5.5).[158] This protective effect was limited to the reduction in cell death in both the cortical lesion site (site of maximal injury) and penumbral areas (e.g., subcortical white matter). GPI 6150-treatment did not significantly affect the number or extent of TUNEL-labeled cells 24 h following injury, a time when TUNEL-positive cells are maximal in the cortex in the lateral fluid percussion model of brain injury.[119] In addition, when morphological criteria for apoptosis were applied to TUNEL-positive cells, no differences in numbers or distribution of apoptotic cells were detected between GPI 6150- and vehicle-treated brains. Given the role of PARP in DNA repair, it cannot be ruled out that while acute inhibition is protective, subsequent activation of PARP in the chronic postinjury period may be beneficial to long-term or remote cellular repair.[159] The observed neuroprotective effects on lesion size, however, warrants further evaluation of PARP inhibition as a means to reduce cellular damage associated with TBI. These studies should include an examination of behavioral function and potential recovery of both motor and cognitive deficits.

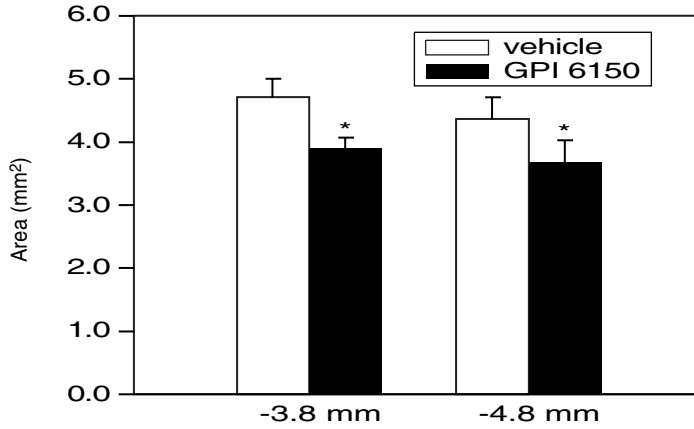

FIGURE 5.5 Acute GPI 6150 treatment reduced lesion size following TBI. Lesion area for vehicle-treated vs. GPI 6150-treated animals in the lesion area (cortical lesion + extended subcortical white matter damage) at –3.8 and -4.8 mm from Bregma, presented as mean ± standard deviation. * Represents groups that are significantly different from vehicle-treated groups, $p < 0.05$. (Figure from LaPlaca, M.C. et al., *J. Neurotrauma,* 18, 369, 2001. With permission.)

5.2.4.2 PARP Null Mice

PARP-deficient mice ($PARP^{-/-}$) have been subjected to experimental TBI to further elucidate the role of PARP in both cell repair and energy conservation following traumatic CNS injury. As stated above, injury paradigms using genetically deleted mice are complementary to pharmacological studies and provide complete inhibition that is not subject to a lack in specificity. Controlled cortical inpact (CCI) brain injury in $PARP^{-/-}$ mice resulted in significant improvement in neurobehavior (motor at 1 day and memory tasks at 14 to 20 days postinjury) vs. $PARP^{+/+}$ mice.[145] In contrast to the reduction in lesion size seen following pharmacologic PARP inhibition in the injured rat brain,[158] Whalen et al. found no difference in contusion volume between $PARP^{-/-}$ (17.57 ± 1.57 mm^3) and $PARP^{+/+}$ (21.01 ± 3.16 mm^3) mice after CCI brain injury at 21 days postinjury.[145] Similarly, preliminary studies with $PARP^{-/-}$ (n = 6), $PARP^{+/-}$ (n = 5), and $PARP^{+/+}$ (n = 6) mice utilizing a weight drop injury method (exposed dura; m = 120 g, h = 40 cm) demonstrated no differences in cortical contusion volume ($PARP^{-/-}$, 5.81 ± 1.22 mm^3; $PARP^{+/-}$, 5.43 ± 2.46 mm^3; $PARP^{+/+}$, 5.22 ± 1.10 mm^3) or number of TUNEL-positive cells in $PARP^{-/-}$ mice vs. WT mice at 1 week postinjury (C. Zhang, unpublished observations). The failure of chronic inhibition of PARP (i.e., genetic deletion) to show a protective effect at the neuroanatomical level suggests that the role of PARP is complex and justifies further study examining pharmacological inhibition windows and dosing schedule. PARP may initially impair cell function by expending energy, but may play a reparative role at later time points.[159] Nitrotyrosine, a marker for $OONO^-$ damage to proteins, has been observed in the damaged cortex following cortical contusion injury in both $PARP^{+/+}$ and $PARP^{-/-}$ mice 24 h following CCI brain injury and may correspond to PARP activation in response to $OONO^-$-damaged DNA.[145] This is consistent with the observation of early PARP activation following TBI in rats prior to widespread DNA damage.[130]

One of the hallmarks of moderate to severe TBI in the mouse is selective neuronal cell loss in the CA_2 and CA_3 subfields of the ipsilateral hippocampus.[160,161] Interestingly, the extent of damage to hippocampal CA_2/CA_3 and dentate granule cell regions has been shown to be greater in brains of injured $PARP^{-/-}$ mice compared with wild-type mice ($p < 0.05$), suggesting a divergent role of PARP in the pathogenesis of hippocampal vulnerability following brain trauma (C. Zhang, unpublished observations). Contrary to the "suicide hypothesis," PARP has been shown to play an essential and positive role during recovery from DNA damage[162] and may assume different roles depending on the type and degree of damage. Reduced blood flow and excitotoxicity may contribute to the secondary injury in hippocampal cells, which are more susceptible to cytotoxic effects from excitatory amino acids and NO.[163] Moreover, blood–brain barrier breakdown was markedly elevated in $PARP^{-/-}$ mice compared to wild-type mice (C. Zhang, unpublished observations), possibly leading to enhanced NO exposure, to which the hippocampus is selectively vulnerable.[163] These results in a traumatic injury model are consistent with the protective effect of PARP activation on hippocampal cell survival following transient global ischemia in normal rats,[159] although there are clear differences between trauma and cerebral ischemia.

The data resulting from the studies of trauma in two different models of experimental TBI suggest that the genetic deletion of PARP may have regionally divergent effects on trauma-induced cell death following experimental brain injury in transgenic mice. The deletion of PARP may be neuroprotective in terms of neurobehavior, but may be detrimental to cell survival in the hippocampus. The observation of improvement in memory function seen in the cortical contusion model at 20 days postinjury, however, is inconsistent with the increased cell death seen in the CA_2/CA_3 region of the hippocampus 1 week after the weight-drop paradigm, although this study did not evaluate behavioral outcome. Although PARP transcripts and PARP activity were undetectable in brain and other tissues of $PARP^{-/-}$ mice,[164] it has been reported that cells derived from $PARP^{-/-}$ animals form ADP-ribose polymers, indicating the presence of enzymatic activity capable of catalyzing ADP-ribose polymer synthesis.[165] Therefore, it cannot be ruled out that ADP-ribose polymers are formed via PARP-independent mechanisms and consuming NAD^+ in regions vulnerable to secondary damage (e.g., hippocampus).

5.3 CONCLUSIONS

Although the involvement of PARP has been implicated in different cellular responses to genomic damage, including cell survival, DNA repair, transformation, and cell death, the exact contribution of PARP to the pattern of cell damage and death observed following ischemia and traumatic injury to the CNS remains unclear. If PARP activation and NAD^+ depletion contribute to cell death, the absence or inhibition of PARP may prevent, in part, the cell death associated with apoptotic cascade and energy deficits. Conversely, if PARP is a passive substrate for the caspases, then an absence of PARP may have no influence on apoptosis, because caspases would continue to act on other pre-apoptotic substrates. The evidence for energy conservation (via NAD^+ conservation) with PARP inhibition is strong for ischemic insults to the brain,[59,67,68,166-168] but requires further investigation in association with TBI. PARP inhibition following TBI in the acute period (<2 h postinjury in the rodent), when PARP activity is elevated, may be neuroprotective. Chronic inhibition of PARP as a neuroprotective strategy results in behavioral recovery, although it may differentially affect brain subregions. The differences in TBI models (as evidenced by difference in injury-induced lesion volumes in the PARP null mouse studies) need to be considered, as well as a detailed time course of energy utilization and DNA damage in the traumatically injured brain. Collectively, these results warrant further study of the effects of PARP inhibition on different brain regions with respect to time and injury severity. In addition, behavioral assessment is important to further characterize in both PARP null and pharmacologically manipulated normal mice in models of ischemia and trauma.

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6 PARP Inhibitors and Cardioprotection

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CONTENTS

6.1 INTRODUCTION

Poly(adenosine 5′-diphosphate ribose) polymerase (PARP), also known as poly(ADP-ribose) synthetase (PARS; EC 2.4.2.30), is a chromatin-bound enzyme, which is abundantly present in the nuclei of numerous cell types. Single-strand breaks in DNA trigger the activation of PARP, which transfers ADP-ribose moieties from NAD^+ to various nuclear proteins including histones and PARP (automodification domain) itself. This reaction leads to the generation of nicotinamide, which is an inhibitor (negative feedback) of PARP activity. Continuous or excessive activation of PARP produces extended chains of ADP-ribose on nuclear proteins and results in a substantial depletion of intracellular NAD^+. As NADH functions as an electron carrier in the mitochondrial respiratory chain, NAD^+ depletion rapidly leads to a fall in intracellular ATP levels. Moreover, nicotinamide can be recycled to NAD^+

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in a reaction that consumes ATP. Thus, excessive activation of PARP leads to a fall in ATP (by two different mechanisms), which may ultimately cause cell death.[1,2] Oxygen-derived radicals including superoxide anions, hydrogen peroxide, or hydroxyl radicals cause strand breaks in DNA, activation of PARP and depletion of NAD^+ and ATP in cultured cells. Peroxynitrite, which is generated when equimolar amounts of nitric oxide (NO) react with superoxide anions, also causes strand breaks in DNA, activation of PARP, and ultimately cell death.[3] In 1997, we discovered that various, chemically distinct inhibitors of PARP reduce the degree of tissue injury caused by ischemia–reperfusion of the heart and skeletal muscle.[4] This chapter reviews the role of PARP in the pathophysiology of ischemia-reperfusion injury of the heart and focuses on the cardioprotective effects of inhibitors of PARP activity in conditions associated with myocardial ischemia and reperfusion.

6.2 DISCOVERY OF THE CARDIOPROTECTIVE EFFECTS OF PARP INHIBITORS

In January 1997, we reported that the administration, prior to reperfusion, of several chemically distinct inhibitors of PARP activity reduces the infarct size caused by regional myocardial ischemia and reperfusion in the anesthetized rabbit.[4] For example, administration of the PARP inhibitor 3-aminobenzamide (3-AB, 10 mg/kg i.a.) 1 min prior to the occlusion (45 min) and before reperfusion (2 h) of the first anterolateral branch of the left coronary artery (LAL) caused a 43% reduction in infarct size, without affecting hemodynamic parameters (Figure 6.1). Single injections of 3-AB either before occlusion or reperfusion caused similar reductions in infarct size (32 or 42%). Thus, activation of PARP during reperfusion contributes to the extension of infarct size in the rabbit. Although 3-AB is a selective inhibitor of PARP activity, it may be argued that the cardioprotection afforded by 3-AB is due to nonspecific effects. This is, however, unlikely, for 3-aminobenzoic acid (3-ABA; 10 mg/kg i.a. at 1 min prior to reperfusion), an analogue of 3-AB, which does not inhibit PARP activity (negative control), did not cause a reduction in infarct size. Moreover, 3-AB prevents the fall in NAD^+ and cell death caused by oxygen-derived free radicals or NO in murine islet cells (wild-type), but not in mutant cells, in which the PARP gene ("knockout") had been inactivated. Our hypothesis that the activation of PARP contributes to injury measured after reperfusion of ischemic tissue was further supported by our findings that nicotinamide (negative-feedback, endogenous PARP inhibitor) and three other chemically distinct inhibitors of PARP activity also reduced infarct size, but did not cause hemodynamic effects. For instance, administration (prior to reperfusion) of nicotinamide (20 mg/kg i.a.) caused a 50% reduction of infarct size (see Figure 6.1), while the PARP inhibitors 4-aminobenzamide (10 mg/kg i.a.), 4-amino-1,8-naphthalimide (1 mg/kg i.a.), or 1,5-dihydroxyisoquinoline (1 mg/kg i.a.) reduced infarct size by 38 to 48% (see Figure 6.1). As nicotinamide enhances the intracellular levels of NAD^+, the cardioprotective effects of nicotinamide may be secondary to an increase in the levels of NAD^+ and, subsequently, ATP. This is, however, unlikely, for nicotinic acid also enhances the intracellular levels of NAD^+, but without forming nicotinamide as an intermediate. Nicotinic acid, however, did not reduce myocardial

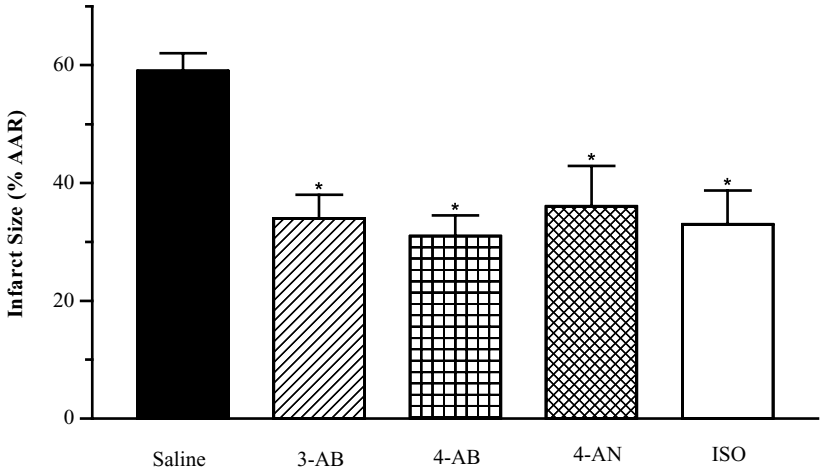

FIGURE 6.1 New Zealand white rabbits were subjected to occlusion (for 45 min) and reperfusion (for 2 h) of the left-anterior lateral coronary artery. Infarct size was determined by staining the tissue with nitroblue tetrazolium and was expressed as percent of the area at risk (AAR). Different groups of animals were treated with saline or the PARP-inhibitors 3-AB, (10 mg/kg), 4-aminobenzamide (4-AB, 10 mg/kg), 4-amino-1,8-naphthalimide (4-AN, 1 mg/kg), or 1,5-dihydroxyisoquinoline (ISO 1 mg/kg) given at 1 min prior to the onset of reperfusion. *$P < 0.05$ when compared to control.

infarct size (when given at the same dose as nicotinamide) (see Figure 6.1). As nicotinamide, but not nicotinic acid,[5] inhibits the activity of PARP, these results support our hypothesis that the cardioprotective effects of nicotinamide are, indeed, due to inhibition of PARP activity and not due to an augmentation by nicotinamide of the intracellular levels of NAD^+.

To elucidate whether inhibition of PARP activity also attenuates the myocardial contractile dysfunction caused by ischemia–reperfusion, we investigated the effects of 3-AB on the alterations in cardiac function caused by global myocardial ischemia and reperfusion in the isolated heart of the rabbit. Global ischemia (30 min) and reperfusion (2 h) resulted in an impairment (of 60%) in left ventricular developed pressure and a substantial rise in left ventricular end-diastolic pressure. Reperfusion of the heart with buffer containing 3-AB (100 μM for the first 60 min of the reperfusion period) largely attenuated this contractile dysfunction (Figure 6.2) and also reduced infarct size (Figure 6.3). Thus, (1) 3-AB interferes with a process leading to the death of cardiomyocytes during reperfusion, and (2) the mechanism of its cardioprotection is independent of alterations in myocardial blood flow or of the inhibition of the function of platelets or neutrophils.

In our first study, we have also investigated whether inhibition of PARP activity reduces the skeletal muscle necrosis caused by ischemia–reperfusion in the rabbit hind limb. Occlusion of the aorta (4 h) and reperfusion (3 h) resulted in an infarct size

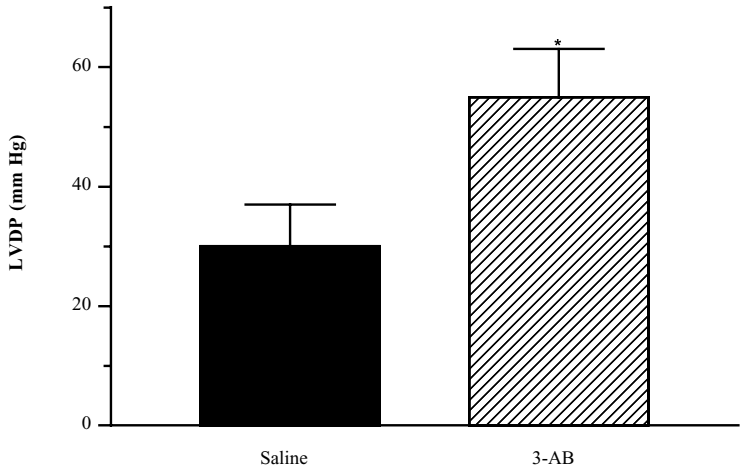

FIGURE 6.2 Hearts of New Zealand white rabbits were perfused *ex vivo* (Langendorff technique) and subjected to 30 min of global myocardial ischemia followed by 2 h of reperfusion. This resulted in a substantial impairment in left ventricular function as measured by a decline in left ventricular developed pressure (LVDP). Different groups of hearts were treated with saline or the PARP-inhibitors 3-AB (100 μ*M*). *P < 0.05 when compared to control.

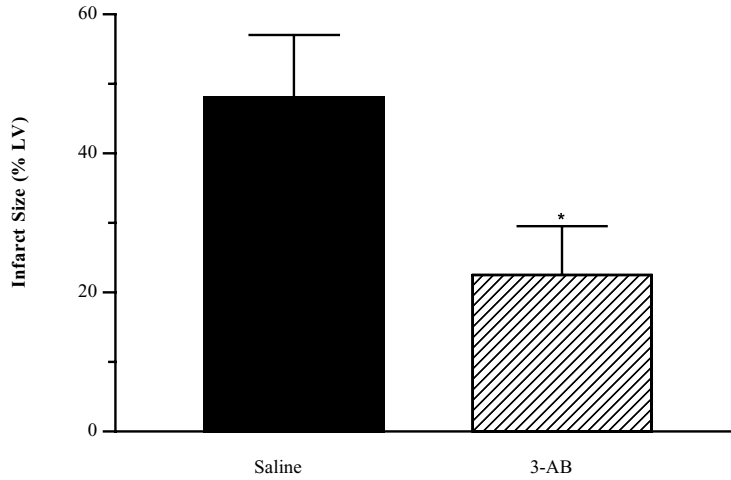

FIGURE 6.3 Hearts of New Zealand white rabbits were perfused *ex vivo* (Langendorff technique) and subjected to 30 min of global myocardial ischemia followed by 2 h of reperfusion. This resulted in a substantial infarct size (determined as percent of the left ventricle, LV). Different groups of hearts were treated with saline or the PARP inhibitor 3-AB (100 μ*M*). *P < 0.05 when compared to control.

of 52% of the gracilis muscle. Intravenous injection (1 min) prior to reperfusion of the PARP inhibitor 3-AB (10 mg/kg), but not of its inactive analogue 3-ABA (10 mg/kg), caused a significant reduction in the degree of skeletal muscle necrosis caused by ischemia-reperfusion of the hind limb. Similarly, nicotinamide, but not nicotinic acid (both at 20 mg/kg), reduced skeletal muscle necrosis when given 1 min prior to reperfusion. In addition, benzamide (1 mg/kg), 4-amino-1,8-naphthalimide (1 mg/kg), or 1,5-dihydroxyisoquinoline (1 mg/kg), which are potent inhibitors of PARP activity,[5] resulted in a 40 to 50% reduction in infarct size in skeletal muscle. Thus, activation of PARP during reperfusion not only contributes to the death of cardiomyocytes, but also to the death of skeletal muscle myocytes challenged with ischemia–reperfusion.

In January 1997, we therefore proposed that the DNA damage caused by reactive radicals during the reperfusion of previously ischemic myocardium or skeletal muscle leads to the excessive activation of the DNA-repair enzyme PARP, which in turn causes cell death.[4] PARP is essential for DNA repair and, hence, contributes to the efficient maintenance of genome integrity, when cells are challenged by genotoxic agents. Thus, a moderate activation of PARP protects genome integrity, whereas its excessive activation (e.g., during reperfusion of ischemic tissue) may lead to cell death. Although chronic inhibition of the activity of PARP may well lead to side effects, we proposed that a transient inhibition of PARP activity is a novel approach for the therapy of ischemia-reperfusion injury of the heart or skeletal muscle (and potentially other organs/tissues).[4] When compared to other drugs, which reduce infarct size, PARP inhibitors may offer the advantages that they are able to salvage previously ischemic tissue even when administered with the onset of reperfusion without causing any hemodynamic effects.[4]

In an independent investigation published in November 1997, Zingarelli and colleagues[6] also reported that the PARP inhibitor 3-AB reduces the myocardial injury caused by regional myocardial ischemia and reperfusion in the anesthetized rat. In this study, occlusion of the left main coronary artery (1 h) followed by reperfusion (1 h) in the anesthetized rat caused severe cardiac necrosis, neutrophil infiltration, and an increase in plasma creatine phosphokinase activity. Myocardial ischemia and reperfusion was also associated with significant peroxynitrite formation (measured as increase in nitrotyrosine staining). Administration of the PARP inhibitor 3-AB (10 mg/kg IV) reduced myocardial infarct size and blunted the increase in plasma creatine phosphokinase activity and myeloperoxidase activity in the ischemic-reperfused hearts. Taken together, the above studies from our own laboratory[4] as well as the study by Zingarelli and colleagues[6] provide the first evidence that the activation of PARP plays a pivotal role in the pathophysiology of ischemia–reperfusion injury of the heart.

6.3 PARP INHIBITORS REDUCE MYOCARDIAL INFARCT SIZE IN A LARGE ANIMALS

Although there is good evidence that inhibitors of PARP activity reduce the degree of myocardial tissue injury caused by regional myocardial ischemia and reperfusion in rodents, there is limited information regarding the efficacy of these compounds in

larger animals. In 1998, we investigated the effects of the PARP inhibitor 3-AB on the infarct size and the contractile dysfunction caused by regional myocardial ischemia and reperfusion in the anesthetized pig.[7] Of the 24 pigs that underwent LAD occlusion, 8 died during the course of the experiment (5 controls, 1 treated with 3-AB and 2 treated with 3-ABA) due to ventricular fibrillation. The data obtained from these animals were excluded from data analysis. In the animals that survived the study, the area at risk ranged from 39 to 43% of the left ventricle (including the septum) and was not different between any of the experimental groups studied. In control pigs, LAD occlusion (60 min) and reperfusion (3 h) resulted in an infarct size of 66 ± 3% of the area at risk. Although the areas at risk (nonperfused myocardium, which potentially can undergo necrosis) were similar in control and treatment group (Figure 6.4), injection upon reperfusion of the PARP inhibitor 3-AB (10 mg/kg) caused a significant (~30%) reduction in infarct size (Figure 6.5). In contrast, the inactive structural analogue of this PARP inhibitor, 3-ABA, did not have any effect on infarct size (when compared to control). In these experiments, the mean baseline data for myocardial contractility (dP/dt_{max}) (control: 1750 ± 136 mmHg/s, 3-AB: 1383 ± 164 mmHg/s, 3-ABA: 1688 ± 171 mmHg/s) were not significantly different between groups. Coronary artery occlusion resulted in a 16 to 20% fall in dP/dt_{max}. There was no difference in dP/dt_{max} prior to the administration of vehicle or drugs between any of the groups studied. In vehicle-treated, control animals, reperfusion was associated with a progressive decline in left ventricular contractility. Administration of the PARP inhibitor 3-AB attenuated this fall in dP/dt_{max}. In contrast, the inactive analogue of this PARP inhibitor, 3-ABA, was without effect on the fall in dP/dt_{max}, which occurred during the reperfusion period. These studies provide convincing evidence that the administration prior to reperfusion of an inhibitor of PARP activity will also reduce the tissue injury (infarct size) as well as the impairment in contractile function caused by regional myocardial ischemia and reperfusion in the anesthetized pig.

6.4 MECHANISM OF THE CARDIOPROTECTIVE EFFECT OF PARP INHIBITORS *IN VIVO*

6.4.1 Preservation of High-Energy Phosphates

To investigate the effects of the inhibition of PARP activity on the tissue levels of high-energy phosphates, isolated rat hearts were perfused in the Langendorff mode and subjected to 23 min total global ischemia and reperfused for 60 min.[8] Left ventricular function was assessed by means of an intraventricular balloon. High-energy phosphates were measured by [^{31}P]-nuclear magnetic resonance (NMR) spectroscopy. Intracellular levels of NAD^+ were measured by capillary electrophoresis of perchloric acid extracts of hearts at the end of reperfusion. Reperfusion in the presence of the PARP inhibitor 1,5-dihydroxyisoquinoline (ISO, 100 μ*M*) attenuated the mechanical dysfunction observed following 1 h of reperfusion; 27 ± 13 and 65 ± 8% recovery of preischemic rate pressure product for control and 100 μ*M* ISO, respectively. This cardioprotection was accompanied by a preservation of intracellular high-energy phosphates during reperfusion; 38 ± 2 vs. 58 ± 4% ($P < 0.05$) of

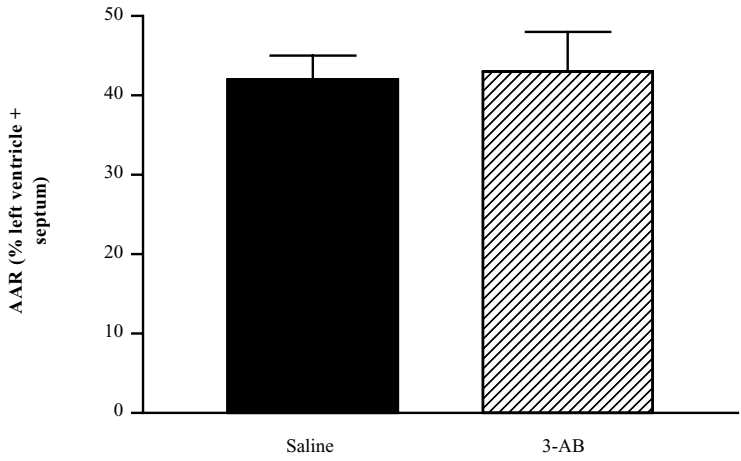

FIGURE 6.4 Mature pigs were anesthetized and subjected to occlusion (for 1 h) and reperfusion (for 2 h) of the left-anterior descending coronary artery. The AAR was determined after injection of Evans blue dye. Different groups of animals were treated with saline or the PARP inhibitor 3-AB (10 mg/kg) given at 1 min prior to the onset of reperfusion.

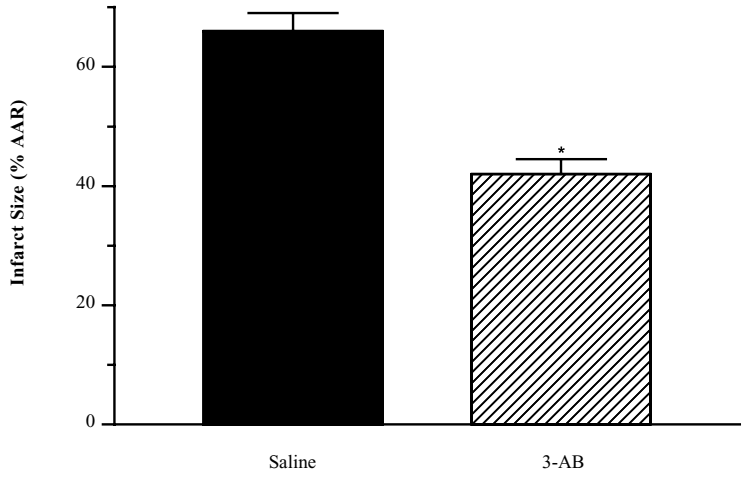

FIGURE 6.5 Mature pigs were anesthetized and subjected to occlusion (for 1 h) and reperfusion (for 2 h) of the left-anterior descending coronary artery. Infarct size was determined by staining of the tissue with nitroblue tetrazolium and expressed as percent of the AAR. Different groups of animals were treated with saline or the PARP inhibitor 3-AB (10 mg/kg) given at 1 min prior to the onset of reperfusion. *$P < 0.05$ when compared to control.

preischemic levels of phosphocreatine (PCr) for control and 100 μ*M* ISO, respectively, and 23 ± 1 vs. 31 ± 3% of preischemic levels of ATP for control and 100 μ*M* ISO, respectively. Cellular levels of NAD^+ were higher in ISO-treated hearts at the end of reperfusion; 2.56 ± 0.45 μ*M*/g vs. 4.76 ± 1.12 μ*M*/g dry weight ($P < 0.05$) for control and ISO treated. Most notably, administration of isoproterenol to hearts that had been subjected to global ischemia and treated with ISO (100 μ*M*), the rate-pressure product as well as +*dP/dt* and –*dP/dt* returned to values that were not significantly different from those measured prior to the onset of myocardial ischemia.

It is likely that the beneficial effect of ISO on cardiac energetics contributes to the observed attenuation of contractile dysfunction following reperfusion of the ischemic myocardium. There remains some controversy regarding the correlation between the recovery of function and intracellular ATP levels following myocardial ischemia-reperfusion. Thus, the studies of Neely and Grotyohann[9] suggested that there is no relation between intracellular ATP levels and functional recovery following ischemia–reperfusion. On the other hand, studies by Rovetto[10] suggested that there is indeed a correlation between heart ATP content and work during reperfusion. In the present study we observed a correlation between cardiac energetics at the end of reperfusion and functional recovery. This correlation, although significant, was quite modest and this appears reasonable given the multifactorial nature of ischemia–reperfusion injury. A stronger correlation between the recovery of energetics and function would be observed only if energetic status were the only determinant of the degree of ischemia–reperfusion injury. The correlation between recovery of energetics and function lends support to the notion that the cardioprotective effects of PARP inhibition are, at least in part, due to preservation of energetic status. Nevertheless, this study demonstrates that the cardioprotection afforded by inhibition of PARP activity with ISO is accompanied by a preservation of high-energy phosphates and cellular NAD^+ levels and suggests that the mechanism responsible for this cardioprotection may involve prevention of intracellular ATP depletion.

Studying cardiac energy metabolism in the Langendorff heart perfusion system by [^{31}P]-(NMR), Halmosi and colleagues[11] recently reported that the PARP inhibitors 3-AB, nicotinamide, *O*-(3-piperidino-2-hydroxy-1-propyl) pyridine-3-carboxylic acid amidoxime dihydrochloride (BGP-15), and 4-hydroxyquinazoline improved the recovery of high-energy phosphates (ATP, creatine phosphate) and accelerated the reutilization of inorganic phosphate formed during the ischemic period, showing that PARP inhibitors facilitate the faster and more complete recovery of the energy production. Furthermore, these PARP inhibitors significantly decreased the ischemia–reperfusion-induced increase of lipid peroxidation, protein oxidation, single-strand DNA breaks, and the inactivation of respiratory complexes, which indicates a decreased mitochondrial reactive oxygen species (ROS) production in the reperfusion period. In addition, these PARP inhibitors used, but not 3-ABA (negative control), prevented the hydrogen peroxide-induced inactivation of cytochrome oxidase in isolated heart mitochondria, suggesting the presence of an additional mitochondrial target for PARP inhibitors. Therefore, PARP inhibitors, in addition to their important primary effect of decreasing the activity of nuclear PARP and decreasing

NAD^+ and ATP consumption, reduce ischemia–reperfusion-induced endogenous ROS production and protect the respiratory complexes from ROS-induced inactivation, providing an additional mechanism by which they can protect heart from oxidative damage.[11]

6.4.2 Protection of Cardiac Myocytes

Peroxynitrite and hydroxyl radical are reactive oxidants produced during myocardial reperfusion injury. In various cell types, including macrophages and smooth muscle cells, peroxynitrite and hydrogen peroxide cause DNA single-strand breakage, which triggers the activation of PARP resulting in cytotoxicity. In September 1997, Gilad and colleagues[12] provided the first evidence that inhibitors of PARP-activity protect cardiac myoblasts against oxidative stress.[12] Their study provides evidence that peroxynitrite (100 to 1000 μ*M*), hydrogen peroxide (0.3 to 10 μ*M*), and the NO donors *S*-nitroso-*N*-acetyl-DL-penicillamine (SNAP) and diethyltriamine NONOate all caused a dose-dependent reduction of the mitochondrial respiration of the cells, as measured by the mitochondrial-dependent conversion of MTT to formazan. Peroxynitrite and hydrogen peroxide, but not the NO donors, caused activation of cellular PARP activity. The suppression of mitochondrial respiration by peroxynitrite and hydrogen peroxide, but not by the NO donors, was ameliorated by pharmacological inhibition of PARP with 3-AB or nicotinamide. The protection by these PARP inhibitors diminished at extremely high concentrations of the oxidants. Hypoxia (1 h) followed by reoxygenation (1 to 24 h) also resulted in a significant activation of PARP, and caused a suppression of mitochondrial respiration, which was prevented by inhibition of PARP. Similar to the results obtained with the pharmacological inhibitors of PARP, a fibroblast cell line, which derives from the PARP knockout mouse, was protected against the suppression of mitochondrial respiration in response to peroxynitrite and reoxygenation, but not to NO donors, when compared with the result of cells derived from wild-type animals. Thus, the authors suggested that the activation of PARP plays a role in the cell death caused by oxidant stress in isolated cardiac myocytes.[12]

We have also investigated the role of PARP in the hydrogen peroxide–mediated cell injury/necrosis in rat cardiac myoblasts. Rat cardiac myoblasts (H9c2 cells) were preincubated with the PARP inhibitors 3-AB, nicotinamide, or ISO or the inactive analogues 3-ABA or nicotinic acid (NicA) prior to exposure with hydrogen peroxide (1 μ*M*). Cell injury was assessed by measuring mitochondrial respiration and cell necrosis by measuring the release of lacate dehydrogenase (LDH). PARP activity was determined by measuring the incorporation of NAD^+ into nuclear proteins. Exposure of rat cardiac myoblasts to hydrogen peroxide caused an increase in PARP activity and cell injury/necrosis, which was attenuated by pretreatment with the PARP inhibitors. These findings demonstrate that the inhibition of the activity of PARP (Figure 6.6) attenuates the cell death (Figure 6.7) associated with oxidant stress in rat cardiac myoblasts *in vitro*.[13]

In a subsequent study, we investigated the role of PARP in the cell death of human cardiac myoblasts caused by hydrogen peroxide.[14] Exposure of human cardiac

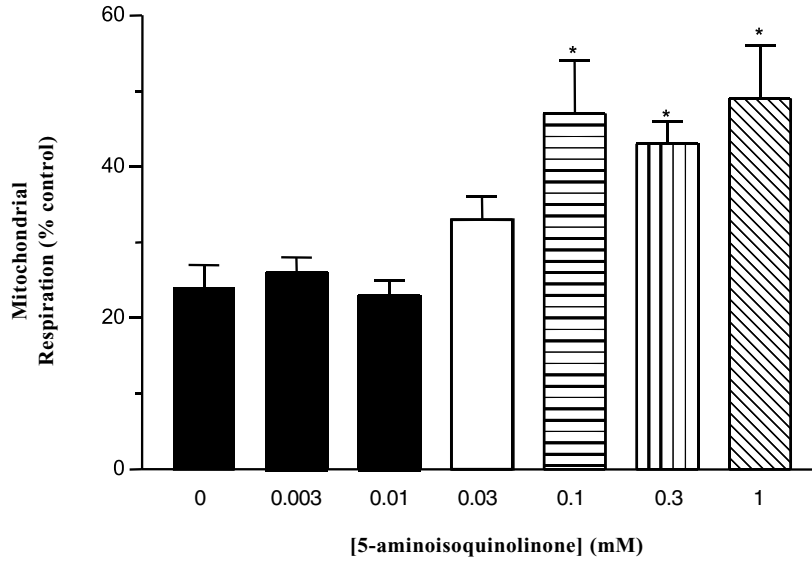

FIGURE 6.6 Impairment in mitochondrial respiration (expressed as percent of control) in human cardiac myoblasts challenged with hydrogen peroxide (1 μ*M* for 4 h). Note that increasing concentrations of the novel PARP inhibitor 5-AIQ caused a concentration-dependent restoration of mitochondrial respiration. **P* < 0.05 when compared to control.

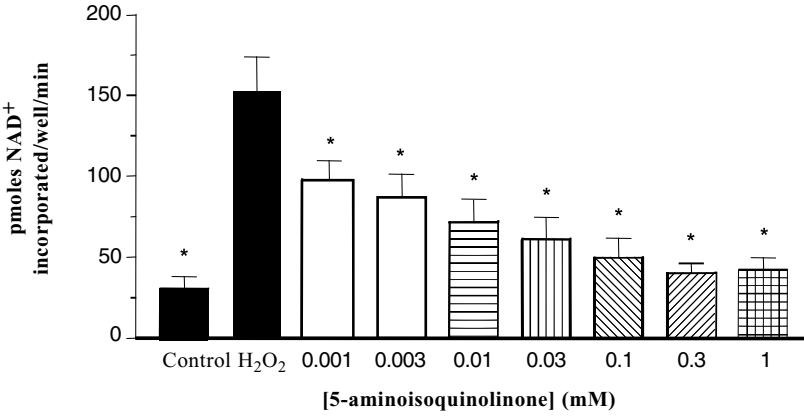

FIGURE 6.7 Increase in PARP activity in human cardiac myoblasts challenged with hydrogen peroxide (1 μ*M* for 4 h). Note that increasing concentrations of the novel PARP inhibitor 5-AIQ caused a concentration-dependent inhibition of PARP activity in this human cell line. **P* < 0.05 when compared to control.

myoblasts to hydrogen peroxide caused a time- and concentration-dependent reduction in mitochondrial respiration (cell injury), an increase in cell death (LDH release), as well as an increase in PARP activity. The PARP inhibitors 3-AB (3 m*M*), ISO (300 μ*M*), or nicotinamide (3 m*M*) attenuated the cell injury and death as well as the increase in PARP activity caused by hydrogen peroxide (3 m*M*; 4 h for cell injury/death, 60 min for PARP activity) in human cardiac myoblasts. In contrast, the inactive analogues 3-ABA (3 m*M*) or nictinic acid (3 m*M*) were without effect. The iron chelator deferoxamine (1 to 10 m*M*) caused a concentration-dependent reduction in the cell injury and death caused by hydrogen peroxide in these human cardiac myoblasts. Thus, the cell injury/death caused by hydrogen peroxide in human cardiac myoblasts is secondary to the formation of hydroxyl radicals and is due to an increase in PARP activity. Taken together, these studies provide very strong evidence that the activation of PARP plays a pivotal role in the pathophysiology of the cell death caused by reactive oxygen and nitrogen species in cardiac myocytes.[14]

6.4.3 Anti-Inflammatory Effects

In 1998, Zingarelli and colleagues[15] reported that the genetic disruption of PARP provides protection against myocardial ischemia and reperfusion injury by inhibiting the expression of *P*-selectin and intercellular adhesion molecule-1 (ICAM-1) and, consequently, by inhibiting the recruitment of neutrophils into the jeopardized tissue.[15] Furthermore, using *in vitro* studies, these authors showed that in fibroblasts lacking a functional gene for PARP, the cytokine-stimulated expression of ICAM-1 is significantly reduced compared with fibroblasts from animals with a normal genotype. Similarly, in cultured human endothelial cells, the oxidative- or cytokine-dependent expression of *P*-selectin and ICAM-1 is reduced by pharmacological inhibition of PARP by 3-AB. These findings provided the first direct evidence that PARP activation participates in neutrophil-mediated myocardial damage by regulating the expression of *P*-selectin and ICAM-1 in ischemic and reperfused myocardium.[15] In a subsequent study, Yang and colleagues[16] investigated whether inactivation of PARP influences the delayed myocardial necrosis and the production of the proinflammatory cytokine tumor necrosis factor-α, the anti-inflammatory cytokine interleukin-10, and the free radical NO in the late stage of myocardial reperfusion injury. They found that genetic disruption of PARP provides marked protection against the delayed myocardial ischemia and reperfusion injury. In addition, in the absence of functional PARP protein, the formation of interleukin-10, tumor necrosis factor-α, and NO was reduced.[16]

6.4.4 Effects in PARP-Deficient Mice

Recent studies utilizing PARP knockout mice have lent support to the notion that activation of PARP at reperfusion contributes to myocardial reperfusion injury. Studies on ischemia–reperfusion injury *in vivo* in PARP knockout mice suggested that this cardioprotection may be — in part — due to a decreased recruitment of neutrophils secondary to an inhibition of the expression of P-selectin and ICAM-1.[15] It should be

noted that the latter study does not rule out additional direct effects of the PARP inhibitors used on the myocardium. Indeed, isolated, buffer-perfused hearts from PARP knockout mice were similarly protected against the injury/dysfunction caused by total global ischemia.[17] In this preliminary report, the improved functional recovery was accompanied by a preservation of cardiac energetics.[17]

In a recent study, Piper and colleagues[18] investigated the activation of PARP in hearts subjected to ischemia and reperfusion by measuring the conversion of [^{33}P]NAD^+ into poly(ADP-ribose) (PAR) and also by immunohistochemical staining for PAR.[18] Cardiac contractility, nitric oxide, ROS, NAD^+, and ATP levels were examined in wild-type and in PARP-1 gene-deleted (PARP-$1^{-/-}$) isolated, perfused mouse hearts. In this study, ischemia-reperfusion augmented the formation of NO, ROS, and PARP activity. The decrease in cardiac contractility and NAD^+ levels caused by ischemia and reperfusion was less pronounced in hearts of PARP-$1^{-/-}$ mice. Residual poly(ADP-ribosyl)ation in PARP-$1^{-/-}$ hearts may reflect alternative forms of PARP. These findings support the view that the poly(ADP-ribosyl)ation caused by PARP-1 or other sources of enzymatic PAR synthesis is associated with cardiac damage following myocardial ischemia.[18]

Grupp and colleagues[19] have investigated whether the absence of PARP protein alters the functional response to hypoxia–reoxygenation.[19] Isolated work-performing mouse hearts were stabilized under the same loading condition (cardiac minute work of 250 mmHg × ml/min, an afterload of 50 mmHg aortic pressure, and similar venous return of 5 ml/min, resulting in the same preload). After 30 min equilibration the hearts were subjected to 30 min hypoxia followed by 30 min of reoxygenation. At the end of the reoxygenation, in hearts from wild-type animals, there was a significant suppression in the rate of intraventricular pressure development (+*dP/dt*) from 3523 to 2907 mmHg. There was also a significant suppression in the rate of relaxation (-*dP/dt*) in the wild-type hearts from 3123 to 2168 mmHg. The time to peak pressure (TPP) increased from 0.48 to 0.59 ms/mmHg and the half-time of relaxation (RT1/2) increased from 0.59 to 0.74 ms/mmHg. In contrast, in the hearts from the PARP knockout animals, no significant suppression of +*dP/dt* (from 3654 to 3419 mmHg), and no significant increase in the TPP (from 0.462 to 0.448 ms/mmHg) were found, and the decrease in -*dP/dt* was partially ameliorated (from 3399 to 2687 mmHg) as well as the half-time of relaxation (from 0.507 to 0.55 ms/mmHg) when compared with the response to the wild-type hearts. These findings demonstrate that the reoxygenation induced suppression of the myocardial contractility is dependent on the functional integrity of PARP.[19]

6.4.5 Mechanism of PARP Activation

What, then, is responsible for the activation of PARP during reperfusion? In addition to ROS, including hydrogen peroxide, hydroxyl radicals, and superoxide anions, the formation of NO and presumably peroxynitrite is enhanced during reperfusion of the previously ischemic myocardium. In cultured cells, all of these radicals cause DNA strand breaks and activate PARP.[1-3] Inhibition of NO synthase (NOS) prevents the formation of NO and peroxynitrite (even in the presence of superoxide). To elucidate the

potential contribution of NO or peroxynitrite to the activation of PARP in heart and skeletal muscle subjected to ischemia–reperfusion injury, we have investigated the effects of various NOS inhibitors on infarct size. Administration (1 min before reperfusion) of the NOS inhibitors N^G-methyl-L-arginine (3 mg/kg i.a.; a nonselective inhibitor of all isoforms of NOS), 7-nitroindazole (7-NI, 30 mg/kg i.p.; a selective inhibitor of neuronal NOS; nNOS or NOS I), or aminoethyl-isothiourea (AE-ITU, 10 mg/kg i.a.; a potent inhibitor of nNOS and inducible NOS; iNOS or NOS II) did not cause a reduction in myocardial infarct size. In contrast to the heart, inhibition of NOS activity with 7-NI (30 mg/kg i.p.) or AC-ITU (10 mg/kg IV) caused a substantial (~50%) reduction in infarct size caused by ischemia–reperfusion in the gracilis muscle, without causing a significant increase in blood pressure (an indicator of inhibition of eNOS activity). Skeletal muscle contains large amounts of nNOS.[20] We show here that 7-NI, a potent and selective inhibitor of nNOS activity, reduces skeletal muscle necrosis suggesting that NO from nNOS contributes to the death of skeletal muscle cells, presumably by causing PARP activation in this tissue. This hypothesis is supported by our findings that AE-ITU, which is a potent inhibitor of both iNOS and nNOS activity, also reduced skeletal muscle necrosis, although there was no induction of iNOS protein in skeletal muscle subjected to ischemia and reperfusion. In principle, activation of nNOS (e.g., with N-methyl-D-aspartate (NMDA)) results in the generation of amounts of NO, which are sufficient to cause PARP activation and neuronal cell death.[21] Thus, we have proposed[4] that an enhanced formation of NO from nNOS contributes to the death of skeletal muscle cells, presumably via PARP activation in this tissue. In contrast, an enhanced formation of oxygen-derived free radicals, but not NO, contributes to cell death and PARP activation in cardiomyocytes subjected to ischemia–reperfusion injury.

6.5 NOVEL PARP INHIBITORS

In contrast to certain isoquinoline derivatives, such as 1,5-dihydroxyisoquinoline and DPQ[3,4-dihydro-5-[4-(piperidin-1-yl)butoxy]isoquinolin-1(2*H*)-one],[22,23] 3-AB is a weak inhibitor of PARP activity that does not readily cross cell membranes.[13,14] Although ISO and DPQ are more potent inhibitor of PARP activity than is 3-AB, ISO and DPQ both have to be dissolved in dimethylsulfoxide (DMSO, 10% w/v). DMSO itself is a potent scavenger of hydroxyl radicals. Thus, it is not surprising that DMSO itself reduces the organ injury in conditions associated with organ ischemia and reperfusion.[24,25] Thus, there is still a great need for the development of potent, water-soluble inhibitors of PARP activity.

In 1991, Suto and colleagues[22] reported that 5-aminoisoquinolin-1(2*H*)-one (5-AIQ) is a water-soluble inhibitor of PARP activity in a cell-free preparation (enzyme purified 900-fold from calf thymus). We have recently optimized the synthetic route as previously described[26,27] for 5-AIQ. We have then evaluated the effects of 5-AIQ on PARP activity in rat cardiac myoblasts and investigated the effects of 5-AIQ on the infarct size caused by regional myocardial ischemia and reperfusion in the anesthetized rat. Exposure of rat cardiac myoblasts to H_2O_2 resulted in a significant

increase in PARP activity. Pretreatment of these cells with 5-AIQ caused a concentration-dependent inhibition of PARP activity (IC_{50}: ~4.5 μ*M*). Exposure of H9c2 cells to H_2O_2 caused a 80 to 90% reduction in mitochondrial respiration, which was significantly attenuated by 5-AIQ (EC_{50}: ~4.45 μ*M*). These findings demonstrate that 5-AIQ is a water-soluble inhibitor of PARP activity in cardiac myoblasts (Figures 6.5 and 6.6). To investigate the effects of 5-AIQ on myocardial infarct size, rats were anesthetized (thiopentone), ventilated (inspiratory oxygen concentration: 30%), and subjected to 25 min of LAD occlusion followed by 2 h of reperfusion. Area at risk was determined with Evans blue and infarct size was determined by staining of the area at risk with P-nitro-blue tetrazolium. LAD occlusion and reperfusion resulted in an infarct size of 50 ± 3% (control). Administration (5 min prior to reperfusion) of 5-AIQ (0.03 mg/kg IV followed by an infusion of 0.03 mg/kg/h, 0.1 mg/kg/h IV followed by an infusion of 0.1 mg/kg/h and 0.3 mg/kg/h IV followed by an infusion of 0.3 mg/kg/h) reduced myocardial infarct size in a dose-related fashion. Thus, 5-AIQ is a potent inhibitor of PARP activity in cardiac myoblasts and reduces myocardial infarct size in the rat *in vivo*.

The protective effect of BGP-15 against ischemia–reperfusion-induced injury were first studied in the Langendorff heart perfusion system. To understand the molecular mechanism of the cardioprotection observed, the effect of BGP-15 on ischemia–reperfusion-induced ROS formation, lipid peroxidation single-strand DNA break formation, NAD^+ catabolism, and endogenous ADP-ribosylation reactions were investigated. These studies showed that BGP-15 significantly attenuated the release of lactate dehydrogenase, creatine kinase, and aspartate aminotransferase in reperfused hearts, and reduced the rate of NAD^+ catabolism. In addition, BGP-15 dramatically decreased the ischemia–reperfusion-induced self-ADP-ribosylation of PARP and the mono-ADP-ribosylation of an endoplasmic reticulum chaperone GRP78. These data suggest that BGP-15 may have a direct inhibitory effect on PARP. This hypothesis was tested on isolated enzyme, and kinetic analysis showed a mixed-type (noncompetitive) inhibition with a $K_i = 57 \pm 6$ μ*M*. Furthermore, BGP-15 decreased the formation of ROS, the degree of lipid peroxidation, and the number of single-strand DNA breaks in hearts subjected to ischemia and reperfusion.[28]

6.6 CONCLUDING REMARKS

The discovery in 1997 that various inhibitors of PARP activity reduce myocardial infarct size was rapidly followed by the discoveries that these agents also reduce the tissue injury associated with ischemia-reperfusion of the intestine,[29] brain,[23,30] and, more recently, the kidney[31] (see Chapter 8). The protective effects of PARP inhibitors are likely to be due to the inhibition of PARP activity (rather than nonspecific effects), as the tissue injury caused by ischemia–reperfusion of the heart,[15-17] brain,[30] and gut[32] are significantly reduced in PARP knockout mice. Given that inhibitors of PARP activity cause a substantial reduction in infarct size in many organs including the heart and brain, it is not surprising that several pharmaceutical companies have selected PARP as a target for drug development. There is a great need for potent, water-soluble PARP-inhibitors for the following reasons:

1. The IC_{50} values of currently used PARP inhibitors (e.g., benzamide analogues) range from 100 μ*M* to 1 μ*M*.
2. Several more potent PARP inhibitors including ISO and DPQ need to be dissolved in DMSO, which not only scavenges hydroxyl radicals, but also inhibits PARP activity.

We expect that we will learn much about the pharmacology of potent, water-soluble inhibitors of PARP activity within the next 2 years. These agents will be very useful to determine whether inhibitors of PARP activity may ultimately be useful in conditions associated with ischemia–reperfusion injury. There is, however, one further issue (at least) that will complicate the development of PARP inhibitors as therapeutic agents. In the last few years, five distinct members of the PARP family have been identified,[32] and it is possible that further isoforms of this protein will be discovered in the years to come. Thus, it will be important to determine which of the isoforms of PARP plays a role in a specific pathology to aid the rational design of novel, isoform-selective, and potent inhibitors of PARP activity for the therapy of ischemia–reperfusion injury, diabetes, shock, and inflammation.

ACKNOWLEDGMENTS

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7 Neuroprotection of the Retina with 3-Aminobenzamide, a PARP Inhibitor

Tim T. Lam

CONTENTS

7.1 CELL DEATH IN OCULAR DISEASES

Since the first publication in 1993 by Chang et al.[1] using biochemical and immunohistochemical methods to document apoptosis in several mice models of retinitis pigmentosa (RP) — a hereditary blinding disease — apoptosis has been shown to be involved in many neurodegenerative diseases involving retinal neurons such as age-related macular degeneration,[2] glaucoma,[3] traumatic retinal detachment,[4] retinoblastoma,[5] and retinal degeneration in pathologic myopia.[2] Experimentally, apoptosis has been demonstrated in the retinas in photic retinopathy in rats and mice,[6] retinal ischemia–reperfusion injury,[7] retinal excitotoxicity,[8] optic nerve crush injury,[9,10] optic nerve transection,[11] and glaucoma,[10,12] as well as RP mimicking animals such as the Royal College of Surgeon (RCS) rats,[13] and the *rd* mice.[1] Hence, apoptosis may be the common pathway of neuronal degeneration in the retina in many of these degenerative diseases that lead to blindness, and the apoptotic pathway becomes a target of exploration for new therapeutic approaches in preserving retinal neurons.

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7.2 PARP AND CELL DEATH

Poly(ADP-ribose) polymerase (PARP; EC 2.4.2.30) is an abundant nuclear enzyme known to be activated by DNA strand breaks. Activated PARP acts to transfer the ADP-ribose moiety of NAD^+ to various proteins including PARP itself and hence modulates their activities. One of its most noted activities is to stimulate DNA excision repair. Although PARP may not be essential for DNA repair, it may play an important part in cell recovery after the initial injury. Consistent with this hypothesis, PARP inhibition[14-16] and PARP null mice[17,18] studies suggest that inhibition or loss of PARP activities may render cells more susceptible to cell death after DNA damages. However, one group using PARP null mice found no effect on DNA damage-induced apoptosis or DNA repair[19] and Kruman et al.[20] showed that PARP is proapoptotic in DNA damage–induced apoptosis by activating caspases and p53. As early as 1993 Kaufmann et al.[21] demonstrated that PARP was cleaved early on during chemotherapy-induced apoptosis and suggested an important role of PARP in apoptotic cell death. Subsequently, many reports confirmed the activation of PARP by caspases during apoptosis.[22-25] These reports suggest an opposite role of PARP, proapoptosis vs. pro-DNA repair (recovery). Furthermore, an earlier report by Zhang et al.[26] in 1994 demonstrated the involvement of PARP in excitotoxic cell death of cultured cerebral neurons. The authors proposed that PARP was activated by DNA strand breaks and utilized intracellular NAD^+ in its enzymatic reactions draining the energy source of the cell leading to cell death ultimately. A recent report by Mandir et al.[27] further demonstrated that *N*-methyl-D-aspartate (NMDA), but not non-NMDA excitotoxicity, was mediated by PARP. These findings suggest a possible neuroprotective role of PARP inhibitors in various neurodegenerative diseases in which NMDA-mediated excitotoxicity is involved such as stroke, Alzheimer's disease, and Parkinson's disease. Consistent with this hypothesis, Ducrocq et al.[28] and others[29,30] demonstrated a neuroprotective effect of a PARP inhibitor, 3-aminobenzamide, on ischemic injury to the brain. On the other hand, Nagayama et al.[31] showed that activation of PARP is beneficial to neurons after ischemic insult. One possible explanation for these apparently opposite roles of PARP is that the role of PARP in neuronal injury/survival may depend on the conditions of the tissues, such as the state and supply of NAD^+.[31]

7.3 NEUROPROTECTION IN RETINAL ISCHEMIA–REPERFUSION INJURY

Loss of retinal ganglion cells and other inner retinal elements is characteristic of retinal ischemic responses and is noted in various retinal diseases such as glaucoma, diabetic retinopathy, retinal arterial or venous occlusion, hypertensive retinopathy, radiation retinopathy, and others.[32] Retinal ischemia and the consequential loss of neurons are considered a most common cause of visual loss.[33] Currently, there is no treatment to protect or rescue these retinal neurons.

Experimentally, retinal ischemia injury can be generated by ligation of retinal vessels at the optic nerve,[34,35] by elevating the intraocular pressure (IOP) above the perfusion pressure of ocular vessels through a liquid column connected to the

anterior chamber of the eye thereby occluding the ocular vessels,[36-38] or more recently by injection or perfusion of endothelin, the most potent vasoconstrictor, into the optic disk area causing occlusion of retinal vessels to create an ischemic insult.[39] Using the elevated IOP method in rabbit eyes and electroretinogram as end point measurement, Yoon and Marmor[36] were able to protect neuronal loss in retinal ischemia-reperfusion injury by prophylactic administration of dextromethorphan, a partial NMDA antagonist. This early study not only demonstrated the feasibility of neuroprotection in retinal ischemia–reperfusion injury but also suggested the involvement of glutamate receptor-mediated cell loss. Hughes[38] introduced morphometric means to quantify neuronal loss in retinal ischemia–reperfusion injury in rat retinas using the elevated IOP method, enabling a quantitative assessment of the injury and the efficacy of various neuroprotectants. Using these methods, we demonstrated the protective effect of MK-801,[40] an NMDA receptor antagonist, flunarizine, a calcium overload blocker,[41] *N*-nitro-L-arginine and *N*-monomethyl-L-arginine,[42] nitric oxide synthase (NOS) inhibitor, naloxone,[43] an opioid antagonist, and basic fibroblast growth factor.[44] Most of these agents are given after the ischemia–reperfusion insult. These later studies not only strengthened the argument that NMDA-mediated excitotoxicity may be involved in retinal ischemia–reperfusion injury but also extended the possibility of neuroprotection to post injurious drug intervention.

The role of apoptosis in cerebral ischemia–reperfusion injury and excitotoxicity remains controversial. However, using the classical criteria of apoptosis: histology, ultrastructural changes, and biochemical documentation of internucleosomal DNA fragmentation by demonstrating a ladder pattern in DNA electrophoretic gels (Figure 7.1), we were the first to note an important role of apoptosis in retinal ischemia–reperfusion injury.[45,46] Interestingly, the maximum expression of these apoptotic changes appeared at 18 to 24 h after the insult. Using a general caspase inhibitor, YVAD, we demonstrated its beneficial effect even when it was given at 4 h after the insult but not after 6 or 8 h (Figure 7.2A) and that YVAD inhibited the formation of the ladder pattern (Figure 7.2B), a hallmark of apoptosis, suggesting that YVAD works by inhibiting the apoptotic pathway. In addition, immunohistochemistry of caspase-3 showed an increased labeling of inner retinal cells at 4 h after the injury, suggesting a possible upregulation of caspase-3 after the insult. Hence, we conclusively demonstrated apoptosis in retinal ischemia–reperfusion injury as well as an early involvement of caspases, especially caspase-3, in the pathway. Our study further extends the therapeutic window of rescuing retinal elements after the ischemia–reperfusion insult to 4 h post injury. Our subsequent studies using two antiapoptotic modulators — cyclosporin A[47], a mitochondrial permeability transition pore (MPTP) inhibitor — and aurintricarboxylic acid,[48] an endonuclease inhibitor, also showed beneficial effects suggesting that apoptotic loss of retinal elements after ischemia–reperfusion injury may involve the activation of caspases by cytochrome *c* release from mitochondrial inner membrane owing to the opening of MPTP[49] and the involvement of endonuclease.[50] These studies indicate that targeting the apoptotic pathway for neuroprotection of the inner retina is a promising approach.

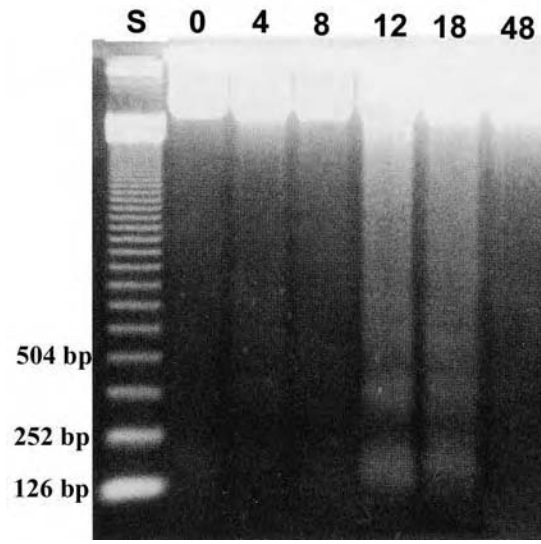

FIGURE 7.1 Agarose gel electrophoresis of retinal DNA from animals at various times of reperfusion after 60 min of ischemia. Lanes: S = DNA standard marker; and 0, 4, 8, 12, 18, 48 hours of reperfusion (10 μg DNA per lane), respectively. Note intense ladder pattern at 12 and 18 h. (From Lam, T. T. et al., *Invest. Ophthalmol. Vis. Sci.*, 40, 967–975, 1999. With permission.)

7.4 3-AMINOBENZAMIDE IN NEUROPROTECTION OF INNER RETINA AFTER RETINAL ISCHEMIA–REPERFUSION INJURY AND EXCITOTOXICITY

Although the caspase inhibitor YVAD was effective in rescuing retinal elements under our hands even when given 4 h after the injury, the therapeutic time window was still too small to be of any practical use. Previously, we demonstrated a dose-dependent beneficial effect of 3-aminobenzamide,[51] a PARP inhibitor, given immediately after ischemia–reperfusion injury (Figure 7.3A) and that it inhibited apoptosis in retinal ischemia–reperfusion injury as demonstrated by its inhibition of ladder pattern formation (Figure 7.3B). These findings are consistent with the hypothesis that PARP plays an important role in apoptotic loss of inner retinal elements after ischemia–reperfusion injury. In a follow-up study,[52] immunohistochemical labeling of poly(ADP-ribose), a reaction product of PARP activity, showed enhanced PARP activity between 12 and 18 h after the insult (Figure 7.4), at a time slightly ahead of the maximum apoptotic changes (see Figure 7.1). Hence, we speculated that PARP activation may be a late event in the apoptotic pathway (contrary to Kaufmann's finding[21]) and that inhibition of PARP may provide a larger time window of treatment in retinal ischemia–reperfusion injury provided that the apoptotic events preceding PARP are reversible. To examine this possibility, we gave intravitreal injection of

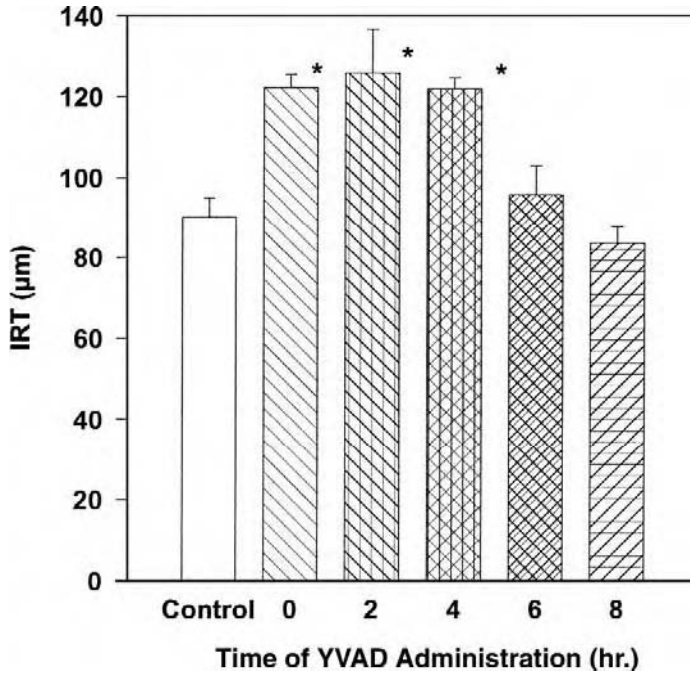

FIGURE 7.2(A) Effect of time of intravitreal treatment with YVAD.CMK (1 n*M* intravitreally) on inner retinal thickness (IRT; the average thickness between the inner limiting membrane and the outer plexiform layer) measured at 7 days of reperfusion. Note the significant beneficial effect of YVAD.CMK administered 0, 2, or 4 h after reperfusion ($P < 0.002$; compared with control) but no effect after 6 or 8 h. (From Lam, T. T. et al., *Invest. Ophthalmol. Vis. Sci.,* 40, 967-975, 1999. With permission.)

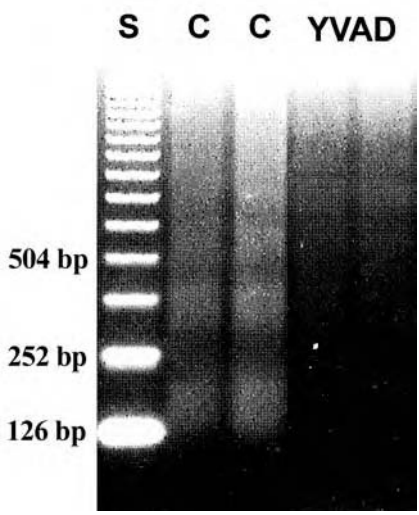

FIGURE 7.2(B) Effect of YVAD.CMK (100 μ*M*, intracameral infusion during ischemia) on internucleosomal fragmentation of retinal DNA collected 12h after reperfusion. Lanes: S = DNA marker; C = control ischemia-reperfused retinas; YVAD = ischemia-reperfused retinas with YVAD treatment. Note inhibition of ladder pattern formation in the YVAD-treated retinas (10 μg DNA per lane; each lane represents a sample from one retina). (From Lam, T.T et al., *Invest. Ophthalmol. Vis. Sci.*, 40, 967–975, 1999. With permission.)

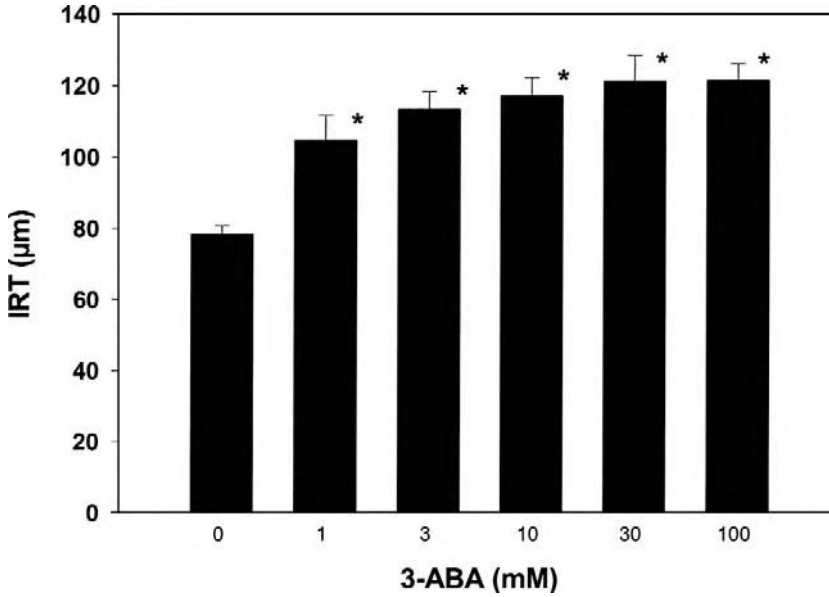

FIGURE 7.3(A) The effect of 3-aminobenzamide (various concentrations, intracameral infusion during ischemia) on inner retinal thickness of the ischemia-reperfused retinas (* $P < 0.02$). Note significantly beneficial effect of 3-aminobenzamide. (From Lam, T. T., *Res. Commun. Mol. Pathol. Pharmacol.,* 95, 241–252, 1997. With permission.)

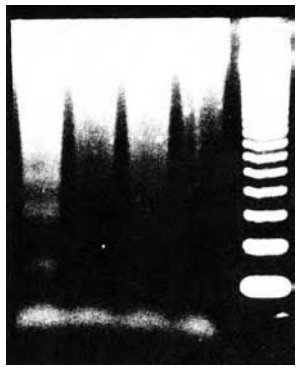

FIGURE 7.3(B) Effect of 3-aminobenzamide (10 m*M*, intracameral infusion during ischemia) on retinal DNA fragmentation at 18 h after reperfusion. Lanes: 1 = vehicle; 2 and 3: 10 m*M* 3-aminobenzamide; 4 = MK-801 treated; and 5 = DNA standard. Note inhibition of ladder pattern by 3-aminobenzamide and MK-801. (From Lam, T. T., *Res. Commun. Mol. Pathol. Pharmacol.,* 95, 241–252, 1997. With permission.)

3-aminobenzamide at various times after retinal ischemia–reperfusion injury and evaluated its effect at 7 days after reperfusion, a time when the degenerative changes stabilized, using morphologic (Figure 7.5) and morphometric (Figure 7.6) criteria. Under both criteria intravitreal 3-aminobenzamide injection was beneficial when given 12 or 18 h after the insult but not earlier or afterward.[52] This finding, together with our earlier study using systemic administration of 3-aminobenzamide, suggested that 3-aminobenzamide, when given appropriately, can have a window of treatment of up to 12 to 18 h after the injury.

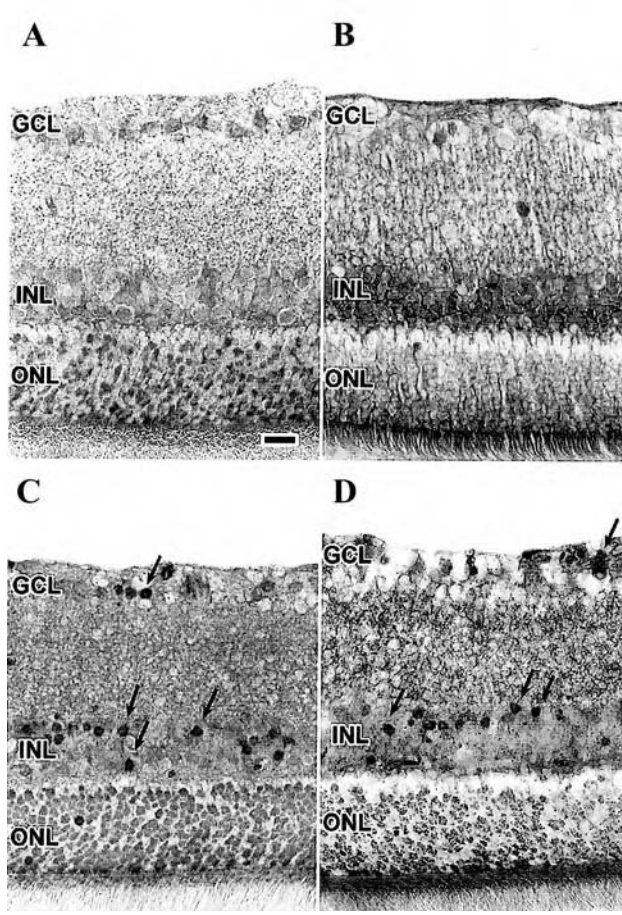

FIGURE 7.4 Immunoreactivity of poly(ADP-ribose) in rat retinas after reperfusion. (A) Normal retina, (B) 4 h, (C) 12 h, and (D) 18 h after reperfusion. Positive immunoreactivity (arrows) was noted in the retinal ganglion cell layer (RGCL) and the inner nuclear layer (INL) at 12 (C) and 18 (D) h after reperfusion. Scale bar, 20 μm. (From Chiang, K. S. and Lam, T. T., *Invest. Ophthalmol. Vis. Sci.*, 41, 3210–314, 2000. With permission.)

To confirm the reported activation/cleavage of PARP from 113k-Da to its 89kDa fragment during apoptosis, retinal samples at various times after ischemia–reperfusion injury were subjected to Western blotting. Unexpectedly, we were unable to detect the expected 89-kDa fragment at all times after the insult. Instead, we noted a gradual increase in the parent 113-kDa band reaching a maximum at 12 or 18 h after injury, suggesting a possible upregulation or suppressed degradation of PARP or both (Figure 7.7). This temporal change in PARP coincided with our time window of treatment and was very similar to the maximum apoptotic changes as measured by the

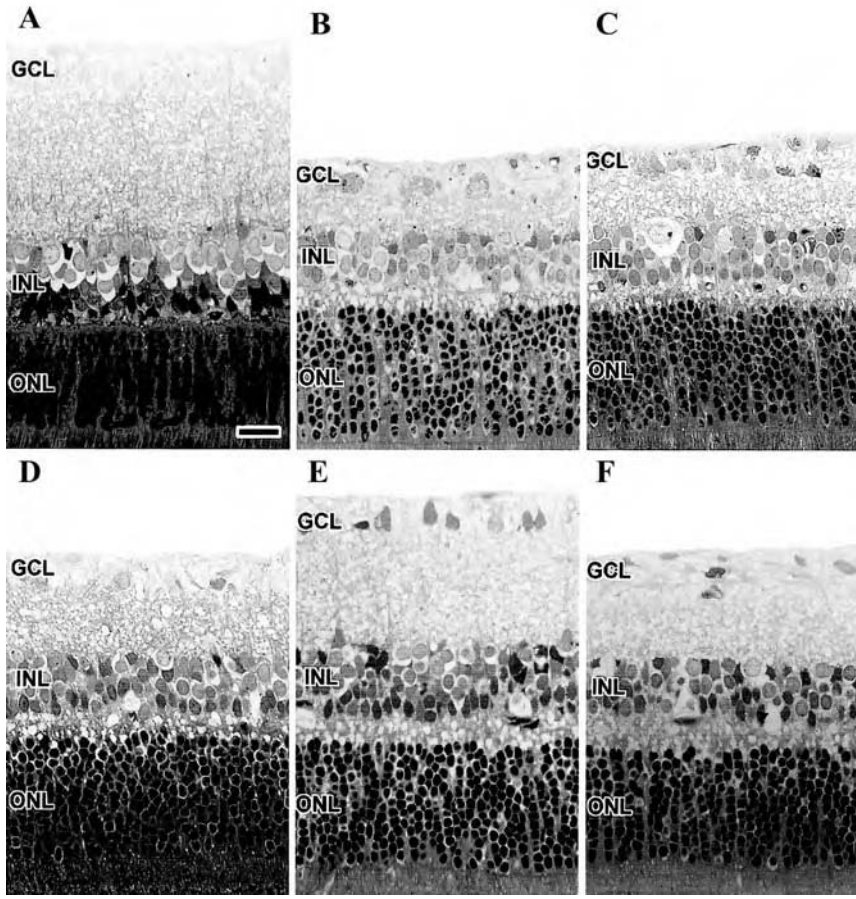

FIGURE 7.5 Retinas at 7 days of reperfusion with 3-aminobenzamide (0.4 n*M*, intravitreally) administered at various times after ischemia. (A) Normal retina. (B) ischemia-reperfused retina without treatment; and retinas treated with 3-aminobenzamide at (C) 4 h, (D) 2 h, (E) 18 h, and (F) 24 h. Note better preserved retinas when 3-aminobenzamide was administered at 12 (D), 18 (E), or 24 (F) h. Toluidine blue. Scale bar, 20 μm. (From Chiang, K. S. and Lam, T. T., *Invest. Ophthalmol. Vis. Sci.,* 41, 3210–3214, 2000. With permission.)

intensity of the ladder pattern (see Figure 7.1) and morphometry of Tdt-mediated biotin-dVTP nick end label (TUNEL). Taken together, our findings indicate an elevated activity of PARP together with elevated PARP protein levels after the insult and are supportive of a pivotal, downstream post-caspase role of PARP in apoptotic loss of inner retinal elements after retinal ischemia–reperfusion injury. In support of its role as neuroprotectant for inner retinal neurons, we also tested the effect of 3-aminobenzamide on NMDA-induced excitotoxicity in the retina using an established protocol.[53] 3-Aminobenzamide given systemically after intravitreal injection of NMDA

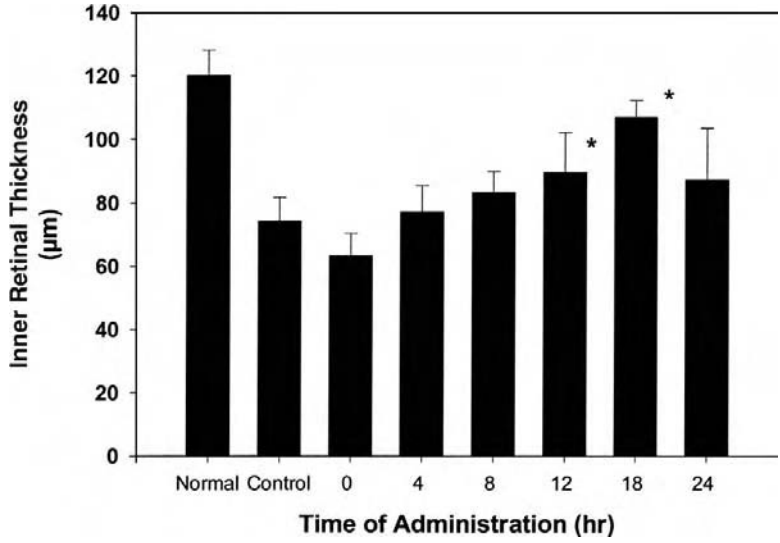

FIGURE 7.6 Morphometry of inner retinal thickness with 3-aminobenzamide (0.4 n*M*, intravitreally) administered at various times after ischemia at 7 days of reperfusion. Note significant improvement in inner retinal thickness with 3-aminobenzamide treatment at 12 or 18 h (*P < 0.05, Tukey's test) after ischemia. (From Chiang, K. S. and Lam, T. T., *Invest. Ophthalmol. Vis. Sci.*, 41, 3210–3214, 2000. With permission.)

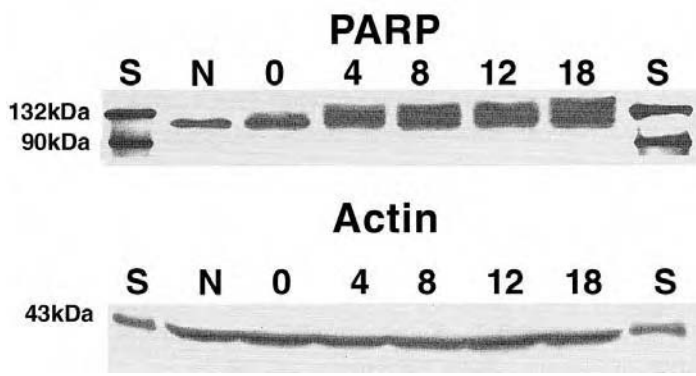

FIGURE 7.7 Western blot analysis of total cell lysates from rat retinas at various times after reperfusion using antibody to the C-terminus of PARP. Top left to right: standard (s), normal (N) retina, and retinas at 0, 4, 8, 12, or 18 h after reperfusion. Note a significant increase in the 116-kDa band between 4 and 18 h. Actin showed no change in protein levels. (From Chiang, K. S. and Lam, T. T., *Invest. Ophthalmol. Vis. Sci.,* 41, 3210–3214, 2000. With permission.)

into the rat eye to induce inner retinal neuronal degeneration ameliorated the loss of inner retinal elements. This later observation is consistent with earlier report by Zhang et al.,[26] who demonstrated a neuroprotective effect of 3-aminobenzamide in cultured cerebral neurons exposed to glutamate. Our data on the neuroprotective effect of 3-aminobenzamide in retinal ischemia–reperfusion injury as well as in retinal excitotoxicity are encouraging although different from some other reports on different cells and tissues.

7.5 3-AMINOBENZAMIDE AND GLAUCOMATOUS LOSS OF INNER RETINAL NEURONS

Glaucoma is a group of blinding diseases affecting over 65 million worldwide with 1.2 million patients with glaucoma in the United States contributing to 62,000 legally blind annually.[54] The common feature of glaucoma is optic nerve damage showing optic disk cupping. Histologically, there is a loss of retinal ganglion cells in the retina and their axons in the optic nerve. The etiology of the diseases is not known except for a small subset of the diseases that it may due to mutations in the (TIGR) protein.[55] Elevated IOP is the major risk factor. Ischemic damages to the optic nerve head may contribute to the loss of retinal ganglion cells and their axons in glaucoma. Currently, there is no treatment in preserving or protecting the retinal ganglion cells and their axons from degenerative changes except medical or surgical means to lower IOP, the major risk factor. Even with IOP controlled within the normal range, some individuals show continued progression of the disease.[56] Apoptosis appears to play a major role in the degeneration of retinal ganglion cells and their axons.[3,10,12] Since our retinal ischemia–reperfusion injury model in rats showed histopathological features resembling those in the retinas of patients with glaucoma and the damage was induced through an acute elevation of IOP, we extended our studies of PARP inhibitor 3-aminobenzamide to animal models of glaucoma.

Previously, evaluation of neuroprotectants for glaucoma had been hampered by the high cost of acquiring and maintaining nonhuman primate models for glaucoma. In addition, because of the inherent large variations, these models require a large sample size for any quantitative measurements. In 1995, Garcia-Valenzuela et al.[12] described a glaucoma-mimicking rat model with chronic elevated IOP, in which some of the limbal/vortex veins are cauterized to block part of the aqueous outflow to produce a chronic elevation of IOP. Loss of retinal ganglion cells and their axons were noted with cupping and apoptotic cell death. Morrison et al.[57] also described similar glaucomatous changes in rats after an injection of hypertonic saline through limbal veins to disrupt part of the outflow facilities causing a chronic elevation of IOP. This was followed by Ueda et al.,[58] who used laser trabecular photocoagulation after a prior injection of India ink in rats to produce a transient elevation of IOP for 2 weeks (without repeated laser treatment). The availability of these low-cost, relatively reproducible, small-animal, glaucoma-mimicking models allows large sample size to compensate for the large individual variations. Quantitative assessment by counting the retinal ganglion cells becomes feasible for evaluating the efficacy of

neuroprotectants for glaucoma. Some of these animal models have been successfully applied in efficacy studies.[59,60]

We decided to examine PARP activity and the effectiveness of 3-aminobenzamide in two rat models of experimental glaucoma: the Garcia-Valenzuela model[12] and the Ueda model.[58] With both models, PARP activity as demonstrated by increased immunoreactivity of poly(ADP-ribose) elevated PARP activity after elevated IOP was demonstrated and systemic administration of 3-aminobenzamide ameliorated retinal ganglion cell loss as assessed by cell counts with whole-mount flat preparations of the retinas at 2 weeks or longer after the induction of elevated IOP.[61] These later findings are consistent with our retinal ischemia–reperfusion studies and are suggestive of a common pathway and common therapeutic approaches to neuroprotection of inner retinal neurons.

7.6 CONCLUSION

Using a rat model of retinal ischemia–reperfusion injury in which apoptosis plays an important role, we demonstrated a neuroprotective effect of 3-aminobenzamide, a PARP inhibitor, showing a relatively large window of treatment of up to 18 h postinjury. Similar neuroprotective effect is also noted in two different rat models of experimental glaucoma in which apoptosis also played an important role. These findings strongly indicate that PARP may play important roles in inner retinal neuronal degeneration and PARP inhibitors are promising neuroprotectants for the retina.

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8 Poly(ADP-Ribose) Polymerase Inhibitors and Acute Renal Failure

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CONTENTS

8.1 INTRODUCTION

Poly(adenosine 5′-diphosphate-ribose) polymerase (PARP, EC 2.4.2.30), also known as poly(adenosine 5′-diphosphate-ribose) synthetase, (PARS), or poly(adenosine 5′-diphosphate-ribose) transferase, (PADRT), is an abundant chromatin-bound enzyme that is present in the nuclei of most cell types. The physiological role of PARP involves its activation by strand breaks in DNA after which it transfers ADP-ribose moieties from nicotinamide adenine dinucleotide (NAD^+) to various nuclear proteins including histones and even to PARP itself (automodification), forming extended

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chains of ADP-ribose or poly(ADP-ribose) (pADPr) polymers. This reaction leads to the generation of nicotinamide, which, via negative feedback, inhibits PARP activity. However, continuous or excessive activation of PARP during conditions of oxidant stress, e.g., during ischemia–reperfusion (I/R), leads to an excessive activation of PARP resulting in a substantial depletion in intracellular levels of NAD^+. As NAD^+ functions as an electron carrier in the mitochondrial respiratory chain, its depletion rapidly leads to a fall in intracellular levels of adenosine triphosphate (ATP). Moreover, nicotinamide can be recycled back to NAD^+ in a reaction that consumes ATP. Thus, continuous excessive activation of PARP results in a fall in ATP via two different mechanisms ultimately leading to cell death — a pathophysiological process commonly referred to as the PARP suicide hypothesis (Figure 8.1).

Reactive oxygen species (ROS) such as superoxide anions ($O_2^{\bullet -}$) and hydroxyl radicals ($OH^{\bullet}$) cause strand breaks in DNA as does the reactive nitrogen species (RNS) peroxynitrite ($ONOO^-$), which is generated when equimolar amounts of nitric oxide (NO) react with $O_2^{\bullet -}$. DNA damage and consequent PARP activation result in depletion of NAD^+ and ATP, ultimately leading to cell death. We have recently discovered that inhibitors of PARP activity reduce the cellular injury and death caused by oxidative stress to primary cultures of rat and human renal proximal tubular (PT) cells. We have also demonstrated that PARP inhibitors reduce the renal dysfunction caused by I/R of the kidney of the rat *in vivo*. This chapter aims to provide the reader with an introduction to the possible roles of PARP in the kidney, and reviews the current evidence that PARP activation plays an important role in renal I/R, which is a major contributor to acute renal failure (ARF).

8.1.1 Role of PARP in the Response to DNA Injury

A constant, low level of DNA strand breakage routinely occurs due to environmental or normal physiological processes such as cell differentiation, resulting in a minor decline in NAD^+ levels, which slows the rate of glycolysis and is reversible.[1] Under these conditions of mild DNA damage, PARP maintains genome integrity by detecting DNA strand breakage and initiating repair.[1] However, under conditions of oxidative stress (e.g., that prevail during I/R), severe damage to DNA results in excessive PARP activation, and in its attempt to repair DNA strand breaks, PARP depletes NAD^+ levels, which is closely followed by depletion of ATP levels leading to cytotoxicity.[2] In 1985, Berger termed this phenomenon the PARP Suicide Hypothesis[2] (see Figure 8.1).

8.1.2 PARP Suicide Hypothesis

Evidence from numerous studies using many different cell types have strongly suggested a role for PARP activation in the cytotoxicity mediated by agents that damage DNA, such as ROS, RNS, radiation, and alkylating agents.[3] ROS, including hydrogen peroxide (H_2O_2), from which $OH^{\bullet}$ is formed via the Haber–Weiss or Fenton reactions, and the RNS $ONOO^-$ can damage DNA within seconds of exposure resulting in excessive activation of PARP and a consequent depletion of NAD^+ within

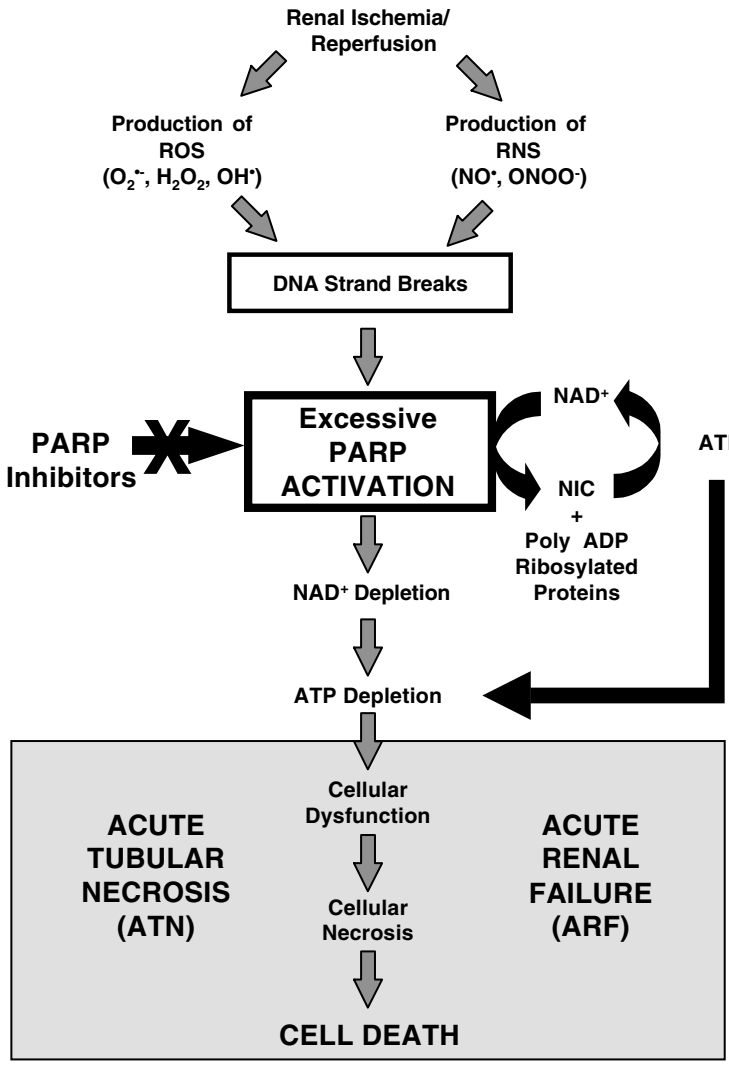

FIGURE 8.1 The role of renal I/R, production of ROS, and PARP activation in acute tubular necrosis and acute renal failure.

minutes. It has been suggested that this marked depletion in the NAD^+ pool is the intracellular mechanism by which the nucleus communicates physiological changes to other cellular compartments, such as the mitochondria, via modulation of the redox state of the cell.[2]

The major consequence of the depletion of the NAD^+ pool is ATP depletion. NAD^+ is an essential co-factor in glycolysis where it is required for the synthesis of

1,3-diphosphoglycerate from glyceraldehyde-3-phosphate and inorganic phosphate.[1] NAD^+ is also required for fatty acid oxidation.[1] In addition, nicotinamide, which is produced during ADP-ribosylation, is converted back into NAD^+ in a reaction that also consumes ATP.[4] Thus, excessive PARP activation leads to a fall in ATP via two different, but related, mechanisms, leading to cellular dysfunction and ultimately, cell death (see Figure 8.1).

The presence of this pathway has been demonstrated in many different cell types[3], ranging from neurons,[5] myoblasts,[6,7] macrophages,[8] fibroblasts,[9] lymphocytes,[10] and hepatocytes,[11] to smooth muscle cells[12] and endothelial cells.[13] This pathway is also present in epithelial cells of many tissues including gut[14,15] and kidney.[16, 17]

8.2 ACUTE RENAL FAILURE

Despite significant advances in critical care medicine, acute renal failure (ARF) remains a major clinical problem, and associated mortality has not decreased significantly over the last 50 years.[18,19] The mortality rate of ARF remains between 50 and 70% among patients in intensive care who require dialysis and ranges between 25 and 100% in postoperative patients suffering ARF.[20-22] Additionally, ARF is frequently implicated in the pathophysiology of other life-threatening complications including sepsis and multiorgan failure (MOF).[23,24] Thus, motivated by the fact that previous interventions against ARF have proved to be largely negative and that dialysis still remains the only effective therapy,[19] development of novel therapeutic interventions with which to reduce renal dysfunction and injury mediated by I/R of the kidney, and associated ARF, have been topics of intense research interest.

8.2.1 Renal Ischemia–Reperfusion Injury

Renal ischemia is a major cause of ischemic ARF.[25] Significant renal ischemia is a common problem during aortic surgery and renal transplantation or subsequent to cardiovascular anesthesia, leading to renal dysfunction and injury.[22,26,27] Surgical procedures that involve clamping of the aorta or renal arteries (e.g., surgery for supra- and juxtarenal abdominal aortic aneurysms and renal transplantation) are particularly liable to produce renal ischemia, leading to significant postoperative complications including ARF.[23,24,26,27] The incidence of renal dysfunction in high-risk patients undergoing this type of surgery has been reported to be as high as 50%.[27] The prognosis is complicated by the fact that reperfusion, although essential for the survival of ischemic tissue, causes additional cellular injury (reperfusion injury)[28] and is a major contributor to early allograft rejection subsequent to renal transplantation, and adversely affects the long-term survival of the allograft.[29] Thus, early therapeutic intervention is likely to reduce the incidence of ARF and associated mortality following surgical manipulations that involve renal I/R.

8.2.2 Role of Reactive Oxygen Species and Reactive Nitrogen Species in Renal Ischemia–Reperfusion Injury

Renal ischemia initiates a complex and interrelated sequence of events, resulting in injury to and the eventual death of renal cells[18,30] with the cells of the PT demonstrating particular susceptibility.[28] Furthermore, reperfusion injury, which has been attributed, *inter alia*, to the generation ROS[28,31,32] contributes significantly to the renal dysfunction and injury associated with I/R of the kidney.[18,28,30] It is now clear that the production of ROS and RNS contribute to renal I/R injury (and associated ARF).[28, 31-35] Furthermore, the susceptibility of the PT to renal I/R injury leads to ischemic acute tubular necrosis (ATN), which plays a pivotal part in the pathogenesis of ARF.[18,34]

Traditionally, ROS have been considered to exert their effects through a direct toxic action on target cells. For example, ROS cause DNA damage during renal I/R and oxidative stress leading to PARP activation, depletion of NAD^+ and ATP levels, and ultimately cell death.[16,36-38] However, recent studies have also suggested a contributory role for ROS in gene induction and may act as signal transduction molecules for transcription factors including NF-κB and activator protein-1 (AP-1).[39,40]

The hypothesis that the generation of ROS contributes to renal I/R injury is supported by several studies that demonstrate the beneficial effects of various interventions, which reduce either the generation of or the effects of ROS.[28] These therapeutic strategies include administration of agents which (i) prevent the generation of ROS, such as deferroxamine or *N*-acetylcysteine.[37,38,41-44] (ii) inhibit enzymes responsible for production of ROS, e.g., inhibition of xanthine oxidase by allopurinol,[45,46] (iii) degrade ROS such as catalase or superoxide dismutase (SOD),[47-49] or (iv) scavenge ROS such as mannitol[41] and sodium benzoate.[49] Although these interventions have shown promise, the potential benefits of the systemic administration of agents such as catalase and SOD are limited for reasons that include their large structures, their inability to permeate biological membranes and, in the case of SOD, to dismutate $O_2^{\bullet-}$ into H_2O_2, which in turn can generate the highly toxic $OH^{\bullet}$ via the Fenton or Haber–Weiss reactions and which also contributes to renal I/R injury.[25,28,50,51] To overcome these limitations, radical scavengers which are of low molecular weight and which permeate biological membranes, have the potential to be useful in conditions associated with I/R injury of the kidney and associated ARF. To this end, we have recently demonstrated that water-soluble ROS scavengers of low molecular weight such as TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-*N*-oxyl) or TEMPONE (4-oxo-2,2,6,6-tetramethylpiperidine-*N*-oxyl) can provide beneficial actions against renal dysfunction and injury mediated by I/R of the kidney *in vivo* and by oxidative stress in PT cultures *in vitro*.[16,52] Furthermore, by reducing the production or presence of ROS, the PARP activation caused by renal I/R can be markedly reduced.[38,52]

The contribution of RNS such as NO and $ONOO^-$ to renal I/R injury and associated ARF has been investigated and reviewed.[33] NO, produced by nitric oxide synthase (NOS), plays an important role in renal function, under both normal and pathophysiological conditions.[33,53,54] The three isoforms of NOS have been located in the

kidney; the endothelial and neuronal (constitutive) isoforms have been identified in the renal vasculature and macula densa, respectively;[55] and inducible NOS (iNOS) can be induced in the kidney by cytokines and lipopolysaccharide during I/R and under inflammatory conditions leading to renal toxicity.[33,53-58] Several *in vivo* and *in vitro* investigations have demonstrated that inhibition or the absence of iNOS activity reduces renal I/R injury.[59-62] Together, these results suggest that NO, generated by iNOS, contributes to renal I/R injury. Furthermore, the reaction of NO with $O_2^{\bullet-}$ to form $ONOO^-$,[62] causes injury via direct oxidant injury and protein tyrosine nitration[63-65] and consequential renal I/R injury.[33,35]

8.2.3 Role of PARP in Ischemia–Reperfusion Injury

The concept that the activation of PARP contributes to the pathophysiology of I/R injury has evolved over the last few years. In 1994, Zhang and colleagues[5] reported that benzamide and other inhibitors of PARP activity reduce the neurotoxic effects of *N*-methyl-D-aspartate (NMDA) and NO in brain slices of the rat, suggesting that the activation of PARP may contribute to the tissue injury associated with stroke. In January 1997, we reported that inhibitors of PARP activity including 3-aminobenzamide (3-AB), ISO, benzamide, and nicotinamide, when administered prior to reperfusion, reduces the I/R injury of the heart of the rabbit *in vivo*[12] and was supported by the findings of Zingarelli et al. using 3-AB who reported similar findings in the anesthetized rat.[66] Further studies demonstrated PARP inhibitors could provide beneficial actions against I/R of the heart of the anesthetized pig[67] and against oxidative stress–mediated injury of human cardiac myoblasts[6] (see also Chapter 6; PARP Inhibition and Cardioprotection). We, therefore, proposed that (1) the activation of PARP contributes to the pathophysiology of I/R injury, and (2) that inhibitors of this enzyme may be useful in the therapy of I/R injury not only of the heart but of other organs.[12] Subsequently, we demonstrated that PARP inhibitors could significantly reduce skeletal muscle necrosis caused by prolonged periods of hind limb I/R[12] and these findings were rapidly followed by the discoveries that these agents could also reduce the tissue injury associated with I/R of the brain,[68,69] gut,[70] and the kidney,[16,37] The protective effects of PARP inhibitors are likely to be due to the inhibition of PARP activity (rather than nonspecific effects), as the tissue injury caused by I/R of the brain,[69] heart,[71] and gut[72] were significantly reduced in genetically modified mice in which PARP is absent (PARP knockout or $PARP^{-/-}$ mice).

8.2.4 Role of PARP in the Pathophysiology of the Kidney

The full role of PARP within the kidney is still unclear and, to date, there has been little research into its role within the kidney or the PT under normal and pathophysiological conditions. Early investigations reported that renal carcinogens such as dichlorovinylcysteine activated PARP subsequent to DNA damage in a renal PT cell line ($LLC\text{-}PK_1$) and in the rat kidney.[73]

However, the results of one study using suspensions of rabbit renal proximal tubular cells suggested that PARP activation did not play a major role in acute renal

proximal cell injury and death induced by agents that cause oxidative stress, mitochondrial dysfunction, or increase in cytosolic free calcium.[74] In further detail, suspensions of rabbit renal proximal tubular cells were incubated with agents known to cause cell injury and death including antimycin A and the protonophore carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) (both inhibitors of mitochondrial function), the calcium ionophore ionomycin, and the oxidant *t*-butyl hydroxyperoxide (TBHP). Lactate dehydrogehase (LDH) release was used as a marker of cell death and demonstrated that the presence of the PARP inhibitor 3-AB (5 m*M*) had no effect on LDH release produced by exposure to antimycin A, FCCP, ionomycin, or TBHP. It was therefore suggested that PARP activation did not play a significant role in the proximal tubule cell death produced by these compounds[74] Furthermore, there was no evidence of generalized DNA breakage under any of the conditions examined.[74]

One unusual investigation demonstrated that exposure of a monolayer of T1 cells cultured from human kidney to a strong static magnetic field (1.4 T, 30 min at 37°C) reduced PARP activity by 40%.[75] Inhibition of PARP was transient and dependent on the time of exposure and the strength of the magnetic field applied. It was suggested that the magnetic field interacted with cell membrane components affecting the transfer of signals that control poly(ADP-ribosyl)ation of proteins.[75]

8.2.5 Role of PARP in Renal Ischemia–Reperfusion Injury

As discussed above, ROS and RNS produce cellular injury and necrosis via several mechanisms including DNA damage, peroxidation of membrane lipids, protein denaturation, and tyrosine nitration. Evidence obtained from studies using cultured cells has demonstrated that ROS produce strand breaks in DNA, triggering PARP activation.[3] However, there has been very little research into the role of PARP within the kidney under normal or pathophysiological conditions and, to date, there are few reports investigating the activation of PARP or the possible beneficial role of PARP inhibitors in the kidney or in renal cells under conditions of oxidative stress such as I/R where the generation and actions of ROS and RNS play a major role.[28,31-35] The possibility of pharmacological inhibition of PARP activation in the kidney could lead to the development of novel therapies for the treatment of renal I/R and associated ARF either alone or in combination with other recognized therapies as (1) renal I/R plays a major role in clinical ARF, (2) PARP activation has been implicated in I/R injury, and (3) the beneficial effects of PARP inhibition have been reported in other models of oxidant stress.

8.2.5.1 Evidence from *In Vitro* Studies

In early 1999, we hypothesized that oxidative stress would result in DNA damage to renal cells, produce excessive activation of PARP, and thus lead to cellular injury and death. Therefore, using confluent primary cultures of rat PT cells, isolated from the cortex of male Wistar rats using a combination of enzymatic digestion, mechanical sieving, and differential centrifugation in Percoll solution,[16,38] we subsequently demonstrated that exposure of rat PT cells to 1 m*M* H_2O_2 for 1 h resulted in

significant increase in DNA strand breaks, PARP activation (Figure 8.2A), and after 4 h, increased cellular injury (Figure 8.2B) and cell death,[16] with a positive correlation ($r = 0.93$) between PARP activation and cytotoxicity.[16] We subsequently demonstrated that the PARP inhibitors 3-AB (3 m*M*), ISO (0.3 m*M*), and nicotinamide (3 m*M*) could significantly (1) inhibit PARP activation (Figure 8.2A) and (2) reduce H_2O_2-mediated PT cell injury (Figure 8.2B) and cell death.[16] Compared to the benzamide analogues (3-AB and nicotinamide), the isoquinolinone compound ISO provided beneficial actions at ten times lower concentration. However, unlike 3-AB or nicotinamide, stock solutions of ISO had to be dissolved in dimethyl sulfoxide (DMSO), which at higher concentrations can exert nonspecific effects.[76]

The respective structural analogues of 3-AB and nicotinamide, 3-aminobenzoic acid (3-ABA) (3 m*M*) and nicotinic acid (3 m*M*), which did not inhibit PARP activity (Figure 8.2A) did not provide any protection against H_2O_2-mediated cellular injury (Figure 8.2B) or cell death.[16] Furthermore, 3-AB and ISO did not reduce the incidence of DNA strand breaks caused by exposure of PT cells to H_2O_2 suggesting a lack of ROS-scavenging or ROS-degrading activity,[16] a feature of 3-AB and ISO demonstrated in a previous study.[6]

In the same study, we demonstrated that OH? (derived from H_2O_2 via the Haber–Weiss and Fenton reactions) plays a major role in renal oxidative stress. Both the ROS scavenger TEMPOL (3 m*M*) and the iron-chelating agent deferroxamine (3 m*M*) also provided significant protection against H_2O_2-mediated cellular injury (Figure 8.2B) and cell death.[16,38] Furthermore, incubation of PT cell cultures with catalase (3 U/ml), which effectively reduces H_2O_2 to H_2O, also provided significant protection against H_2O_2-mediated cellular dysfunction and necrosis (Figure 8.2B). In contrast to the PARP inhibitors, both deferroxamine and catalase significantly reduced H_2O_2-mediated DNA damage.[16] However, the degree of protection against oxidative stress–mediated PT cell injury obtained using the PARP inhibitors was comparable to that obtained using TEMPOL and deferroxamine, whereas catalase provided a higher level of protection (Figure 8.2B).

In a similar study using a porcine renal cell line (LLC-PK[1] cells), exposure of cultures to 1 m*M* H_2O_2 increased PARP activity and produced decreases in NAD^+ and ATP levels.[17] In that study, 3-AB (10 m*M*) significantly inhibited these changes but, as expected, 3-AB did not have any effect on H_2O_2-mediated DNA damage.[17] At this dose of 3-AB, H_2O_2-mediated PARP activation and cell death were completely inhibited.[17] Interestingly, although treatment of LLC-PK_1 cells with 3-AB protected against H_2O_2-mediated cell death, the cells were found to undergo apoptosis (as determined using DNA fragmentation and bisbenzamide staining), and it was concluded that inhibition of PARP activity targeted the cells toward apoptosis.[17]

In another, later study by Jung and colleagues,[77] the effects of H_2O_2 and the organic hydroperoxide, tert-butyl hydroperoxide (t-BHP) on PARP activation and cell death or cultures of an opossum kidney (OK) cell line were investigated. Both H^2O^2 and t-BHP were found to produce significant cellular death at a concentration of 1 m*M* but only H_2O_2 was found to increase PARP activity, which was completely inhibited by 0.5 m*M* 3-AB.[77] Both H_2O_2 and t-BHP reduced cellular ATP levels but only

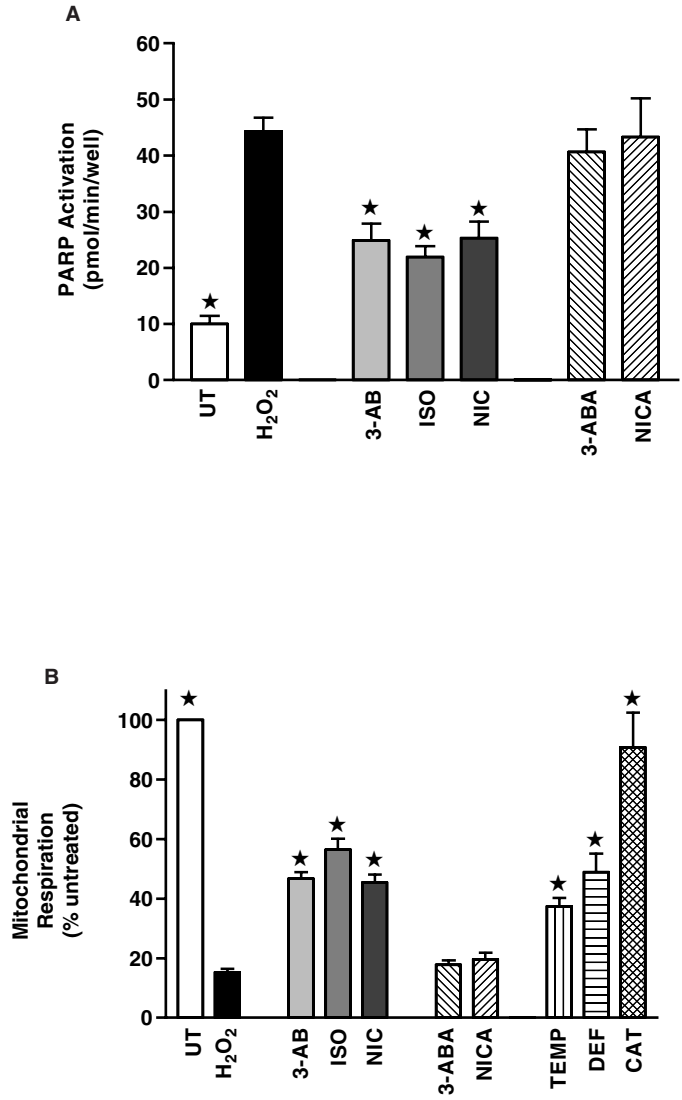

FIGURE 8.2 Effect of PARP inhibitors on (A) H_2O_2-mediated PARP activation and (B) H_2O_2-mediated cellular injury of rat PT cells. Rat PT cells were incubated in the absence (UT = untreated) or presence of H_2O_2) (1 m*M* for 4 h). PARP inhibitors used: 3-AB (3 m*M*), ISO (0.3 m*M*), NIC (3 m*M*). Inactive structural analogues (negative controls) used: 3-ABA (3 m*M*) and NIC A (3 m*M*). The effects of the ROS scavenger TEMPOL (3 m*M*), the iron chelator DEF (3 m*M*), and catalase (3 U/ml) were also investigated. ★ $P < 0.05$ vs. H_2O_2-only, N = 6 to 12.

H_2O_2-mediated ATP depletion could be attenuated by 3-AB.[77] Furthermore, results from a fluorimetric assay involving 2′,7′-dichlorodihydrofluorescein (DCFH), indicated that H_2O_2 and t-BHP might have different mechanisms for promoting cell death. Interestingly, t-BHP, when added in combination with H_2O_2, inhibited H_2O_2-mediated increase in PARP activity, suggesting that t-BHP could have a direct inhibitory effect on PARP activation. This was reflected in the finding that in the presence of H_2O_2, t-BHP had an additive protective effect on cell death.[77] In a subsequent study, the same group investigated the role of PARP activation in oxidant-induced membrane transport dysfunction in the OK cell line.[78] Specifically, H_2O_2 was found to inhibit Na^+-dependent phosphate uptake into OK cells in a dose-dependent manner, an effect that was suppressed by dithiothreitol and glutathione.[78] However, the potent antioxidant DPPD (*N,N′*-diphenyl-*p*-phenylenediamine) did not have any effect on Na^+-dependent phosphate uptake although it completely inhibited H_2O_2-mediated lipid peroxidation.[78] However, the PARP inhibitor 3-AB was able to completely inhibit H_2O_2-mediated reduction of Na^+-dependent phosphate uptake (as well as H_2O_2-mediated ATP depletion) leading to the conclusion that the effect of H_2O_2 on membrane transport function in OK cells is mediated by PARP activation but not with lipid peroxidation[78]

8.2.5.2 Evidence from *In Vivo* Studies

Cold renal ischemia produced *ex vivo* by storage of mouse kidneys at 0°C for up to 72 h, was found to increase PARP activity.[79] In the same study, mouse kidneys were exposed to ischemic insults both *in vitro* by warm ischemia (37°C), to assess the effects of blood loss at normal body temperature and *in vivo* after depletion of blood supply by arterial clamping. Kidneys treated using both methods exhibited decreased levels of PARP activity.[79] However, kidneys exposed to cold ischemia *in vitro* (0°C, to assess the effects of organ storage as utilized for transplantation) exhibited elevated levels of PARP activity.[79] Furthermore, DNA isolated from ischemic kidneys had a stimulatory effect upon exogenous PARP extracted from calf thymus. In the same study, analysis of DNA extracted from "cold-storage" kidneys using electron microscopy revealed large (~500 b.p.) single-stranded regions. It was thus suggested that PARP activity was related to the nature of DNA damage resulting from an ischemic insult.[79]

In a recent study we investigated whether the PARP inhibitors 3-AB, ISO, or nicotinamide could provide protection against renal I/R injury in the rat *in vivo.*[37] Male Wistar rats were subjected to 45 min bilateral clamping of the renal pedicles followed by 6 h reperfusion. Renal I/R produced significant increases in plasma concentrations of urea and creatinine (Figure 8.3A), and a significant reduction in creatinine clearance (C_{CL}) indicating significant glomerular dysfunction.[37] Renal I/R also produced a significant increase in fractional excretion of Na^+ (FE_{Na})(Figure 8.3B), indicating significant tubular dysfunction.[37] Rats were administered the following PARP inhibitors as a bolus 1 min prior to beginning reperfusion, and an infusion was maintained throughout reperfusion: 3-AB (10 mg/kg bolus followed by 5 mg/kg/h), ISO (1 mg/kg bolus followed by 0.5 mg/kg/h), and nicotinamide (10 mg/kg bolus

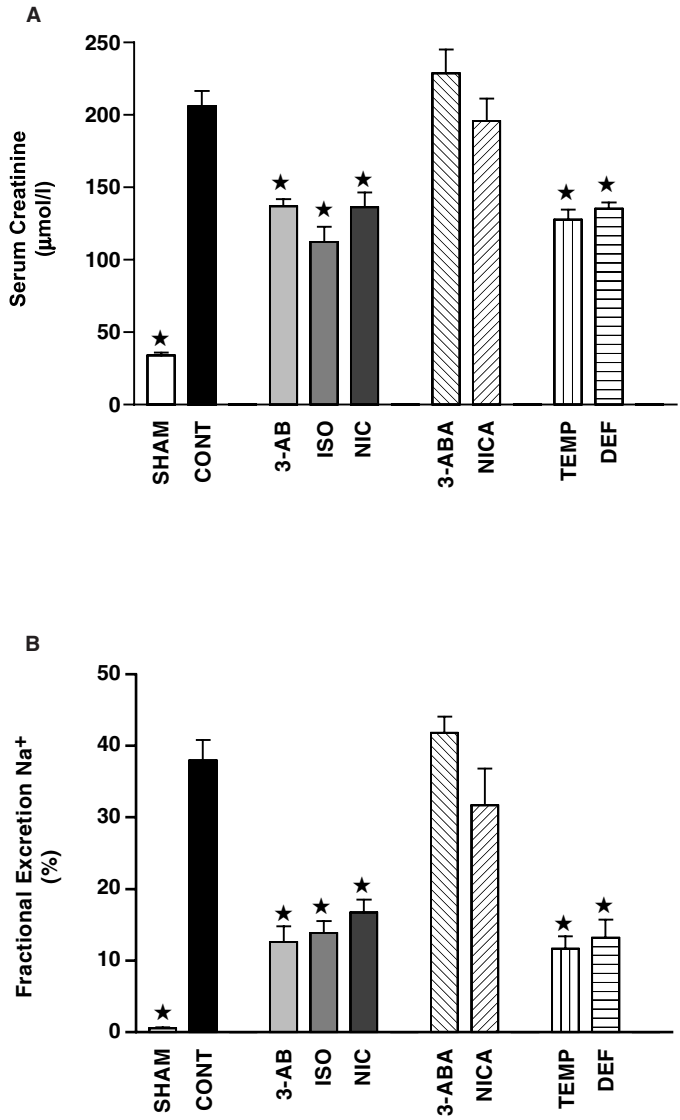

FIGURE 8.3 Effect of PARP inhibitors TEMPOL and deferroxamine on renal dysfunction mediated by I/R of the rat kidney. Alterations in (A) serum creatinine (marker of glomerular dysfunction) and (B) fractional excretion of Na^+ (marker of tubular dysfunction) after administration of the following PARP inhibitors immediately prior to and during reperfusion: 3-AB (10 mg/kg bolus + 5 mg/kg/h), ISO (1 mg/kg bolus + 0.5 mg/kg/h), or NIC (10 mg/kg bolus + 5 mg/kg/h). Inactive structural analogues, 3-ABA (10 mg/kg bolus + 5 mg/kg/h) and NIC A (10 mg/kg bolus + 5 mg/kg/h) were used as negative controls. The effects of the ROS-scavenger TEMPOL (30 mg/kg bolus + 30 mg/kg/h) and iron chelator DEF (40 mg/kg bolus + 40 mg/kg/h) were also investigated. ★ $P < 0.05$ vs. control (renal I/R-only), N = 6 to 12.

followed by 5 mg/kg/h). Rats that were administered the PARP inhibitors during reperfusion had significantly reduced plasma urea and creatinine concentrations (Figure 8.3A), and increased C_{CL}, suggesting improved glomerular function.[37] The PARP inhibitors also significantly increased FE_{Na}, suggesting recovery of tubular function (Figure 8.3B). Overall, recovery of tubular function was more pronounced than that of glomerular function, which was in line with *in vitro* data obtained using PT cell cultures demonstrating the beneficial actions of PARP inhibitors against oxidative stress.[16] As in those studies, the isoquinolinone ISO provided beneficial actions at a dose ten times lower than that of the benzamide analogues (3-AB and nicotinamide). In kidneys from animals subjected to I/R only, histological examination revealed severe PT damage and immunohistochemical localization demonstrated PARP activation in the PT.[37] Both histological evidence of PT damage and immunohistochemical evidence of PARP activation were markedly attenuated by administration of PARP inhibitors.[37]

Importantly, the beneficial effects were obtained when the PARP inhibitors were administered just prior to and during reperfusion, which may prove to be useful in the clinical scenarios in which cases of ARF are encountered. The reduction of I/R-mediated renal dysfunction was similar in magnitude to that obtained using the iron-chelating agent deferroxamine or the ROS-scavenger TEMPOL, which both also demonstrated beneficial actions against renal I/R-mediated glomerular (Figure 8.3A[37,38]) and tubular dysfunction (Figure 8.3B). Furthermore, both deferroxamine and TEMPOL reduced immunohistochemical evidence of PARP activation.[38] Thus, when compared with other drugs that demonstrate beneficial actions against renal I/R and ARF, PARP inhibitors may offer the advantage that they are able to salvage previously ischemic renal tissue even when administered at the onset of reperfusion without causing any hemodynamic effects.[37]

In a recent study by Martin and colleagues,[80] male Sprague-Dawley rats were subjected to bilateral renal clamping for 60 min followed by recovery. PARP expression was enhanced in the S3 segment of the PT (which also demonstrated histological signs of I/R injury) between 6 and 12 h after renal ischemia. Compared with untreated animals, intraperitoneal administration of benzamide (40 mg/kg) or 3-AB (10 mg/kg) increased ATP levels after 24 h and accelerated recovery of renal function 6 days later.[80] Interestingly, the effects of PARP inhibition on PT regeneration were also investigated by assessment of proliferating cell nuclear antigen (PCNA) expression. Regeneration of the PT subsequent to I/R injury plays a fundamental part in restoring structure and physiological function and is initiated by noninjured or sublethally injured PT cells.[81] PARP inhibition was found to increase cell proliferation (compared with vehicle controls) 24 h after ischemia, suggesting that PARP inhibition contributes to PT regeneration.[80] This was supported by the observation of increased levels of ATP 24 h after ischemia, which is required for the initiation of the cellular repair process. Overall, PARP inhibition improved the histological appearance of kidneys 7 days after ischemia. The authors concluded that inhibition of PARP activity ameliorated the course of ARF.[80]

Thus, taken together, the results of studies investigating the beneficial role of PARP inhibitors against oxidative stress-mediated injury to renal cells *in vitro* and against

renal I/R injury *in vivo* suggest that (1) PARP activation contributes to renal I/R injury (and is therefore likely to contribute to ARF) and (2) PARP inhibitors may prove to be beneficial in renal disorders associated with oxidative stress–mediated injury.

8.3 FUTURE DIRECTIONS

ARF is a common, life-threatening disease, the mortality of which has remained high over the last 40 years despite advances in supportive care.[82] ARF affects up to 5% of the hospitalized population[82] and is recognized as an independent risk factor for death with mortality rates exceeding 50% when it occurs in clinical settings involving trauma, surgery, or multiple organ dysfunction.[82] ARF remains an extremely difficult disease to treat as its pathophysiology is not well understood, and the therapeutic options are therefore limited.[19] The course of ARF is highly variable, ranging from a transient illness lasting less than 1 week and associated with full recovery of renal function to a disease persisting for longer than 1 month and requiring dialysis and intensive care management.[83] There is a positive correlation between the duration of dialysis (and/or renal dysfunction) and mortality from ARF.[83] Despite major advances in the understanding of the epidemiology of ARF, the results of recent clinical trials investigating the beneficial actions of novel interventions have been largely negative.[19] Thus, novel interventions, such as potent and specific inhibition of the PARP "Suicide Pathway" in the kidney, that ultimately prevent the need for dialysis, shorten the course of ARF, and improve survival in the 21st century are required.

8.3.1 PARP Inhibition and Acute Renal Failure

The question posed here is, "Could inhibition of PARP be useful against ARF in humans?" We have recently performed experiments using primary cultures of human PT cells isolated from the normal cortex of kidneys removed by nephrectomy for focal, polar tumors.

As for rat PT cells, pure populations of human PT cells were isolated using a combination of enzymatic digestion, mechanical sieving, and differential centrifugation in Percoll solution.[84] Recently, we have discovered that exposure of human PT cells to 3 m*M* H_2O_2 for 1 h results in a significant increase in PARP activation (Figure 8.4A), and, after 4 h, increased cellular injury (Figure 8.4B). We subsequently demonstrated that the PARP inhibitors 3-AB (3 m*M*), ISO (0.3 m*M*), and nicotinamide (3 m*M*) could significantly (1) inhibit PARP activity (Figure 8.4A) and (2) reduce H_2O_2-mediated PT cellular injury (Figure 8.4B), with ISO providing beneficial actions at ten times lower concentration than 3-AB or nicotinamide. The respective structural analogues of 3-AB and nicotinamide, 3-ABA (3 m*M*) and nicotinic acid (3 m*M*), which did not inhibit PARP activity (Figure 8.4A) did not provide any protection against H_2O_2-mediated cellular injury (Figure 8.4B). Interestingly, compared with the results obtained using rat PT cells, comparable concentrations of the PARP inhibitors provided human PT cells with a greater degree of protection against a higher level of oxidative stress (i.e., 3 m*M* H_2O_2 for human PT cells vs. 1 m*M* H_2O_2 for rat PT cells). For example, ISO (0.3 m*M*) increased mitochondrial respiration

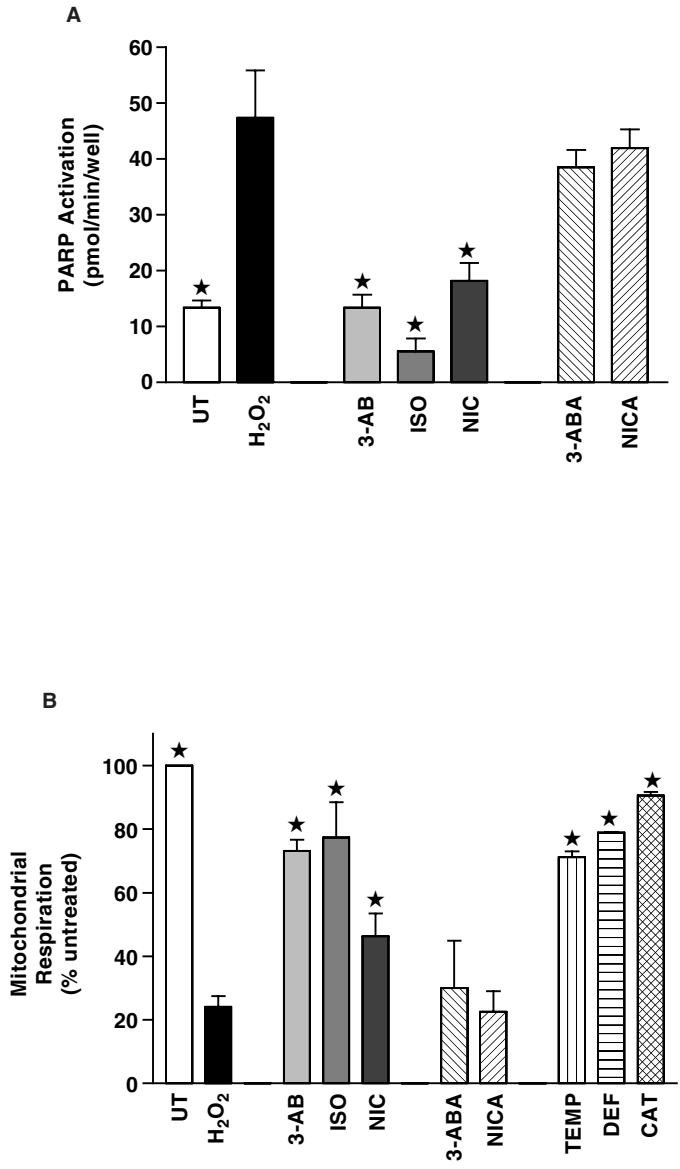

FIGURE 8.4 Effect of PARP inhibitors on (A) H_2O_2-mediated PARP activation and (B) H_2O_2-mediated cellular injury of human PT cells. Human PT cells were incubated in the absence (UT = untreated) or presence of H_2O_2 (1 m*M* for 4 h). PARP inhibitors used: 3-AB (3 m*M*), ISO (0.3 m*M*), NIC (3 m*M*). Inactive structural analogues (negative controls) used: 3-ABA (3 m*M*) and NIC A (3 m*M*). The effects of the ROS-scavenger TEMPOL (3 m*M*), the iron chelator DEF (3 m*M*), and catalase (3 U/ml) were also investigated. ★ $P < 0.05$ vs. H_2O_2-only, N = 2.

from 15% (H_2O_2 only) to 57% in rat PT cell cultures, whereas the same concentration of ISO increased mitochondrial respiration from 24% (H_2O_2 only) to 77% in primary cultures of human PT cells. The beneficial actions provided by PARP inhibitors against oxidative stress were similar in magnitude to those obtained using TEMPOL (3 m*M*), deferroxamine (3 m*M*) and catalase (3 U/ml).

However, several of the "traditional" PARP inhibitors used in previous studies have encountered problems with specificity and potency (benzamide analogues) and solubility (isoquinolines).[3] Specifically, 3-AB is a weak inhibitor of PARP activity that does not readily cross cell membranes.[6,7] Although the isoquinolines such as ISO and DPQ (3,4-dihydro-5-[4-(piperidin-1-yl)butoxyisoquinolin-1(2*H*)-one] are more potent inhibitors of PARP activity, both have to be initially dissolved in DMSO, which itself is a potent scavenger of hydroxyl radicals at higher concentrations.[76] Thus, there is still a great need for the development of new inhibitors which are selective, potent, and water soluble. Recently, 5-aminoisoquinolin-1(2*H*)-one (5-AIQ), a water-soluble inhibitor of PARP with high potency and enzyme selectivity *in vitro* has produced promising results from studies in which the effects of 5-AIQ on PARP activity in human cardiac myoblasts were investigated.[85] 5-AIQ was found to be a potent inhibitor of PARP activity *in vitro*, even in comparison with ISO[85] and significantly reduced H_2O_2-mediated cellular injury.[85] Subsequently, 5-AIQ reduced glomerular dysfunction caused by severe hemorrhage and resuscitation in an *in vivo* model of hemorrhagic shock in the anesthetized rat.[85] We are currently investigating if 5-AIQ can provide beneficial actions against renal dysfunction and injury mediated by I/R of the kidney.

8.4 CONCLUDING REMARKS

Taken together, our findings, and those of others, suggest that PARP activation contributes to the renal dysfunction and injury caused by I/R injury of the kidney and plays a major role in the development of ARF. It is now clear that inhibition of PARP activity provides beneficial actions against oxidative stress of renal cells *in vitro* and against renal I/R-mediated dysfunction *in vivo*. It is highly likely that novel, more potent, and soluble inhibitors of PARP activity will provide beneficial actions against renal I/R injury and will lead to the development of a novel therapies for patients suffering I/R injury of the kidney and who are at risk of developing ARF. However, a further issue that will complicate the development of PARP inhibitors for the therapy of ARF is the recent discovery of several different isoforms of PARP.[86] It will be extremely important and interesting to discover what role the different PARP isoforms play in the etiology of renal I/R dysfunction/injury and ARF and whether the development of novel, isoform-specific PARP inhibitors will provide useful therapeutic agents.

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9 Role of Poly(ADP-Ribose) Polymerase Activation in the Pathogenesis of Shock and Inflammation

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CONTENTS

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9.1 THE ROLE OF PARP ACTIVATION IN CELL DEATH

9.1.1 The poly(ADP-ribose) Polymerase Suicide Pathway

Poly(ADP-ribose) polymerase (PARP) is a protein-modifying and nucleotide-polymerizing enzyme that is abundantly present in the nucleus. PARP consists of the DNA-binding N-terminal domain, the central automodification domain, and the C-terminal catalytic domain. The DNA-binding domain utilizes two zinc fingers, which recognize breaks in double-stranded DNA. The central, highly conserved domain can be auto-poly-ADP-ribosylated by PARP. The C-terminal catalytic domain is involved in the synthesis of poly(ADP-ribose) polymer.[1,2] Recent work identifies several isoforms of PARP. For the current review, "PARP" generally refers to the firstly identified, common isoform, which is now also termed PARP-1.

The obligatory trigger of PARP activation is DNA single-strand breaks, which can be induced by a variety of environmental stimuli and free radical/oxidants, most notably hydroxyl radical and peroxynitrite (see below). In response to DNA damage, PARP becomes activated and, using NAD^+ as a substrate, catalyzes the building of homopolymers of adenosine diphosphate ribose units. Poly(ADP-ribose) acceptors include histones, topoisomerases I and II, DNA polymerases, and DNA ligase 2, as well as PARP itself. Poly(ADP-ribose) catabolism and metabolism is a dynamic process, with poly(ADP-ribose) glycohydrolase catalyzing the degradation of the polymer.[1,2]

Cellular NAD^+ levels are known to regulate an array of vital cellular processes. NAD^+ serves as a cofactor for glycolysis and the tricarboxylic acid cycle, thus providing ATP for most cellular processes. NAD^+ also serves as the precursor for NADP, which acts as a co-factor for the pentose shunt, for bioreductive synthetic pathways, and is involved in the maintenance of reduced glutathione pools. The observation that activation of PARP can lead to massive NAD^+ utilization and changes in the cellular NAD^+ levels led Berger et al.[3,4] to propose that consumption of NAD^+ due to DNA damage and activation of PARP can affect cellular energetics and function. In the 1980s, a variety of *in vitro* studies demonstrated that rapid depletion of NAD^+ due to PARP activation leading to cellular ATP depletion and functional alterations of the cell, with eventual cell death.[3-9] The relative contribution of PARP to the cellular metabolic changes and cell injury is dependent on the cell type studied. In endothelial cells, epithelial cells, and fibroblasts, PARP appears to play a greater role in the oxidant damage, whereas in hepatocytes inhibition of PARP has lesser influence on oxidant-induced cell damage.[10-20] The initial studies on the role of PARP were performed using pharmacological inhibitors of PARP (most frequently, 3-aminobenzamide and nicotinamide). These agents can have additional actions, for example, as free radical scavengers. More recent studies using cells from PARP knockout animals confirmed the role of the PARP pathway in oxidant-mediated cell injury. In the first such study, Heller and co-workers observed[21] that islets of the $PARP^{-/-}$ mice are resistant to NO and oxidant-related injury, when compared to the response in islets of the wild-type mice. Similarly, we observed that pulmonary fibroblasts from the $PARP^{-/-}$ mice are protected from peroxynitrite-induced cell injury when compared to

the fibroblasts of the corresponding wild-type animals.[17] Furthermore, Eliasson and colleagues[22] demonstrated protection by PARP-negative phenotype in brain slices exposed to various oxidants. Thus, the more definitive studies utilizing PARP knockout cells have now fully confirmed the conclusions of the earlier pharmacological studies. The mode of cell death by PARP overactivation has been clarified in recent studies.[23-25] It appears that the mechanism by which PARP activation leads to cell death is related to the triggering of cell necrosis (rather than apoptosis), which occurs because of the severe energetic crisis of the cell.[23-25] In fact, pharmacological inhibition of PARP shifts the necrotic cell population into the normal, as well as the apoptotic population, as determined by flow cytometry studies in thymocytes exposed to peroxynitrite or hydrogen peroxide.[23] The shift of cell death from necrotic to apoptotic, in the absence of functional PARP, is considered beneficial in inflammation, for necrotic cells (but not apoptotic cells) release their content into the extracellular space, thereby further triggering the inflammatory process (see also below).

9.1.2 Hydroxyl Radical and Peroxynitrite, as Triggers of DNA Single-Strand Breakage and PARP Activation

DNA single-strand breakage is an obligatory trigger of activation of the nuclear enzyme poly(ADP-ribose) polymearse (PARP). Peroxynitrite is a labile, toxic oxidant species produced from the reaction of superoxide and nitric oxide (NO).[26,27] This species, as well as hydroxyl radical, are the key pathophysiologically relevant triggers of DNA single-strand breakage.[14,17] The fact that hydroxyl radical, produced during oxidant stress or radiation injury, is able to induce DNA single strand breakage has been well known for longer than a decade.[28] In recent years, peroxynitrite emerged as another major trigger of DNA single strand breakage. In 1992, King and colleagues[29] demonstrated that potassium peroxynitrite induces DNA cleavage in solutions of end-labeled DNA restriction fragments. Over the past years, several groups have independently demonstrated the occurrence of DNA single strand breakage in various types of intact cells upon exposure to peroxynitrite. For example, in calf thymus DNA, and in the bacteriophage PM2, DNA strand breakage has been reported after exposure to a peroxynitrite or peroxynitrite-generating compounds.[30-32] Moreover, DNA single-strand breakage has been demonstrated in our laboratory in cultured macrophages and smooth muscle cells exposed to peroxynitrite.[14,17,33] Also nitroxyl anion (NO^-), a no-derived reactive species can trigger DNA single strand breakage and PARP activation *in vitro*.[34] The mechanism of the DNA strand breakage is probably related to abstraction of hydrogen atoms from the ribose of the DNA moiety, thereby opening the sugar ring.[30]

9.1.3 Endogenously Produced Oxidants Can Also Trigger DNA Single-Strand Breakage and PARP Activation

Earlier than 10 years ago it was generally assumed that triggers of DNA single-strand breakage are restricted to severe, environmental toxic agents e.g., genotoxic or cytotoxic drugs or various forms of ionizing radiation.[28,35-37] The research into the suicidal

role of PARP gained a new momentum in the mid-1990s by studies linking the formation of NO — an endogenously produced, reactive free radical species produced from L-arginine by a family of enzymes termed NO synthases — to DNA single-strand breakage and PARP activation, with subsequent energetic changes in the cell.[38,39] Subsequent studies clarified that the actual trigger of DNA single-strand breakage is peroxynitrite, rather than NO per se:[14] "pure" NO donors, even at high concentrations, are relatively weak inducers of DNA single-strand breakage, when compared to the effect of peroxynitrite (see References 14, 15, and 40).

The identification of NO and peroxynitrite as important mediators of the cellular damage in various forms of inflammation stimulated significant interest into the role of the PARP-related suicide pathway in various pathophysiological conditions. Endogenous production of peroxynitrite and other oxidants has been shown to lead to DNA single-strand breakage and PARP activation. For example, in immunostimulated macrophages and smooth muscle cells, which simultaneously produce NO and superoxide, and thus peroxynitrite from endogenous sources,[14,33,41,42] DNA single-strand breakage has been demonstrated, and the time course of the strand breakage was shown to parallel the time course of NO and peroxynitrite production.[14,33] Similarly in brain slices, activation of NMDA receptors (a trigger for enhanced NO, superoxide, and peroxynitrite production) led to peroxynitrite-mediated DNA single strand breakage and PARP related cell injury.[39,40] In a recent study, using co-culture of activated macrophages and hepatocytes, it was concluded that activated macrophage-derived NO and its oxidative metabolite, peroxynitrite, play key roles in hepatocyte injury during inflammation, and cause subsequent DNA damage (including a significant degree of DNA single-strand breakage, but also other types of DNA base modifications) in surviving hepatocytes.[43] Similarly, the ability of activated neutrophils to induce DNA single-strand breakage in neighboring cells is well documented.[44]

Interestingly, recent work indicates that in intact mammalian cells the process of DNA single-strand breakage by peroxynitrite may not be a direct result of peroxynitrite interacting with nuclear DNA, but may also be related, at least in part, to endogenous production of oxidants. For example, in thymocytes exposed to peroxynitrite, there is a time-dependent gradual increase in mitochondria-derived reactive oxygen species generation.[24] In a study in human hepatocytes, there is a persistent and marked increase in DNA damage following the treatment with NO or peroxynitrite generators, which appears to arise from the disruption of electron transport in the mitochondria.[45] Cantoni and colleagues[46] proposed that peroxynitrite mediates inhibition of mitochondrial complex III and, under these conditions, electrons are directly transferred from ubisemiquinone to molecular oxygen. Hydrogen peroxide is produced by the dismutation of superoxides, and this process was proposed as the actual species mediating the peroxynitrite-dependent DNA cleavage. A calcium-dependent step may also be important in the processes triggering peroxynitrite-induced DNA single-strand breakage. Our studies in thymocytes exposed to low, pathophysiologically relevant concentrations (20 μM) of peroxynitrite demonstrated a rapid (1 to 3 min) Ca^{2+} mobilization. Inhibition of this early calcium signaling by

cell-permeable Ca^{2+} chelators abolished cytotoxicity as measured by propidium iodide uptake.[47] Intracellular Ca^{2+} chelators also inhibited DNA single-strand breakage and activation of PARP in the same experimental model.[47]

The time course of PARP activation, in relation to the time course of various other free radical-induced cytotoxic processes, has been investigated in detail by Schraufstratter and colleagues.[7-9] Various cell types were exposed to oxidants that are generated from stimulated leukocytes, including H_2O_2, superoxide, and HOCl. The target cells used were P388D1 murine macrophage-like tumour cells, human peripheral lymphocytes, GM 1380 human fibroblasts, and rabbit alveolar macrophages. The oxidants used were H_2O_2 and phorbol ester-stimulated neutrophils. In this experimental system, cell lysis could only be prevented when catalase was added within the first 30 to 40 min of H_2O_2 exposure, indicating that early metabolic changes determined the fate of the cell. Within seconds after the addition of H_2O_2 to the cells, activation of the hexose monophosphate shunt was observed, indicative of increased glutathione cycle activity. At the same time, DNA strand breaks (determined by an alkaline unwinding technique) were detected. They resulted in the rapid activation of PARP (within minutes after the addition of H_2O_2). At the same time, ATP and NAD^+ concentrations dropped and nicotinamide accumulated extracellularly. Approximately 15 min after oxidant exposure, free intracellular Ca^{2+} concentrations, as determined by Quin 2 fluorescence, started to increase due to release of calcium from intracellular stores.[7-9] These findings, collectively, indicate the rapid activation and central role of PARP in the pathogenesis of oxidant-induced cell injury.

As mentioned above, peroxynitrite and hydrogen peroxide are potent oxidants, whereas NO is not. It is noteworthy that, although NO per se does not directly and acutely cause DNA single-strand breaks in most mammalian cells, several hypotheses have been proposed for indirect mechanisms. Unrepaired abasic sites (due to NO-mediated injury) may lead to the development of DNA single-strand breaks.[48] The development of these DNA strand breaks may involve AP endonucleases, excision repair, topoisomerase-mediated repair, Ca^{2+}/Mg^{2+} dependent endonucleases.[48] Furthermore, it has been proposed that inhibition of ribonucleotide reductase by NO may reduce the supply of deoxyribonucleotides for DNA synthesis and repair, which, in turn, may cause a delay in the repair process and lead to the prevalence of DNA single-strand breaks.[49] Similarly, it is possible that inhibition by NO of a variety of DNA repair processes, as demonstrated, for example, for the Fpg protein and for O6-methylguanine-DNA-methyltransferase,[50,51] may increase the degree of DNA single-strand breakage in cells challenged with oxidants. Even if inhibition of NO-mediated inhibition of 8-oxodG base excision DNA repair processes may not be sufficient to induce DNA injury per se, they may well be considered as a mechanism potentiating DNA damage in human inflammatory diseases, as shown, among others, in recent studies involving the biliary tract.[52] Additional mechanisms of NO-related injury may involve the production of oxyradicals by the mitochondrial chain. In such scenario, NO may first inhibit the activity of mitochondrial enzymes, which subsequently triggers increased oxyradical generation from the mitochondria,[53] (see also below). One can hypothesize that this process may lead to the generation of peroxynitrite, and

subsequent DNA single-strand breakage. An example of such a mechanism has recently been described in relation to tumor necrosis factor–induced inhibition of mitochondrial respiration, oxyradical production, DNA injury, and PARP activation in L929 cells.[54]

9.1.4 The Nature of PARP-Related Cell Death in Inflammation

In recent years, PARP (or specifically, its cleavage) has been implicated in apoptotic cell death (programmed cell death). In this scheme, it is proposed that the role of PARP is to act as a "death substrate" for caspases.[55-57] Since PARP is assumed to be an enzyme important in DNA repair, cleavage (i.e., inactivation) of PARP, in turn, would lead to enhanced apoptosis, because it would end the "basal" poly(ADP-ribosyl)ation of Ca^{2+}/Mg^{2+}–dependent endonuclease, and the ADP-ribosylation of histone H1, which are required for a "basal" suppression of apoptosis.[58,59] In this context, the PARP-related mechanisms of apoptotic cell death are perceived as terminal, delayed effectors.[60,61] Pharmacological inhibition of PARP is not expected to modulate the PARP-cleavage and related cell death processes: the cleaved PARP enzyme is catalytically inactive.

In contrast, in the scheme of PARP activation the process of "DNA single-strand breakage >> PARP activation >> energy depletion" induces necrotic, rather than apoptotic cell death, characterized by rapid cell injury, changes in membrane integrity, release of lactate dehydrogenase from the cells, and mitochondrial injury.[21-25,39,62] In fact, in cells challenged with peroxynitrite or hydrogen peroxide, inhibition of PARP diverts the mode of cell death from the necrotic toward the apoptotic mode, or protects against cell injury altogether.[23,63] In these latter studies, strong, oxidant-induced triggers of cell death were used, which induce a substantial degree of energetic changes and a substantial degree of necrosis-like cell death, while in many previous studies investigating the role of PARP in apoptosis, the stimuli used mainly induced milder cell injury and delayed apoptosis.

Recently, we have also investigated the role PARP in intestinal epithelial barrier function, an active process that is highly dependent upon cellular ATP concentration. Exposure of human Caco-2BBe enterocyte cell monolayers to peroxynitrite rapidly induced DNA strand breaks and triggered an energy-consuming pathway catalyzed by PARP.[64] The consequent reduction of cellular stores of ATP and NAD^+ was associated with the development of hyperpermeability of the epithelial monolayer to a fluorescent anionic tracer. Pharmacological inhibition of PARP activity had no effect on the development of peroxynitrite-induced DNA single-strand breaks, but attenuated the decrease in intracellular stores of NAD^+ and ATP and the functional loss of intestinal barrier function. Similar results pertain to epithelial cells exposed to peroxynitrite.[19]

Many studies implicate mitochondrial alterations in the process of necrotic cell death. In fact, damage to the mitochondria appears to be the primary early event in necrosis.[65,66] At least two pathways may be involved. In the first, inhibition of oxidative phosphorylation in the absence of the mitochondrial permeability transition

leads to ATP depletion, ion dysregulation, and enhanced degradative hydrolase activity. If oxygen is present, toxic oxygen species may be generated and lipid peroxidation can occur. Subsequent cytoskeleton and plasma membrane damage result in plasma membrane bleb formation. If injury continues, bleb rupture and cell lysis occur. In the second pathway, mitochondrial damage results in a mitochondrial permeability transition. This step is believed irreversible and leads to cell death.[65,67] It is believed that mitochondrial permeability transition occurs secondary to changes in the first pathway e.g., oxidative stress, increased intracellular Ca^{2+}, and ATP depletion, and that all the "downstream events" occurring in the first pathway may result from permeability transition. Mitochondrial permeability transition involves the formation of proteaceous, regulated pores, probably by apposition of inner and outer mitochondrial membrane proteins, which cooperate to form the mitochondrial megachannel, also known as the mitochondrial permeability transition pore. The permeability transition has important metabolic consequences, namely, the collapse of the mitochondrial transmembrane potential, uncoupling of the respiratory chain, hyperproduction of superoxide anions, disruption of mitochondrial biogenesis, outflow of matrix Ca^{2+} and glutathione, and release of soluble intermembrane proteins.[65,68] Recent studies have demonstrated the occurrence of the above-listed mitochondrial alterations in various cell types challenged with peroxynitrite.[24,69] Importantly, the changes in mitochondrial membrane potential, the mitochondrial permeability transition, the increase in reactive oxygen intermediate production, the increased Ca^{2+} mobilization, and the destruction of mitochondrial structure are attenuated by inhibition of the activation of PARP.[24] Because cellular NAD^+ and ATP are important regulators of mitochondrial functions,[70-74] maintenance of cellular energetic pools in cells where PARP is inhibited may explain the improvement of the mitochondrial function.

A widely held view is that necrosis is a process that cannot be influenced by pharmacological means, and apoptosis is the process that is under the control of a sophisticated cellular machinery. Recent reports, demonstrating protection against cell injury by inhibition of PARP, however, prove that the necrotic process, indeed, is amenable to pharmacological interventions. Additional mechanisms that can affect necrotic cell death include Bcl-2, which can protect against necrosis.[75-78] The most likely mode of Bcl-2's action is inhibition of secondary free radical generation in the mitochondria of cells exposed to peroxynitrite. Similarly, heat shock protein 2775 and certain free radical scavengers that inhibit secondary, mitochondria-related free radical generation) can protect against necrotic cell death.[65,67] In some systems, inhibition of interleukin-1 converting enzyme can protect against necrotic cell death.[76] Thus, according to the more current models, necrosis does not equal overwhelming injury, but is controlled and can be modulated (i.e., suppressed by antioxidants, Bcl-2, heat shock protein 27, or PARP) to achieve survival benefit.

Although the end result of the PARP-overactivation pathway is a necrotic cell, it may be worthwhile introducing the concept of a "pre-necrotic" stage of cell function. This stage may describe a cell with free radical-mediated or oxidant-mediated damage, DNA injury, PARP activation, compromised energetic status, and compromised cell function, without actually reaching the stage of cell death. We believe that

pharmacological inhibition of PARP, by restoring cellular energetics, may rescue cells from this pre-necrotic stage, and thereby can restore cell function. Evidence for such an effect was found, for example, in endothelial cells producing high levels of endogenous oxidants,[79] and also in intestinal epithelial cells from colitic guts (see below).

Necrotic (or pre-necrotic) cell death appears to be an important pathway of cell death, which has direct relevance for the pathophysiology of various forms of inflammation. Under inflammatory conditions, overwhelming oxidant production can occur, and cells rapidly die via necrosis. The importance of PARP in mediating this process is underlined by recent *in vivo* experiments, where pharmacological inhibition or inactivation of PARP protects against cell necrosis in various forms of inflammation and reperfusion injury (see below).

9.2 ROLE OF THE PARP PATHWAY IN VARIOUS FORMS OF INFLAMMATION

9.2.1 Zymosan- and Carrageenan-induced Inflammatory Models

Recent studies have clearly demonstrated the role of PARP activation in various forms of local or systemic inflammation induced by the prototypical inflammatory stimuli zymosan and carrageenan. For example, in carrageenan-induced paw edema, inhibition of PARP with 3-aminobenzamide reduced paw swelling and inhibited the infiltration of neutrophils into the inflamed paw.[80] Furthermore, in a model of acute local inflammation (carrageenan-induced pleurisy), 3-aminobenzamide inhibits the inflammatory response (pleural exudate formation, mononuclear cell infiltration, histological injury).[81,82] Similar to the effect of the pharmacological inhibitors, $PARP^{-/-}$ animals are resistant against zymosan-induced inflammation and multiple organ failure when compared to the response of wild-type mice.[80] GPI 6150 (1,11b-dihydro-[2*H*]benzopyrano[*4,3,2-de*]isoquinolin-3-one), a novel potent PARP inhibitor, was also found very effective in attenuating joint swelling and various parameters of inflammation in rodent models of carrageenan-induced paw edema and zymosan-induced multiple organ failure.[83]

Inhibition of PARP also reduced the formation of nitrotyrosine — an indicator of the formation of peroxynitrite — in the inflamed tissues.[80,82] This finding was at first unexpected because PARP activation is distal to the generation of oxidants. The explanation for this finding is likely related to the fact that $PARP^{-/-}$ phenotype or pharmacological inhibition of PARP reduces the infiltration of neutrophils into inflammatory sites.[17,80,82] Thus, the reduction in tissue injury by PARP inhibitors may result from a decreased inflammatory infiltrate, which would be associated with a reduction in both oxygen- and nitrogen-centered free radical production (hence, reduced nitrotyrosine staining). The basis for PARP-inhibitable neutrophil infiltration is not yet defined, but may relate to the effect of PARP activation on the expression of intercellular adhesion molecules or may be due to modulation by PARP of a postadhesion event.[80-86] Other mechanisms by which PARP modulates neutrophil tissue infiltration cannot be excluded, including an effect on endothelial integrity.[16,79]

9.2.2 Arthritis

Oxygen-derived free radicals and oxidants are massively overproduced in arthritis.[87-90] Furthermore, several lines of evidence suggest a role for NO overproduction in the pathogenesis of arthritis. The expression of the inducible isoform of NO synthase and the production of large amounts of NO have been demonstrated in chondrocytes from experimental animals and humans.[91-95] An increase in the circulating levels of nitrite/nitrate the breakdown products of NO has been demonstrated in patients with arthritis.[96,97] Increased plasma and synovial fluid levels of nitrotyrosine, a marker of peroxynitrite formation, have been demonstrated in patients with arthritis.[98] Similarly, increased nitrotyrosine formation was observed in the joints of mice suffering from collagen-induced arthritis.[17] The development of the disease has been shown to be ameliorated by various, non-isoform-selective inhibitors of NO synthase in various animal models of adjuvant-induced arthritis.[99-106] Mercaptoethylguanidine, an anti-inflammatory agent with a combined mechanism of action — inhibition of the inducible isoform of NO synthase, scavenging pathophysiological free radicals and inhibition of cyclooxygenase — also provided marked beneficial effects in a collagen-induced arthritis.[107]

Several series of experiments directly implicate the key role of PARP activation in the pathophysiology of arthritis. In murine models of arthritis, inhibition of PARP with nicotinamide, or with nicotinic acid amide, reduced the onset of the disease.[108-110] The onset, progression, and remission of arthritis positively correlated with the phorbol ester–activated respiratory burst of neutrophils and monocytes.[108] Inhibition of PARP not only prevented the development of arthritis, but also inhibited the progress of established collagen-induced arthritis.[17,110] The combined application of thalidomide (as a drug that inhibits tumor necrosis factor-α expression in arthritis) and nicotinic acid amide provided a powerful synergistic inhibition of arthritis.[110] Furthermore, recent studies with 5-iodo-6-amino-1,2-benzopyrone, a novel PARP inhibitor that lacks oxyradical-scavenging properties also protected in a mouse model of collagen-induced arthritis: the PARP inhibitor reduces both the incidence of arthritis and the severity of the disease throughout the experimental period.[17] Histological evaluation of the paws in the vehicle-treated arthritic animals revealed signs of severe suppurative arthritis, with massive mixed (neutrophil, macrophage, and lymphocyte) infiltration. In the animals treated with the PARP inhibitors, the degree of arthritis was significantly reduced: a moderate, primarily neutrophil infiltration into several of the larger joints, coupled with mild to moderate necrosis and hyperplasia of the synovium.[17] GPI 6150, another potent PARP inhibitor, was also found highly effective in a rodent model of adjuvant-induced arthritis.[83] PJ 34, another potent, novel PARP inhibitor was found highly effective in a murine model of collagen-induced arthritis[111] (Figure 9.1). Finally, PARP-1 deficient mice are largely resistant to collagen-induced arthritis (joint swelling and inflammatory parameters) when compared to mild-type animals (F. Mabley, C. Szabó, unpublished observations, 2001). As in the other forms of inflammation, hydroxyl radical and peroxynitrite are the most likely triggers of PARP activation.

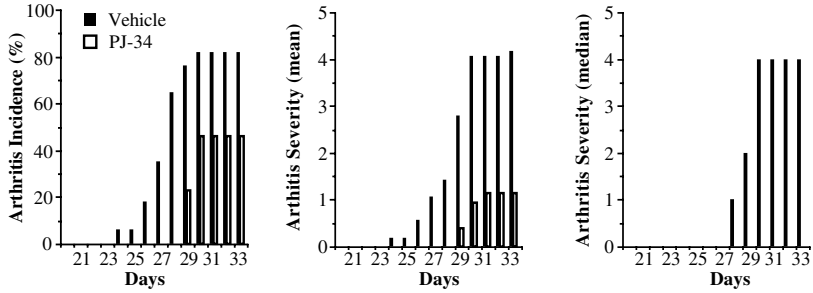

FIGURE 9.1 Antiarthritic effect of the phenantridinone PARP inhibitor PJ 34. The PARP inhibitor compound (or appropriate vehicle in the control group) was administered at 10 mg/kg oral gavage, twice a day, starting simultaneously with the second injection of collagen on day 21 on arthritis development in a mouse model of collagen-induced arthritis in male DBA/1J mice. Mice were evaluated daily for arthritis by using a macroscopic scoring system (0 to 4 scale). The arthritic index for each mouse was calculated by adding the four scores of individual paws. N = 16 animals per group. PJ 34 provided a significant protection against the development of arthritis in this model.

PARP activation has not yet been directly demonstrated in samples from patients with arthritis. Nevertheless, in a human study analyzing DNA single-strand breakage in peripheral mononuclear cells from patients with arthritis, a significant elevation was found, when compared to normal volunteers.[112] Studies conducted about a decade ago in humans reported increased frequency of circulating antibodies against PARP, chiefly in patients suffering from systemic lupus erythematosus (SLE) as well as rheumatoid arthritis.[113-115] It is likely that these antibodies do not directly reflect on the potential PARP activation in these patients, but, rather, may be related to disease-associated increased cell necrosis, followed by systemic spillage of PARP from the nucleus, and a subsequent immune reaction against PARP (as well as numerous other components of nuclear and cytoplasmatic debris) in these patients. In another study, patients with SLE showed an approximately 70% decrease in poly(ADP-ribose) synthesis; this decreased synthesis persisted even with the addition of histones or DNase.[115] The findings of increased DNA strand breakage, coupled with decreased *ex vivo* PARP activity, may be related to either (1) an earlier increase in PARP activation, followed by auto-ADP-ribosylation of the enzyme, and eventual inactivation or (2) massive cleavage and inactivation of PARP due to apoptotic processes.

9.2.3 Inflammatory Bowel Disease

It is well established that inflammatory bowel disease is associated with the production of oxygen-derived free radicals and oxidants.[116-119] Increased NO production from the inducible NO synthase has also been proposed to be responsible for various experimental models of inflammatory bowel disease[120-127] and ulcerative colitis in humans, where inducible NO synthase activity and elevated levels of luminal nitrite

have been detected in rectal dialysates and in biopsy specimens.[128-131] During inflammatory bowel disease, the simultaneous production of superoxide and NO is likely to produce peroxynitrite and to promote oxidative reactions. Biochemical evidence for the formation of peroxynitrite has been provided in an experimental model of ileitis in guinea pigs by immunohistochemical staining of nitrotyrosine in epithelial cells.[132] Similarly, in human samples of active Crohn's lesions, massive nitrotyrosine staining has been reported.[133] The role of peroxynitrite in the pathogenesis of colitis is further supported by the fact that intracolonic administration of exogenous peroxynitrite produces a severe mucosal damage in rats.[134] Experimental studies have shown that the inflammatory response can be reduced by administration of NO synthase inhibitors, such as N^G-nitro-L-arginine methyl ester and aminoguanidine.[120-127]

Recent studies in rodent models of experimental colitis support the role of PARP activation in the pathogenesis of the disease.[86,135,136] Intraluminal administration of the hapten trinitrobenzene sulfonic acid in 50% ethanol induced mucosal erosion and ulceration associated with increased neutrophil infiltration, lipid peroxidation, an intense staining for nitrotyrosine, and progressive weight loss. Genetic ablation of the PARP gene or pharmacological inhibition of PARP with 3-aminobenzamide resulted in significant resistance to the damage induced by trinitrobenzene sulfonic acid administration, reduced nitrotyrosine formation and tissue levels of malondialdehyde, and reduced neutrophil recruitment into the injured tissue.[86,135] These *in vivo* data are in good agreement with recent *in vitro* studies demonstrating protection by pharmacological inhibition of PARP against intestinal epithelial cell injury (necrosis) induced by hydrogen peroxide[62] or peroxynitrite.[64] The latest study assessed the role of PARP in the colitis seen in interleukin (IL)-10 gene-deficient mice. IL-10 gene-deficient mice demonstrated significant alterations in colonic cellular energy status in conjunction with increased permeability, proinflammatory cytokine release, and nitrosative stress.[136] After 14 days of treatment with 3-aminobenzamide, IL-10 gene-deficient mice demonstrated normalized colonic permeability; reduced tumor necrosis factor-α and interferon-γ secretion, inducible NO synthase expression, and nitrotyrosine levels; and significantly attenuated inflammation. Time course studies demonstrated that 3-aminobenzamide rapidly altered cellular metabolic activity and decreased cellular lactate levels. This was associated with normalization of colonic permeability and followed by a downregulation of proinflammatory cytokine release.[136] Importantly and unexpectedly, not only was the deterioration of the intestinal epithelial function prevented by PARP inhibition *in vitro*, but *ex vivo* incubation of intestinal segments was able to restore some of the intestinal epithelial function.[136] This latter finding may indicate that the intestinal epithelial cell population is not yet necrotic or dead, but, rather, persists in a state of metabolic and functional suppression (pre-necrosis, see above), which can be reversed by inhibition of PARP.

9.2.4 Inflammatory Disease of the Central Nervous System: Allergic Encephalomyelitis and Multiple Sclerosis

Increased oxygen-derived free radical production and oxidative injury have been reported in central nervous system (CNS) tissues from animals subjected to

experimental allergic encephalomyelitis[137-141] and oxidative injury has been implicated in the pathogenesis of chronic CNS inflammatory disorders such as multiple sclerosis. The production of NO by invading macrophages or CNS-resident cells of the macrophage/monocyte lineage has also been implicated in the pathogenesis of chronic diseases of the CNS, such as multiple sclerosis.[142-146] The overproduction of NO in inflammatory diseases of the CNS is due to the expression of the inducible NO synthase, which is strongly upregulated in experimental allergic encephalomyelitis and immune-mediated viral diseases of the CNS. Similarly, in brain tissue from patients with multiple sclerosis where cells expressing inducible NO synthase–specific mRNA have been identified.[143,147] The overproduction of NO and oxyradicals in experimental allergic encephalomyelitis leads to the generation of peroxynitrite. Accordingly, increased nitrotyrosine staining has been reported in humans with multiple sclerosis, as well as in the active experimental allergic encephalomyelitis lesions.[143,144,148] Furthermore, a putative peroxynitrite scavenger has been shown to improve the outcome of experimental allergic encephalomyelitis in mice.[146]

Recent data directly implicate the role of the peroxynitrite–PARP axis in the pathogenesis of experimental allergic encephalomyelitis (EAE). In a rat model of experimental allergic encephalomyelitis in male Lewis rats, 3-aminobenzamide and the novel, potent PARP inhibitor 5-iodo-6-amino-1,2-benzopyrone delayed the course of the disease.[149] PARP inhibition resulted in both a delay in the onset as well as a reduction in the incidence and severity of disease signs. Increased poly-(ADP-ribose) immunoreactivity was associated with the development of the brain lesions in vehicle-treated rats, whereas inhibition of PARP with 5-iodo-6-amino-1,2-benzopyrone eliminated the development of the lesions and abolished poly(ADP-ribose) immunoreactivity.[149] Similarly, pharmacological inhibition of PARP with the novel potent PARP inhibitor PJ 34 potently reduces neurological signs and improves survival in a murine model of EAE (G. Scott, C. Hooper, and C. Szabó, unpublished observations, 2001).

The mechanism by which inhibition of PARP suppresses the course of experimental allergic encephalomyelitis has not been clarified. In fact, even the exact pathogenesis of experimental allergic encephalomyelitis is unclear at present. Undoubtedly, one of the major features of multiple sclerosis and experimental allergic encephalomyelitis is demyelination. Experimental allergic encephalomyelitis (and presumably multiple sclerosis) is triggered and amplified by a variety of interrelated immunological events. Immunological, clinical, and pathological studies suggest that T lymphocytes directed against myelin antigens are involved in the pathogenesis of multiple sclerosis. It is now clear that myelin basic protein or proteolipid-protein-specific T cells mediate the destruction of CNS myelin in experimental allergic encephalomyelitis. Although the autoimmune disease is initiated by antigen-specific autoreactive T cells, there is accumulating evidence that CNS injury is essentially mediated by CNS-infiltrating inflammatory cells, and inhibition of cell infiltration can suppress the course of experimental allergic encephalomyelitis.[150-152] In addition, it is established that activated inflammatory mononuclear cells contribute to tissue damage in several inflammatory diseases by releasing highly reactive oxygen metabolites[141] and nitrogen metabolites (see above) and by subsequent activation

of matrix metalloproteinases.[153] It is therefore possible that demyelination associated with experimental allergic encephalomyelitis/multiple sclerosis results from oxidative injury caused by a cascade of reactive oxygen and nitrogen metabolites produced by CNS-infiltrating activated macrophages and other inflammatory cells. The infiltration of mononuclear cells into the CNS is a process that is closely linked to the breakdown of the blood-brain barrier, a process related to the production of oxidants and free radicals in experimental allergic encephalomyelitis.[154,155] Once mononuclear cells infiltrate the CNS, and myelin degradation begins, a variety of positive feed-forward cycles initiate. For example, phagocytosis of opsonized myelin can trigger the induction of the inducible NO synthase in macrophages, which can, in turn, further enhance the process of demyelination during multiple sclerosis or experimental allergic encephalomyelitis.[156] Induction of the inducible NO synthase and expression of proinflammatory cytokines may enhance each other during experimental allergic encephalomyelitis. This is supported by the finding that aminoguanidine, an inhibitor of the inducible NO synthase, reduced the expression of tumor necrosis factor-α, in experimental allergic encephalomyelitis.[157] Although the exact cell types involved have not yet been identified, recent data indicate that activation of NMDA receptors also plays an important role in the pathogenesis of experimental allergic encephalomyelitis. In fact, antagonists of these receptors has been shown to suppress the course of disease.[158,159] Possibly this process is related to the decreased metabolism of glutamate in astrocytes during experimental allergic encephalomyelitis.[160] Considering the abundant evidence for a role of PARP activation in the pathogenesis of NMDA-mediated neuroinjury,[39,22] the above studies lend further support to our working hypothesis that PARP activation plays a role in experimental allergic encephalomyelitis, and inhibition of PARP has beneficial effects.

Currently, the cellular and molecular targets where inhibition of PARP would interrupt the inflammatory cascade leading to demyelination in experimental allergic encephalomyelitis are unclear. Nevertheless, several possibilities can be considered by which PARP inhibition prevents myelin degradation in experimental allergic encephalomyelitis, such as (1) protection against oligodendrocyte death, and improved myelin synthesis; (2) protection against astrocyte death; (3) protection against the breakdown of the blood–brain barrier; (4) the related inhibition of mononuclear cell infiltration into the CNS; (5) inhibition of the expression of the inducible NO synthase during experimental allergic encephalomyelitis; and (6) inhibition of NMDA-activation related cell injury. With respect to oligodendrocyte death, there are direct *in vitro* data to show that oligodendrocytes and astrocytes are susceptible to NO-induced or hydrogen peroxide induced mitochondrial damage and death, and the cell death can be partially inhibited by PARP inhibitors.[161,162] Thus, it is conceivable that oligodendrocytes and astrocytes would be injured in a PARP-dependent fashion during the course of experimental allergic encephalomyelitis. Furthermore, and similar to the inflammatory responses of peripheral organs, where inhibition or genetic inactivation of PARP suppresses inflammatory cell recruitment,[80] it can be expected that inhibition of PARP would have similar actions in the CNS as well.

9.2.5 Systemic Inflammatory Response Syndrome — Circulatory Shock

Circulatory shock is associated with the enhanced formation of oxyradicals[163,164] and with the expression of a distinct inducible isoform of NOS, resulting in overproduction of NO.[165,166] NO and superoxide react to form peroxynitrite, which can be demonstrated in various organs and tissues in animals or humans suffering from various forms of systemic inflammation and shock.[167-169] In isolated cells and tissues, authentic peroxynitrite is capable of mimicking many of the pathophysiological alterations associated with shock (endothelial and epithelial dysfunction, vascular hyporeactivity, and cellular dysfunction), and these alterations are, in part, related to PARP activation.[15,16,64]

The vascular contractile failure associated with circulatory shock is closely related to overproduction of NO within the blood vessels. Expression of the inducible NO synthase within the vascular smooth muscle cells has been implicated in the pathogenesis of vascular hyporeactivity during various forms of shock.[166,170] A superoxide dismutase mimetic also offers significant protection against the suppression of the vascular contractility of the thoracic aorta in a rat model of endotoxic shock, suggesting that the vascular hyporeactivity may be related to peroxynitrite generation, rather than NO per se.[171] In studies in anesthetized rats, inhibition of PARP with 3-aminobenzamide and nicotinamide were able to reduce the bacterial lipopolysaccharide (LPS) induced suppression of the vascular contractility of the thoracic aorta in *ex vivo* experiments.[15,172] Similarly, in a murine model of cecal ligation and puncture, inhibition of PARP with 3-aminobenzamide improved microvascular contractility.[173] In another recent study in pigs injected with *Escherichia coli* endotoxin, pretreatment with 3-aminobenzamide eliminated the LPS-induced rise in pulmonary and total respiratory resistance, indicating that PARP activation plays an important role in the changes of lung mechanics associated with endotoxin-induced acute lung injury.[174]

Peroxynitrite production has been suggested to contribute to endothelial injury in ischemia-reperfusion, circulatory shock, and atherosclerosis.[15,171] Peroxynitrite can impair the endothelium-dependent relaxations.[15,175] Current data, demonstrating protective effects of 3-aminobenzamide against the development of endothelial dysfunction in vascular rings obtained from rats with endotoxic shock,[15] suggest that DNA strand breakage and PARP activation occur in endothelial cells during shock and that the subsequent energetic failure reduces the ability of the cells to generate NO in response to acetylcholine-induced activation of the muscarinic receptors on the endothelial membrane. Indeed, several lines of *in vitro* data demonstrate DNA injury, PARP activation, and consequent cytotoxicity in endothelial cells exposed to hydroxyl radical generators,[10,11,13,18] or in response to peroxynitrite.[15,79] The relative contribution of peroxynitrite vs. hydroxyl radical in the PARP activation and endothelial injury in shock remains to be clarified. Both species are produced in shock, and the available scavengers, such as superoxide dismutase analogues would be expected to reduce the production of both peroxynitrite and hydroxyl radical. The molecular mechanism by which oxidant stress and PARP activation induce endothelial dysfunction is likely to be related, at least in part, to a

PARP-mediated downregulation of endothelial NADPH levels. NADPH is an essential co-factor of endothelial NO synthase. In endothelial cells exposed to prooxidant conditions, where PARP activation and concomitant severe NADPH depletion was measured, the NADPH depletion was reversed by pharmacological inhibition or genetic inactivation of PARP.[79]

There is now good evidence suggesting that NO (or a related species, such as peroxynitrite) plays a role in the cellular energetic changes and the related organ dysfunction associated with endotoxic shock. This conclusion is based on the results of pharmacological studies, in which inhibition of NO synthesis, especially by agents that are selective toward the inducible isoform of NO synthase, reduces cellular injury and improves organ function in shock.[176] It is noteworthy that in the same experimental models of rodent endotoxic shock, the cell-permeable superoxide dismutase analogue MnIII tetrakis (4-benzoic acid) porphyrin[177] also reduced the endotoxin-induced depression of mitochondrial respiration in peritoneal macrophages *ex vivo*,[171] thereby suggesting that peroxynitrite, rather than NO per se, plays a role in these alterations. Peroxynitrite-induced activation of the PARP pathway has also been implicated in the pathophysiology of the cellular energetic failure associated with endotoxin shock by demonstration of increased DNA strand breakage, decreased intracellular NAD^+ and ATP levels and mitochondrial respiration in peritoneal macrophages obtained from rats subjected to endotoxin shock.[172,178] This cellular energetic failure was reduced by pretreatment of the animals with the PARP inhibitors 3-aminobenzamide or nicotinamide.[172,178] In contrast to these results in peritoneal macrophages, it appears that the PARP pathway only plays a limited role in the hepatic dysfunction associated with endotoxin shock. In an endotoxic shock model in the rat, inhibition of PARP with 3-aminobenzamide and nicotinamide did not affect the alterations in most parameters of liver injury; inhibition of PARP with 1,5-dihydroxyisoquinoline resulted in a small protective effect;[179] and PJ 34 treatment resulted in significant protection against liver and kidney dysfunction in endotoxic shock in the rat.[180] 5-Aminoisoquinolinone, a water-soluble, potent PARP inhibitor, significantly reduced the circulating aspartate aminotransferase, alanine aminotransferase, and γ–glutamyl-transferase levels (indicators of liver injury and dysfunction) in hemorrhagic shock.[181] The relatively modest degree of protection against hepatotoxicity by PARP inhibition in some of the above-referenced studies is perhaps not surprising when considering the fact that in *in vitro* studies the oxidant-induced injury in cultured hepatocytes is not prevented by pharmacological inhibition of PARP.[11] The exact reason inhibition of PARP does not affect the course of the oxidant injury in hepatocytes remains to be further investigated.

PARP activation appears to play a significant role in the renal failure associated with various forms of shock. Both pharmacological inhibition of PARP[180,181] and genetic inactivation of PARP (Figure 9.2) provides significant protection against the shock-associated increases in circulating BUN and creatinine levels in various forms of shock.

There is a clear and pronounced protection by PARP inhibition against the shock-induced intestinal epithelial permeability changes as well. In endotoxic shock in both rats and mice, inhibition of PARP activation by 3-aminobenzamide or by PJ34[181]

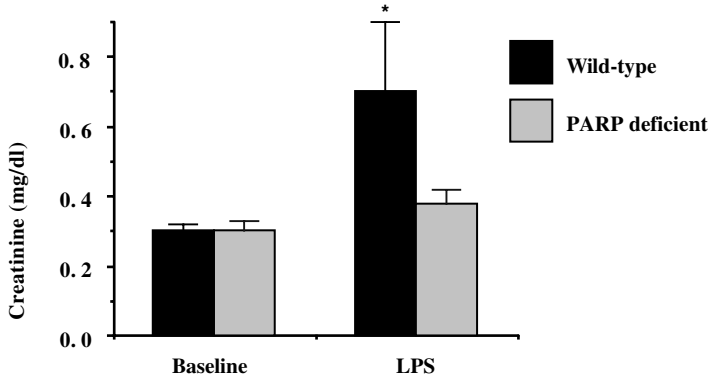

FIGURE 9.2 Plasma levels of BUN and creatinine in wild-type and PARP-deficient mice 24 h after vehicle injection or high-dose intraperitoneal injection of LPS. Please note the emergence of marked kidney failure (*$p < 0.05$ when compared to control) in the wild-type but not the PARP-deficient animals. N = 8 animals per group.

protects against the intestinal hyperpermeability, and so does genetic deletion of PARP in hemorrhagic shock.[182]

The role of PARP activation in the pathogenesis of hemorrhagic shock was recently further investigated in a murine model, by comparing the response to hemorrhage and resuscitation in wild-type and PARP-deficient mice.[182] Animals were bled to a mean blood pressure of 45 mmHg and were subsequently resuscitated after 45 min with isotonic saline (2× volume of shed blood). There was a massive activation of PARP, detected by poly(ADP-ribose) immunohistochemistry, which localized to the areas of the most severe intestinal injury, i.e., the necrotic epithelial cells at the tip of the intestinal villi, and co-localized with tyrosine nitration, an index of peroxynitrite generation.[182] A similar pattern of intestinal PARP activation was detected in shock induced by cecal ligation and puncture in rats (Figure 9.3), as well as in splanchnic occlusion–reperfusion shock (unpublished observations). The finding that in various forms of shock most of the PARP activation localizes to the tip of the villi is especially interesting, because it markedly contrasts with the normal (baseline, noninflammatory) conditions, where the majority of PARP activation in intestinal villi is localized to the lower villi: non-dividing but differentiating and maturing cells in the upper crypts and on the villi contain no more than about 10% of the synthetase activity of lower-crypt cell nuclei.[183] Intestinal PARP activation during hemorrhagic shock results in gut hyperpermeability, which developed in the wild-type but not PARP-deficient mice. PARP-deficient mice were also protected from the rapid decrease in blood pressure after resuscitation and showed an increased survival time, as well as reduced pulmonary neutrophil sequestration.[182] The beneficial effects of PARP suppression were not related to a modulation of the NO pathway or to a modulation of signaling through IL-6, which similarly increased in both wild-type

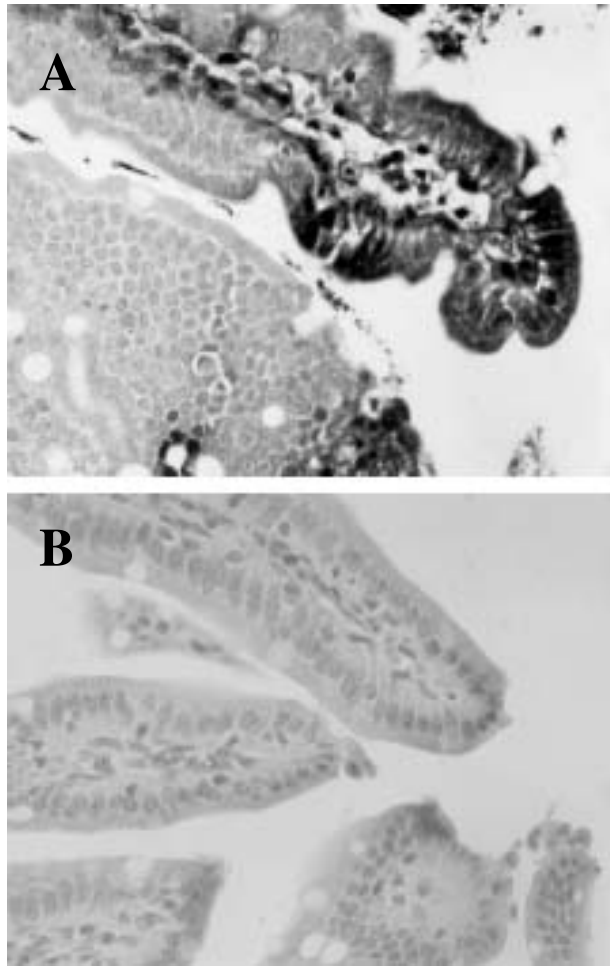

FIGURE 9.3 Immunohistochemical detection of poly(ADP-ribose) in the gut of wild-type (top) and PARP deficient (bottom) mice subjected to cecal ligation and puncture. Guts were removed from wild-type mice after 24 h of cecal ligation and puncture and tissue sections were stained for poly(ADP-ribose). Poly(ADP-ribose) has been detected in the epithelial cells with strongest immuno-positivity at the tip of the villi. Several stromal cells also displayed poly(ADP-ribose) positivity. No PAR staining was noted in the PAR-deficient mice.

and knockout mice exposed to hemorrhagic shock. There was no evidence of severe oxidant stress (no increase in tissue malondialdehyde levels and no depletion in reduced glutathione levels in any of the organs studied),[182] indicative that PARP activation does not require prior oxidant stress or low antioxidant status in this particular experimental model, and it appears to develop at a relatively early stage of shock. In a large animal model of hemorrhagic shock, treatment with 3-aminobenzamide

significantly ameliorated the fall in blood pressure, cardiac output, and stroke work; slightly increased left atrial pressure during resuscitation; and significantly prolonged survival.[184] It is likely that PARP activation and associated cell injury (necrosis or pre-necrosis) play a crucial role in the intestinal injury, cardiovascular failure, and multiple organ damage associated with endotoxic and septic shock, as well as resuscitated hemorrhagic shock.

Pharmacological inhibition of PARP, either with 3-aminobenzamide[184] or with the potent, novel PARP inhibitor[185] 5-iodo-6-amino-1,2,-benzopyrone[20] improves survival rate in mice challenged with high-dose endotoxin. Also, several recent studies compared the survival times of wild-type and PARP-deficient mice in response to high dose endotoxin, and compared the degree and nature of liver damage in the two experimental groups. In one study, all PARP-deficient animals survived high-dose (20 mg/kg) LPS-mediated shock, which killed 60% of wild-type animals.[186] Similar results were obtained by another independent group, led by DeMurcia.[187] In our studies, 100% mortality in the wild-type group and less than 50% mortality in the PARP deficient animals was observed at 48 h after intraperitoneal injection of high-dose (120 mg/kg) *E. coli* endotoxin (Figure 9.4). Moreover, LPS-induced necrotic liver damage was significantly reduced in the PARP-deficient mice.[186] In contrast, when apoptotic liver damage was induced via injection of low concentrations of LPS (30 mg/kg) into D-galactosamine-sensitized mice, or via activation of hepatic cell death receptors, PARP-deficient animals were not protected.[186] Thus, PARP activation is involved in systemic LPS toxicity, while it plays a minor role in apoptotic liver damage mediated by tumor necrosis factor or CD95.

All of the above-described experiments utilized bacterial components, such as endotoxin or hemorrhage and resuscitation. It is generally believed that sepsis induced by live bacteria is more appropriate in mimicking the human septic condition. In a preliminary study, we have compared the survival rates of wild-type and PARP-deficient mice to cecal ligation and puncture (CLP), a commonly used model of polymicrobial sepsis. We found that CLP-induced death was delayed in the PARP deficient mice when compared with wild-type animals (Figure 9.4). The beneficial effects of PARP inhibition in bacterial sepsis were also confirmed in a model of sepsis induced by live *E. coli* sponge implantation in pigs. Pharmacological inhibition of PARP provides marked hemodynamic improvements and massive survival benefit.[188]

Based on these observations, one can conclude that, in response to pharmacological inhibition or genetic deletion of PARP, the improved hemodynamic status in shock and sepsis is due to improved vascular function and, possibly, the improved cellular energetic status in some organs. These improvements, in turn, result in an overall survival benefit in this condition.

9.3 ADDITIONAL ROLES OF PARP IN THE REGULATION OF CELL DEATH AND THE INFLAMMATORY RESPONSE

In addition to the PARP-related energetic depletion and suicidal cycle, PARP may have other important functions in modulating cell death and the inflammatory response. Although highly controversial, PARP (or its cleavage) may have a role in the process

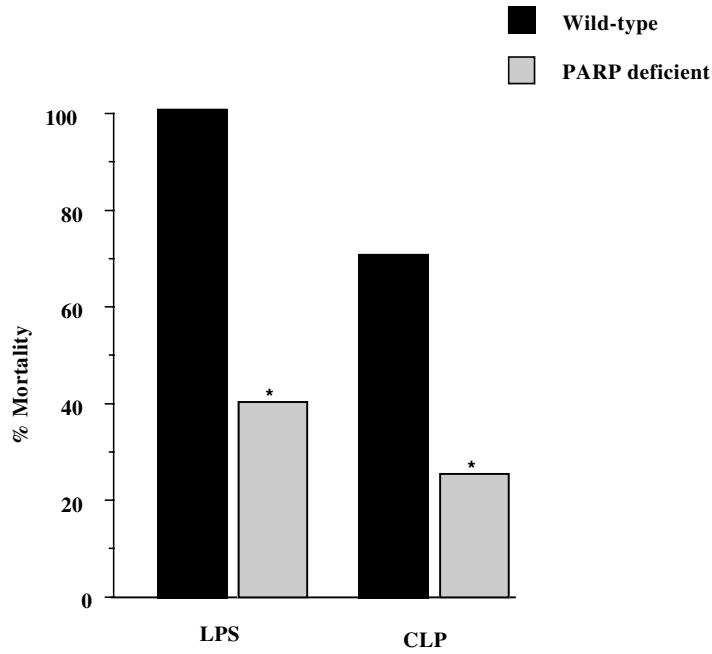

FIGURE 9.4 Survival rates of wild-type and PARP-deficient mice 48 h after high dose intraperitoneal injection of LPS (left panel) or after cecal ligation and puncture (right panel). Note the marked survival benefit (*$p < 0.05$) of the PARP-deficient mice in both experimental models of sepsis. N = 20 to 22 animals per group.

of apoptosis. In addition, PARP appears to regulate directly the expression of a variety of genes, some of them directly related to various inflammatory conditions.

9.3.1 Role of PARP in the Process of Apoptosis

As mentioned above, according to one school of investigation, a potential role of PARP is that it acts as a "death substrate" for caspases during the process of apoptosis.[55-57] Several reports demonstrated that peroxynitrite — as well as hydrogen peroxide and various other oxygen derived oxidants and free radicals — can cause apoptosis in a variety of cell types.[189-191] It appears that sustained exposure or low levels of peroxynitrite cause apoptosis, whereas sudden exposure to high concentrations of peroxynitrite induces cell necrosis. However, the peroxynitrite-induced apoptosis, in all cell types studied so far, cannot be attenuated by pharmacological inhibitors of PARP or $PARP^{-/-}$ phenotype.[24,192-194]

It is important to emphasize that, although NO is one of the precursors of peroxynitrite, the chemical properties and the reactivity of these two species are quite different, and so are the modes of peroxynitrite and NO-induced apoptosis. With respect

to cellular injury, NO can inhibit apoptosis, a process that can occur, at least in part, via a thiol modification of caspases.[195-198] Yet, large amounts of pure NO can also cause apoptosis, via p53 generation, inhibition of mitochondrial respiration, and, perhaps, by PARP cleavage.[199,200] Under these conditions, no DNA strand breakage occurs, there are no changes in cellular NAD^+, and the NO-induced ATP depletion is not affected by inhibitors of PARP.[15,64,199]

Although a role for PARP in the development of apoptosis has previously been proposed in a variety of cell types,[53, 201,202] based on recent studies using PARP knockout cells, several groups concluded that PARP is dispensable for apoptosis.[192,193] The role of PARP in the process of NO-induced apoptosis is also highly controversial. In human leukemia cells, 3-aminobenzamide and nicotinamide reduce NO-induced apoptosis,[203] whereas these inhibitors are ineffective in blocking apoptosis in RAW murine macrophages.[199] It is, nevertheless, clear that the NO-induced apoptosis is mediated through mechanisms that are different from the apoptosis triggered by potent DNA single-strand breaking agents, such as peroxynitrite or hydroxyl radical. The NO-mediated apoptosis may be related to the cleavage of PARP, at least in some systems.[200] In immunostimulated cells the delayed proteolytic cleavage of PARP can be blocked by pharmacological inhibitors of NO synthase. Overexpression of the antiapoptotic protein Bcl-2 in these cells has been shown to block the NO-induced apoptosis, and the NO-induced proteolytic cleavage of PARP.[200] Based on these results, the hypothesis has been proposed that PARP cleavage (with a consequent inhibition of the catalytic activity of PARP) is a process involved in endonuclease activation and apoptosis in NO-treated cells.[200] However, this proposal is somewhat in contrast to the finding that pharmacological inhibitors of PARP do not affect the course of immunostimulation-induced (and NO-dependent) apoptotic process in macrophages.[194,200]

9.3.2 Role of PARP in the Regulation of Proinflammatory Gene Expression

It appears that PARP plays an important role in the regulation of gene expression and cell differentiation.[204,205] Under basal conditions, PARP is closely associated to DNA, with preference to regions of cruciform DNA, bent DNA, and in A-T rich regions.[206] PARP also appears to be more frequently associated with transcriptionally active regions of chromatin.[207,208] Basal PARP activity has been proposed to regulate histone shuttling and nucleosomal unfolding.[209] By using pharmacological inhibitors of PARP, it has been demonstrated that the activity of PARP is required for the expression of the major histocompatibility complex class II gene,[210-212] DNA methyltransferase gene,[185] protein kinase C,[185] and collagenase.[186] Moreover, in several independent lines of investigations, it has been demonstrated that pharmacological inhibition of PARP (with nicotinamide, 3-aminobenzamide, and 5-iodo-6-amino-1,2-benzopyrone) suppresses the expression of mRNA of the inducible NO synthase.[17,33,214,215] In studies using 5-iodo-6-amino-1,2-benzopyrone, inhibition of the expression of the inducible NO synthase in RAW macrophages was indicated by the inhibition of nitrite production and the expression of the inducible NO synthase mRNA protein.[20] Similarly, the expression of the inducible NO synthase mRNA and protein and the

production of nitrite/nitrate was reduced in $PARP^{-/-}$ fibroblasts when compared with wild-type cells.[20] The regulation of the expression of the inducible NO synthase by PARP appears to be related to a specific part of the promoter of the inducible NO synthase. In transfection studies using the PARP inhibitor 5-iodo-6-amino-1,2-benzopyrone in murine RAW macrophages, it was found that this PARP inhibitor suppressed the transcription of the inducible NO synthase, when cells transfected with the full length (-1592 bp) promoter construct with the inhibitor. However, similar cotreatment of cells transfected with the -367 bp deletional construct did not significantly reduce the bacterial lipopolysaccharide-mediated increase in luciferase activity.[20] The regulation by 5-iodo-6-amino-1,2-benzopyrone of the induction of the inducible NO synthase also occurred in whole animals challenged with bacterial lipopolysaccharide. Pretreatment, but not post-treatment, of the animals with the inhibitor suppressed the bacterial lipopolysaccharide-induced increase in plasma nitrite/nitrate concentrations and reduced the bacterial lipopolysaccharide-induced increase in the expression of the inducible NO synthase in the lung.[20]

Although the mode of inhibition of the expression of proinflammatory mediators by inhibition of PARP or the mode of promotion of the expression of these mediators by poly(ADP-ribosyl)ation has not yet been fully explored, recent studies proposed that at a step involving poly(ADP-ribosyl)ation is required to activate the process of NF-κB-mediated gene transcription *in vitro*[79, 216,217] and *in vivo*.[187] In fact, it appears that PARP is required for specific NF-κB transcriptional activation *in vitro*. The activation of the HIV-LTR promoter and an NF-κB-dependent artificial promoter is drastically reduced in PARP-deficient cells, independent of the signaling pathway through which NF-kB was induced. Furthermore NF-κB-dependent gene activation can be restored by the expression of PARP in PARP-deficient cells. It appears that NF-κB and PARP formed a stable immunoprecipitable nuclear complex, and this interaction did not need DNA binding.[218]

From the above experimental data, it appears that PARP, via a not yet fully characterized mechanism, regulates the expression of a variety of genes, including the inducible NO synthase, and intercellular adhesion molecule 1 and collagenase. Meisterernst and colleagues[219] proposed a mechanism whereby PARP acts as one functional component of the positive co-factor 1 activity. PARP enhances transcription by acting during preinitiation complex formation, but at a step after binding of transcription factor IID. The PARP coactivator function is suppressed by NAD^+, probably as a result of auto-ADP-ribosylation. A different mechanism has been proposed in the case of Reg gene, a gene for insulin-producing β-cell regeneration. Poly(ADP-ribose) polymerase appears to bind to the Reg promoter and thereby regulates reg transcription by auto-poly(ADP-ribosyl)ation.[220] Yet another mechanism pertains to the regulation by PARP of E2F-1 promoter activity. PARP plays a role in the induction of E2F-1 promoter activity, which then positively regulates both E2F-1 and pol α expression, with important implications for cell cycle regulation.[221] According to a systematic analysis of gene expression of 11,000 genes monitored by oligonucleotide microarray analysis in wild-type and PARP-deficient fibroblasts,[91] differentially expressed genes were identified.[222] Although the exact molecular

mechanisms are unclear and probably multiple, it is clear that PARP acts as a pluripotent regulator of gene expression, and inhibition of the expression of various proinflammatory genes may represent an additional mode of beneficial action of PARP inhibition in various forms of inflammation.

9.4 PHARMACOLOGICAL INHIBITION OF PARP: A POWERFUL ANTI-INFLAMMATORY APPROACH

Current strategies aimed at limiting free radical–mediated and oxidant-mediated cell/organ injury include agents that catalyze superoxide or peroxynitrite, or inhibit the induction or activity of the inducible NO synthase. Less attention has been directed to strategies that interfere with intracellular cytotoxic pathways initiated by nitrogen- or oxygen-derived free radicals or their toxic derivatives. Direct and indirect experimental evidence presented in this chapter supports the view that peroxynitrite-induced DNA strand breakage and PARP activation importantly contribute to the pathophysiology of various forms of inflammation. The relative contribution of PARP activation to inflammatory cell injury is likely to depend on a variety of factors. One such factor is the endogenous antioxidant status. After the endogenous antioxidants are depleted, cells are sensitized for DNA injury and PARP activation and associated cell death.[223] Also, the nature and relative composition of the free radical and oxidant species produced is important. NO alone does not act as a strong oxidant, whereas simultaneous generation of equal or similar fluxes of NO and superoxide presents strong pro-oxidant conditions. The various oxidants and free radicals produced in inflammation frequently synergize with each other, with respect to PARP activation,[16] and also other (PARP-independent parallel) oxidant and cytotoxic processes. Hypochlorous acid, although not inducing DNA single-strand breakage, is still cytotoxic via PARP-independent pathways. At higher concentrations, hypochlorous acid actually inactivates PARP[224] — and so do high levels of hydrogen peroxide and peroxynitrite (unpublished observations) — which means that under extreme levels of oxidant stress PARP-independent pathways of cell injury take over. In fact, overwhelming cytotoxicity, both in response to peroxynitrite and hydrogen peroxide, is no longer completely inhibitable with PARP inhibitors or PARP deficiency.[21,171] Thus, at extreme levels of oxidant stress, PARP-independent pathways of injury may take over. The concept of combining antioxidants with PARP inhibitors is a viable one and needs to be directly tested in further studies.

What, then, is the reason that a mechanism as potentially deleterious as PARP overactivation has been maintained through the evolutionary tree? One possibility is that PARP serves crucial physiological functions in maintaining genomic integrity — although the fact that PARP-deficient mice are viable and do not present increased incidence of spontaneous tumors speaks against this possibility. It is also possible that, on a small scale, PARP-mediated necrosis, or PARP-mediated pro-inflammatory gene expression is a beneficial process. For example, NAD^+ depletion and cell necrosis may help eliminate "innocent bystander" parenchymal cells with severely damaged DNA, e.g., due to a nearby occurring neutrophil attack on invading microbi.

It is also possible that a low-level, localized inflammatory response may be beneficial in recruiting mononuclear cells to an inflammatory site. For example, invading microorganisms trigger a local neutrophil oxidant burst, and the DNA injury and PARP activation in nearby professional and nonprofessional immune cells triggers proinflammatory cytokine and chemokine production, which recruits additional mononuclear cells to the site of infection, to eliminate the invading microorganisms (Figure 9.5). It is important to note that the only known mammalian cells that do not contain PARP are the neutrophil and eosinophil granulocytes. It is possible that the presence of PARP in these cells is not compatible with the high levels of local oxidant production these cells frequently generate. We hypothetize that PARP overactivation — just like many other inflammatory processes — represents a physiological regulatory process that has amplified itself beyond control (Figure 9.6).

We conclude that pharmacological inactivation of PARP represents a novel, therapeutically viable strategy to limit cellular injury and to improve the outcome of a variety of inflammatory conditions. Unlike many current anti-inflammatory approaches, PARP inhibition is unlikely to interfere with the antimicrobial defense systems, since bacteria do not contain PARP. In life-threatening diseases such as circulatory shock, the potential therapeutic benefit of PARP inhibition is likely to out-

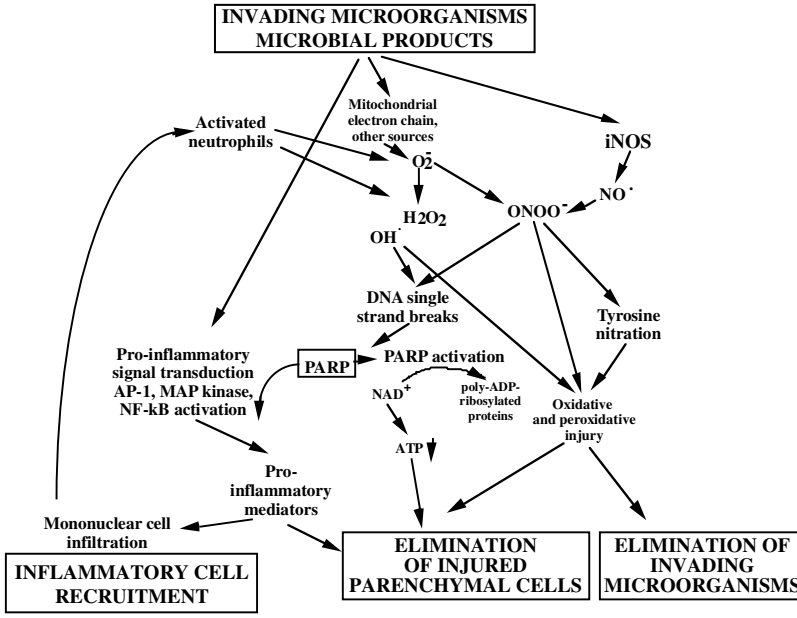

FIGURE 9.5 Proposed physiological functions of PARP activation during the inflammatory process. NAD^+ depletion and cell necrosis may help eliminate "innocent bystander" parenchymal cells with severely damaged DNA, e.g., due to a nearby occurring neutrophil attack on invading microbi. A low-level, localized inflammatory response may be beneficial in recruiting mononuclear cells to an inflammatory site, which will help eliminate the invading microorganisms.

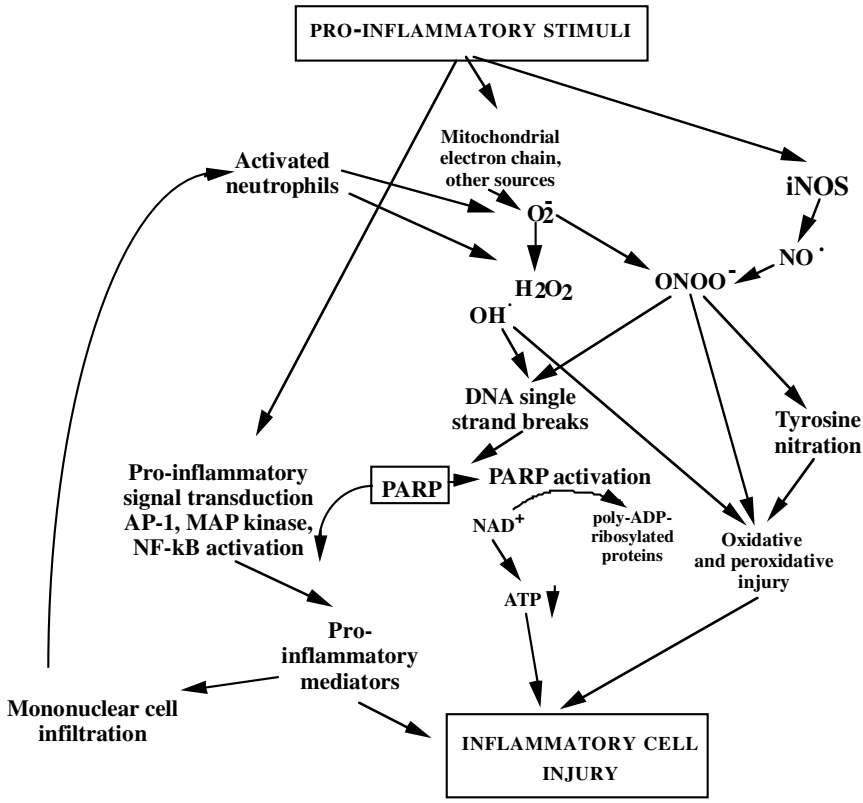

FIGURE 9.6 Proposed scheme of PARP-dependent and PARP-independent cytotoxic pathways involving nitric oxide ($NO^\cdot$), hydroxyl radical ($OH^\cdot$), and peroxynitrite ($ONOO^-$) in local and systemic inflammation. Proinflammatory stimuli trigger the release of proinflammatory mediators, which, in turn, induce the expression of the inducible NO synthase (iNOS). NO, in turn, combines with superoxide to yield peroxynitrite. Hydroxyl radical (produced from superoxide via the iron-catalyzed Haber–Weiss reaction) and peroxynitrite or peroxynitrous acid induce the development of DNA single-strand breakage, with consequent activation of PARP. Depletion of the cellular NAD^+ leads to inhibition of cellular ATP-generating pathways leading to cellular dysfunction. NO alone does not induce DNA single-strand breakage, but may combine with superoxide (produced from the mitochondrial chain or from other cellular sources) to yield peroxynitrite. Under conditions of low cellular L-arginine NOS may produce both superoxide and NO, which then can combine to form peroxynitrite. PARP activation, via a not-yet-characterized fashion, promotes the activation of NF-κB, AP-1, MAP kinases, and the expression of proinflammatory mediators, adhesion molecules, and of iNOS. PARP-independent, parallel pathways of cellular metabolic inhibition can be activated by NO, hydroxyl radical, superoxide, and peroxynitrite.

weigh the risks associated with the temporary inhibition of PARP, such as increased carcinogenic potential due to inhibition of PARP or due to preservation of cells that

contain severely damaged DNA. Careful long-term studies are required to estimate the risks and benefits associated with therapeutic PARP inhibition in chronic indications such as colitis or rheumatoid arthritis.

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10 Clinical Development of the Nicotinamides and Benzamides Involving PARP as a Molecular Target

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CONTENTS

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10.1 INTRODUCTION

The benzamides and nicotinamides represent a class of drugs that have experienced an unusually broad spectrum for clinical development. So far, these agents are being or have already been developed as clinically relevant antipyschotic (sulpiride), antiemetic (metoclopramide), antiarrhythmic (lidocaine), diabetic (nicotinamide), local anesthetic (procainamide), chemosensitizing/antitumor (declopramide), multidrug resistance reversing and anti-inflammatory drugs.[1] This diverse clinical spectrum is paralleled by an equally diverse mode of action profile which includes effects on tumor microcirculation, DNA repair, poly(ADP-ribose) polymerase (PARP), dopamine/hydroxytryptamine receptors, ADP-ribosyl cyclase/Ca homeostasis, apoptosis, NF-κB, P-glycoprotein, and phosphodiesterases (see patents described below). This is an unusual situation for drug development where small informational molecules such as benzamides and nicotinamides could influence so many seemingly unrelated biological processes. To appreciate further this drug discovery area, it is important to understand that nicotinamide is a vitamin not efficiently manufactured by the body, and hence dietary intake is essential to support human health in a disease-free state.

10.2 NICOTINAMIDE

The body readily converts niacin (nicotinic acid) to nicotinamide, and dietary tryptophan can also be converted physiologically into niacin. In the United States the recommended daily dietary allowance for niacin equivalents for adults is 6.6 mg per 1000 kcal or at least 13 mg at caloric intakes of less than 2000 kcal. Needless to say, with such a requirement for nicotinamide involving both metabolic precursors and products, there is inevitably a great deal of signal transduction of biochemical information where the nicotinamide moiety helps in the chemical recognition of the transduced information. Based on this reasoning, it is not so surprising that structural analogues of nicotinamide can mediate quite diverse biological effects.

To appreciate more fully the central role nicotinamide plays in human physiology, it is necessary to understand its metabolism. Both nicotinamide and nicotinic acid are the substrates for the synthesis of nicotinamide nucleotide coenzymes, nicotinamide adenine dinucleotide (NAD) and its phosphorylated derivative NADP.[3] NAD is directly linked to ATP, the primary energy source for cells, so that as cosubstrates or catalysts they control most of the signal transduction essential to life processes. Moreover, NAD is the cosubstrate for PARP, which is activated by DNA strand breaks, and catalyzes the formation of extensive linear and branched polymers of $(ADP\text{-}ribose)_n$ attached to proteins in chromatin.[4] The polymers are short lived and within a few minutes degradation by poly(ADP-ribose) glycohydrolase takes place. Hence, under conditions of DNA damage and repair, NAD is consumed at the expense of energy from ATP to restore genomic stability, avoid mutation, and maintain cellular differentiation and gene expression.[5,6] The importance of structural modifications of nicotinamide in altering cellular control of signal transduction is emphasized by the fact that PARP is inhibited by both nicotinamide and benzamide analogues.[1] Whereas the poly(ADP-ribose) polymer–influenced biochemical reactions

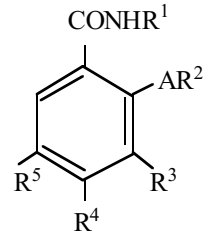
are located primarily in chromatin, mono-ADP-ribose polymers are also abundant and found principally in the plasma membrane where they are involved in transport in and out of cells.[7] Similarly, nicotinamide and benzamide analogues can also inhibit mono-ADP-ribosylation. Viewed from this perspective, it is not unreasonable to postulate that depending on where NAD is binding as a cosubstrate or catalyst, various analogues of nicotinamide or benzamide might very well mediate quite varied biological responses, thus at least suggesting their widespread development as efficacious drugs.

10.3 CHEMICAL CONSIDERATIONS

The benzamides and nicotinamides both can inhibit PARP and thus are examples of analogues with similar enough structural affinity for the enzyme that they can bind and inhibit its activity. Similar reasoning could explain modulation of other nicotinamide-mediated biological effects. This interpretation raises the question of what possible functional groups are shared in common with the benzamides and nicotinamides that may be key to their diverse biological response mediation. In fact, the most obvious structural detail in common is the presence of a carboxamide group. Hence, in this chapter aromatic compounds containing a carboxamide group as a key structural component that supports the diversity and richness of the benzamides and nicotinamides as drug discovery sources are reviewed.

10.3.1 N-Substituted Benzamide Antiemetics

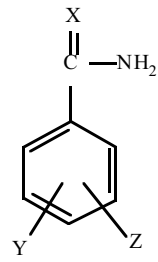
These are novel benzamide structures invented by Ivo Monkoviv and David Willner, which were based on the original discovery of metoclopramide as a powerful antiemetic assigned to Bristol-Myers Squibb Company (New York, NY) and presented in patent U.S. 5,011,992 issued on April 30, 1991. The main claims cover both benzamide compositions and methods for the treatment of nausea, vomiting, and impaired gastric motility. Novel composition of aliphatic N-substituted benzamides of the general formula were claimed:



where R^3 is hydrogen or, when R^4 and R^5 are each hydrogen, R^3 may be (lower) alkoxy; R^4 is hydrogen, amino or (lower)alkoxy; and R^1, R^2, and R^5 are further defined as in the patent.

10.3.2 Substituted Benzamide Radiosensitizers

These are proprietary benzamide derivatives invented by William W. Lee, Edward W. Grange, and J. Martin Brown, assigned to SRI International (Menlo Park, CA) and presented in patents U.S. 5,032,617 and U.S. 5,041,653 issued on July 16, 1991 and August 20, 1991, respcetively. The wording of the main claims are based on both methods and composition containing benzamide analogues used to radiosensitze hypoxic tumor cells having the formula:

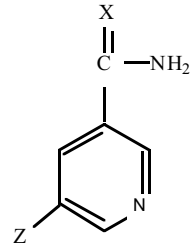


where X is O or S; Y is H, Me, OMe, OEt, acetoxy, or acetamido; and Z is OR, NHR, O(CO)R, or NH(CO)R in which R is H, straight chain alkyl (1-6C) optionally substituted with one or two substituents selected from the group consisting of halo, hydroxy, epoxy, alkoxy (1-4C), amino, acyloxy (1-4C), and acylamido (1-4C), and in which R can optionally be interrupted by a single ether linkage.

10.3.3 Substituted Nicotinamide Radiosensitizers

These are nicotinamide analogues discovered by William W. Lee, Edward W. Grange, J. Martin Brown, and Abelardo P. Martinez, assigned to SRI International (Menlo Park, CA) and presented in patent U.S. 5,215,738 issued on June 1, 1993. The main claim is an invention describing nicotinamide compounds for use as radiosensitizers. Specifically, it is a method to destroy hypoxic tumor cells in a warm-blooded animal; the method comprises:

1. Administering to said warm-blooded animal a compound of the formula:

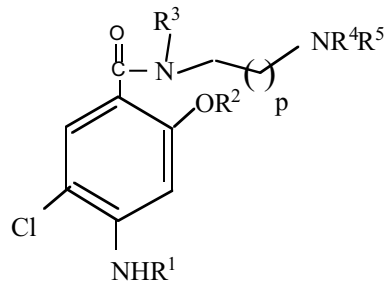


where X is O or S; Z is H, OR, SR, or NHR in which R is H, hydrocarbyl (1-6C) including cyclic and unsaturated hydrocarbyl, optionally substituted with one or two substituents selected from the group consisting of halo, hydroxy, epoxy, alkoxy, alkylthio, amino including morpholino, acyloxy, and acylamido and their thio analogues, alkylsulfonyl or alkylphosphonyl, carboxy or alkoxycarbonyl, or carbamyl or alkylcarbamyl, and in which R can optionally be interrupted by a single ether (–O–) linkage, or Z as O(CO)R, (CO)R, O(SO)R, or O(POR)R in which R is as above defined, in an amount effective to radiosensitize said hypoxic tumor cells.

2. Followed by, after a time period to provide maximum radiosensitization, irradiating these tumor cells with a dose of radiation effective to destroy the cells.

10.3.4 Multidrug Resistance Reversing N-Benzamides

These are benzamide analogues discovered by Ivo Monkovic, Lotte Wang, and David Willner assigned to Bristol Myers Squibb Company (New York, NY) and presented in patent application publication number EP 0529395 A2 that was published on March 3, 1993. The invention claims composition of novel benzamides and their use to reverse multidrug resistance evaluated in tumor cells lacking sensitivity to cytotoxicity induced by adriamycin. The claimed structures have the general formula:

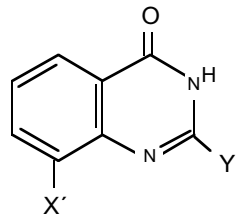


where p is 1 to 3; R^1 is hydrogen or acyl group R^6 CO–, in which R^6 is C1-C6 alkyl, C3-C7 cycloalkyl, C2-C6 alkenyl, aryl or radical of the formula as further defined in the application and for other variants of R^2 to R^5 as well.

10.3.5 DNA Repair-Inhibiting-Benzamides (N-Substituted Carboxamides, Quinazolinones)

These are 3-oxy benzamide analogues and related quinazolinone compounds discovered by a group of inventors working for the Cancer Research Campaign in

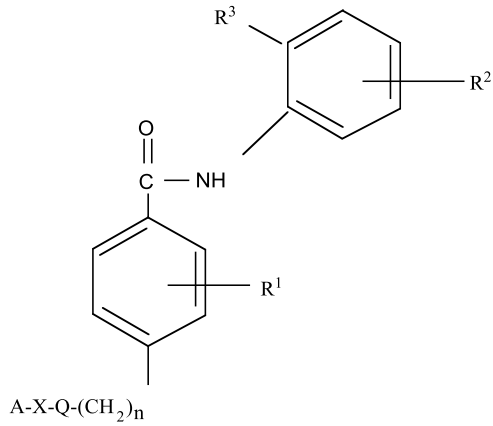
Great Britian. They have been assigned to Cancer Research Campaign Technology Limited (London, U.K.) and presented in international patent application publication number WO 95/24379. It was published on September 14, 1995. The claims are both for novel compositions and use for inhibiting PARP activity and increasing cytotoxic effectiveness of cytotoxic drugs or radiotherapy administered in conjunction with them for the treatment of tumors in mammals. The claimed structures have the general formula:



where X′ represents hydroxy, alkyl, alkoxy, or an optionally substituted aryl (e.g., phenyl) or aralykl (e.g., benzyl) group and Y′ represents hydrogen, alkyl, or an optionally substituted aryl (e.g., phenyl) or aralkyl (e.g., benzyl) group.

10.3.6 Cell Differentiation-Inducing N-Substituted Benzamides

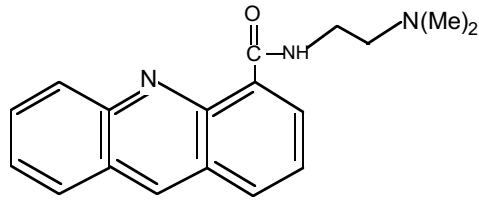
These are benzamide analogues and related anilide compounds discovered by a group of inventors working for Mitsui Chemicals, Inc. (Tokyo, Japan) to whom assignment was made. They were presented in European patent application EP 0847992 A1 that was published on June 17, 1998. The claims are both for novel compositions, and because the invented compounds have differentiation-inducing effects, they are useful as a therapeutic or improving agent for treating malignant tumors, autoimmune diseases, dermatological diseases, and parasitism. The claimed structures have the general formula:



where A is an optionally substitituted phenyl or heterocyclic group that has one to four substituents selected from the group consisting of a halogen atom, a hydroxyl group, an amino group, a nitro group, a cyano group, an alkyl group, having 1 to 4 carbons, an alkoxy group having 1 to 4 carbons, an aminoalkyl group having 1 to 4 carbons, an alkyl amino group having 1 to 4 carbons, an acyl group having 1 to 4 carbons, an acylamino group having 1 to 4 carbons, an alkylthio group having 1 to 4 carbons, a perfluoroalkyl group having 1 to 4 carbons, a perfluoroalkyloxy group having 1 to 4 carbons, a carboxyl group having 1 to 4 carbons, an alkoxycarbonyl group having 1 to 4 carbons, a phenyl group, and a heterocyclic group. X is a bond structure moiety, Q is a funtional moiety structure and R^1, R^2, R^3 are further defined as presented in application.

10.3.7 Antitumor N-Substituted Carboxamides (e.g., Acridine Analogues)

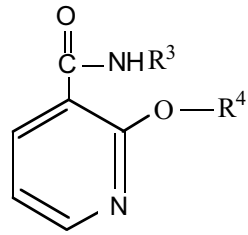
These are N-substituted aryl carboxamides such as acridine analogues discovered by a group of inventors working for the Cancer Research Campaign in Great Britain. They have been assigned to Cancer Research Campaign Technology Limited (London, U.K.) and presented in international patent application publication number WO 93/24096 that was published on December 9, 1993. There are only method claims presented, and they are based on aryl N-substituted carboxamides such as acridine analogues represented by unsubstituted or substituted fused ring systems comprising two or more aromatic rings, where the carboxamide side chain is peri to a nitrogen atom, which is part of an aromatic ring that is a fused ring system, or to an oxygen atom in a non-aromatic or aromatic ring in the fused ring system, and where the carboxamide side chain is present as a substituent of an aromatic ring for the manufacture of a medicament for the treatment of tumors by a divided-dose schedule comprising two or more administrations at frequent intervals. An example of these structural types is given below:



10.3.8 Anti-inflammatory N-Substituted Nicotinamides

These are N-substituted nicotinamide analogues discovered by John B. Cheng, Anthony Marfat, and John W. Watson. They have been assigned to Pfizer, Inc. (New York, NY) and presented in patent application publication number EP 0773024 A2,

which was published August 10, 1992. There are only method claims presented based on compounds with the general formula:

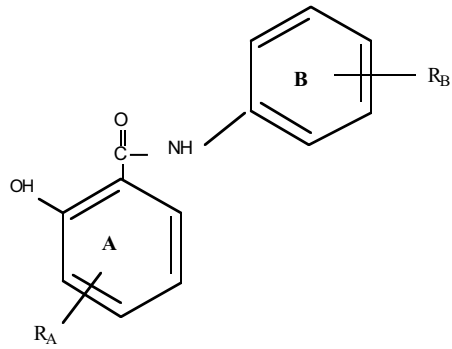


wherein R^3 is 1-piperidyl, 1-(3-indolyl)ethyl, (C1-C4)-alkyl, phenyl, benzyl, 1-(1-phenylethyl) or monosubstituted benzyl where the substituent is chloro, fluoro, methyl, or methoxy and said substituent is on the aromatic ring; R^4 is bicylo(2.2.1)hept-2-yl or as defined further in the application. This invention relates to a method of inhibiting phosphodiesterase type IV or the production of tumor necrosis factor in mammals, particulary humans, comprising administering to these mammals an anti-inflammatory amount of these N-substituted nicotinamides.

Other relevant patents and patent applications supporting anti-inflammatory nicotinamides are U.S. 6,028,111, U.S. 6,100,299, JP 11147874, EP 0706795, and WO 984568.

10.3.9 Anti-inflammatory N-Substituted Benzamides

These are aryl N-substituted benzamide analogues discovered by in the United States by James Calahan and Marie C. Chabot-Fletcher, they have been assigned to Smith Kline Beecham Corporation (Philadelphia, PA) and presented in patent application publication number EP 0773024 A2 that was published on December 23, 1999. The claimed invention provides pharmaceutical compositions of inhibitors of transcription factor NF-κB, and methods for treating diseases in which activation of NF-κB is implicated. Specifically, methods are provided for the treatment of a variety of diseases associated with NF-κB activation including inflammatory disorders, particularly rheumatoid arthritis, inflammatory bowel disease, and asthma; dermatosis, including psoriasis and atopic dermatitis; autoimmune diseases; tissue and organ rejection; Alzheimer's disease; stroke; atherosclerosis; restenosis; cancer, including Hodgkin's disease; certain viral infections, including AIDS; osteoarthritis; osteoporosis; and ataxia telengiestasis by administering to a patient in need of aryl N-substituted benzamide analogues. The claimed structures have the general formula:



where R_A substitutes ring A zero to three times and independently selected from the group consisting of: NO_2 halogen, C1-C6 alkyl, trifluoromethyl, O-C1-C6 alkyl, and S-C1-C6 alkyl; and R_B substitutes ring zero to three times and is independently selected from the group consisting of halogen, C(O)C1-C6 alkyl, C1-C6 alkyl, S-C1-C6 alkyl, CH2-aryl, and aryl.

In addition to the uses of the compounds outlined above, their use as antidepressants has also been claimed in U.S. 4,861,891, which issued August 29, 1989. Other relevant patents and patent applications supporting anti-inflammatory benzamides are U.S. 4,146,637, U.S. 5,350,770, WO 9312782, WO 9959569, U.S. 5,552,439, JP 090059236, JP 02207063, U.S. 5,905,090, EP 0888338, and WO 9732853.

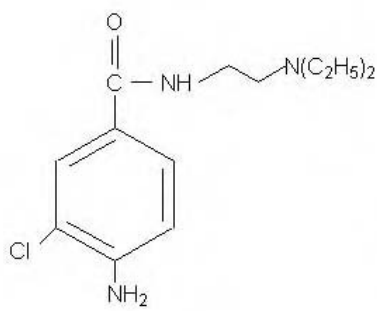
10.4 FUTURE DIRECTION OF DRUG DISCOVERY

This review of the known chemistry of nicotinamides and benzamides, together with their diverse modes of action leading to a variety of clinical indications, should convince most scientific skeptics of the unusual opportunity offered by this class of compounds for drug discovery. In cases where reasonable clinical experience with these compounds has been developed, such as with the local anesthetics, antiarrhythmics, and antiemetics, the problem has been side effects limiting their efficaious indications.[8,9] This is probably quite reasonable because there has already been identifed many nicotinamides and benzamides possesing multiple modes of action; for example, (1) metoclopramide and analogues modulate dopamine and hydroxytryptamine receptors as well as induce apoptosis and radio- and chemosensitization, and (2) N-substituted nicotinamides can inhibit phosphodiesterase IV and tumor necrosis factor, so they are useful in treating inflammation and depression. Hence, the challenge to drug discovery in this area is to identify which mechanisms any particular structure is most sensitive to regulate, and then to develop chemical libraries around the indication to be emphasized. In this way, the structural information leading to the diversity of mechanism and biological response can be mapped into the candidate drug

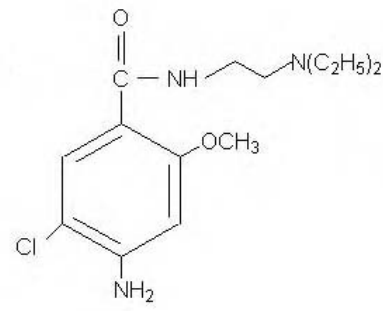
for identifying a more-targeted chemical functionality into the desired specified biological response. One obvious advantage for this approach is that the already available extensive chemistry can then be used to identify previously unrecognized novel uses for existing compounds, and to incorporate this chemical information into new novel drug candidates. As an example of the next generation drug discovery approach for the nicotinamides and benzamides, we present declopramide which is being developed by Oxigene, Inc. (Boston, MA).

10.5 DECLOPRAMIDE

Declopramide is 3-chloro procainamide which is an N-substituted benzamide with the following structure:



Declopramide



Metoclopramide

It was created to be a close analogue of metoclopramide and yet to have severely reduced central nervous system (CNS) side effects without sacrificing any of the radio- and chemosensitizing properties demonstrated by metoclopramide.[10] Several broadly issued patents and published patent applications dealing with N-substituted benzamides as radio- and chemosensitizing agents as well as anti-inflammatory agents in novel compositions of formulation make declopramide an attractive candidate for further clinical development (U.S. 5,340,565, U.S. 5,561,161, U.S. 6,028,111, U.S. 6, 100, 299, WO9732582, WO 9732576, EP 0927030 A1).

10.5.1 Background

The history of this drug development began with the discovery in 1989 that metoclopramide at clinically relevant doses could sensitize cisplatin chemotherapy in nude mice carrying human squamous cell carcinomas.[11] These data were quickly confirmed in rodent models, and because metoclopramide was already in the clinic as an antiemetic, a human study was published in 1995 that basically supported the growing data in animals that metoclopramide was an effective radiosensitizer.[12] Because the human use of metoclopramide was limited by extrapyramidal effects above 2 mg/kg, but yet in animals radiosensitizing effects could be shown at doses of 10 mg/kg or greater, Oxigene scientists embarked on a program to identify a

candidiate drug that would have chemosensitizing efficacy comparable to metoclopramide but without extrapyramidal side effects.

The ortho location of the methoxy group next to the carboxamide group in metoclopramide had already been identified as crucial to planarizing the molecule allowing it to antagonize D_2 and 5-hydroxy tryptamine$_3$ (5-HT_3) receptors.[13] Another version of metoclopramide called Neu-Sensamide was developed by formulating metoclopramide under neutral conditions so that an acid-sensitive hydrogen bond was disrupted resulting in reduced extrapyramidal side effects.[14] This knowledge was also used to create declopramide, which structurally is metoclopramide minus the ortho methoxy group. As predicted from the earlier work, declopramide had severely reduced receptor affinity leading to proportional reductions in extrapyramidal effects such as sedation.[10] On the other hand, declopramide was an effective chemosensitizer and an even more effective antitumor agent *in vitro* and *in vivo* than metoclopramide.[15,16]

Metoclopramide (neu-sensamide second generation version) entered into a Phase 3 trial as a radiosensitizer for non-small-cell lung cancer in March 1997, but the trial was discontinued in April 1999 because of the extrapyramidal side effects associated with the repeated-dose regimen of sensitizer that was necessary when coadministering a fractionated radiation dose over 6 weeks. During this period, Oxigene accelerated the clinical development of its third-generation chemosensitizer, declopramide, as a possible backup to neu-sensamide.

10.5.2 Declopramide Toxicology and Pharmcokinetics

The dose-limiting toxicity *in vivo* was prostration or convulsions in rats, mice, and dogs, according to results in both single-dose and repeat daily dose studies[10] (Oxigene toxicology data). The symptoms reversed within hours. These clinical signs generally became evident after single doses of 200 to 400 mg/kg (i.m. and oral) in rats and 120 mg/kg (oral) in dogs, and after five consecutive daily doses of 350 mg/kg/day (oral) in rats and 90 mg/kg/day (oral) in dogs. These CNS side effects of declopramide were also compared directly with metoclopramide, which occurred at 12.5 to 25 mg/kg when similarly evaluated in the rat, and thus these data showed an improved CNS side effect profile.[10] Overall, based on the results of gross necropsy and antemortem evaluations, declopramide did not appreciably affect the toxicity of 5-FU (5-fluorouracil) administered either with or without leucovorin or increase cisplatin toxicity, and in some cases appeared to slightly ameliorate cisplatin toxicity, especially at the 5.0 mg/kg dose level.

The oral bioavailability of declopramide was about sixfold lower than metoclopramide in the rat and was attributed to formation of the N-acetyl declopramide metabolite.[15] However, this is a very reduced route for metabolism in humans. The drug was readily absorbed through the gastrointestinal track with a $T_{1/2}$ value of 5.85 $\pm$ 0.25 min in the rat. Declopramide had no D_2 affinity up to 1000 μM, and as a result, could be shown to mediate fewer CNS side effects.[10]

10.5.3 Declopramide Clinical Development

DNA repair inhibitors such as declopramide are products that are intended to make cancer cells more receptive to the conventional cancer therapies of radiation and chemotherapy. Declopramide has completed Phase 1 studies in the United States in patients with advanced-stage cancers. These studies combine declopramide with either the traditional chemotherapeutic agent 5-FU or cisplatin.

A total of 40 patients have received declopramide in a dose-escalation study up to total daily doses of 1600 mg, either as a single agent for one cycle and then combined with 5-FU in later cycles (18 patients) or in combination with cisplatin (22 patients). In the 5-FU combination study, one patient has achieved a complete remission (colon cancer) and one patient in the cisplatin combination study has achieved a partial response (melanoma). Declopramide was generally found to be safe in combination with 5-FU and cisplatin at the doses indicating efficacy. Based on these indications Oxigene has initiated a Phase 2 open-label clinical trial evaluating declopramide in combination with 5-FU and leucovorin in patients with advanced colorectal cancer. This trial is expected to be complete in early 2002.

10.5.4 Mode of Action Studies and the Future for Drug Discovery

10.5.4.1 Effects on Apoptosis

The *N*-substituted benzamide analogue declopramide (see structure, section 10.5) has recently been identified as a compound able to induce apoptosis *in vitro*.[2,17] By using different structural benzamide derivatives of procainamide (PA), a structural analysis was performed *in vitro* using a mouse pre-B-cell line 70Z/3. 70Z/3 cells were exposed to declopramide and apoptosis was measured by FACS analysis.[17] Apoptotic cells were identified as cells that excluded the vital dye 7-amino-actinomycin D ($7AAD^-$) but were positive to Annexin V, which recognize phosphatidyl serine moieties on the cell surface of apoptotic cells[18] (Figure 10.1). Thus, the addition of a chloride to the 3′ position of the benzamide ring was sufficient to create a compound (i.e., declopramide) that showed a dose-dependent proapoptotic effect *in vitro* in the dose range of 250 to 500 μ*M*. The presence of a methoxy-group in the 2′ position of the benzamide ring i.e., metocloropramide (MCA) did not significantly change the apoptotic activity. However, an acetyl group added to the 4′-amino group on the benzamide ring (*N*-acetyl declopramide) was sufficient to inhibit totally the proapoptotic effect of the chloride substitution[17] (Figure 10.1). From these data we concluded that an addition of a chloride next to an amino group in the benzamide ring created a structure with the ability to induce apoptosis in the 70Z/3 cells.

The broad caspase inhibitor zVAD-fmk (50 μM)[19] reduced the apoptotic effect of declopramide and improved the viability of 70Z/3 cells when exposed to 500 μ*M* declopramide for 18 h (Table 10.1). Also, the declopramide-induced apoptosis in 70Z/3 cells was significantly reduced by the specific caspase-9 inhibitor zLEDH-fmk (50 μ*M*), whereas the addition of caspase-8 inhibitor Z-IETD-fmk (50 μ*M*) had only a

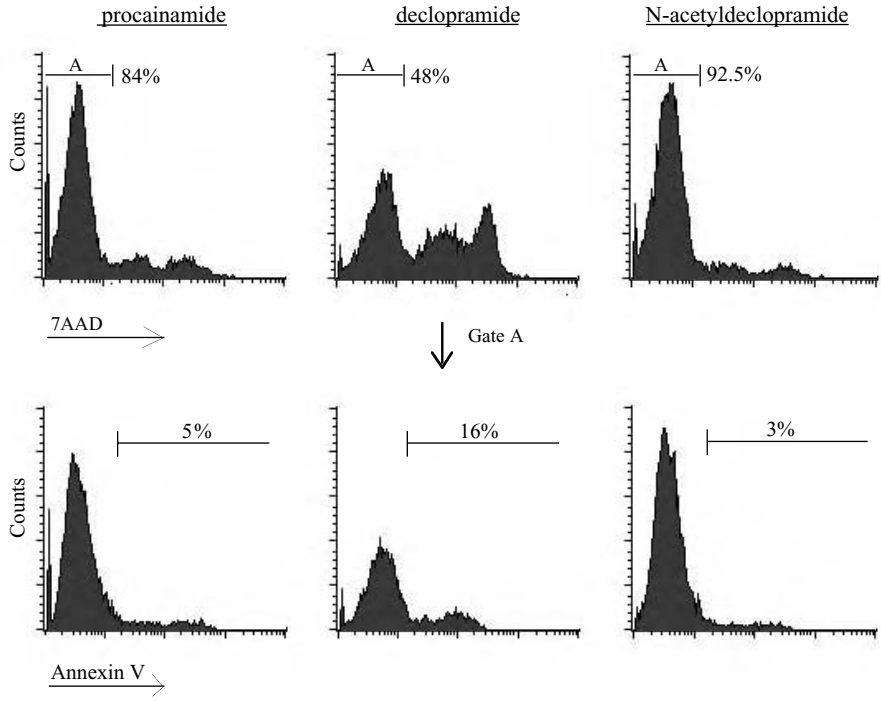


FIGURE 10.1 Declopramide, but not *N*-acetyl declopramide or procainamide, induce apoptosis in mouse pre-B-cells 70Z/3. 7AAD negative (viable) cells were gated (gate A) and analyzed for cell surface Annexin V^+ expression (apoptotic cells) by FACS analysis after exposure to declopramide (500 μ*M*) or procainamide and *N*-acetyl declopramide (1000 μ*M*) for 18 h.

marginal inhibitory effect.[20] These experiments suggested to us that the mechanism of action for the cytotoxic function of *N*-substituted benzamides required a functional caspase-9 activity.

In further studies we have confirmed these results using Western blot analysis of cytosol preparations from 70Z/3 cells exposed to declopramide. Cytochrome *c* release into the cytosol, and processing of procaspase-9 into its active form occurred within 12 to 18 h after incubation with declopramide.[20] Furthermore, both events were inhibited by overexpression of the Bcl-2 oncogene that excerts its antiapoptotic activity by regulating the release of cytochrome *c* into the cytosol.[20]

We conclude in this section that *N*-substituted benzamides can induce an apoptotic response in cells via the mitochondrial pathway and caspase-9 activation. The utilization of this property, either alone or in combination with other therapies, may open novel treatment protocols for malignant diseases. Furthermore, using declopramide as a lead, more potent apoptotic inducers may be developed in future efforts.

TABLE 10.1
The Broad Caspase Inhibitor zVADfmk Counteracts Declopramide-induced Apoptosis (AnnV$^+$7AAD$^-$ Cells) and Improves Cell Viability (7AAD$^-$)

	Apoptosis (%)		Viability (%)	
Drug	**$^-$zVAD**	**$^+$zVAD**	**$^-$zVAD**	**$^+$zVAD**
Procainamide	3	3	84	99
Declopramide	22	9	4	92
N-Acetyl decloprami	2.5	2.5	85	77

70Z/3 cells were pretreated with 50 μ*M* zVADfmk for 1 h before further incubation with 500 μ*M* of procainamide, declopramide, or *N*-acetyldeclopramide.
Data from Liberg et.al.[17]

TABLE 10.2
Transcriptional Activity of Reporter Genes of Different Transcription Factors are Affected by Addition of *N*-Substituted Benzamides

	% of Control		
Inhibitor	**NFkB**	**NFAT**	**AP-1**
Control	100	100	100
Procainamide	99	122	129
Declopramide	109	122	184
N-Acetyl declopramide	51	64	599

Jurkat cells were transiently transfected with different luciferase reporter genes containing multiple binding sites for NFκB, NFAT, or AP-1. At 22 h after transfection the cells were stimulated through the TCR and CD28; 1 h before the stimulation, the different benzamides were added in the concentration of 1 m*M*. After 8 h of stimulation, the cells were harvested and the luciferase activity was measured. The activity is presented as percent of control, that is, luciferase activity of stimulated, but not benzamide-treated cells

Data from Lindgren et.al.[21]

10.5.4.2 Effects on Transcription Factors

Our interest in apoptosis led us to investigate effects of *N*-substituted benzamides on the NFkB signaling pathway, with the rationale that transcription factor kappa B (NFκB) plays an essential role in the salvage pathway induced by apoptotic stimuli in many tissues.[17] We later expanded this analysis to include also other transcription factors involved in the regulation of inflammation and immunity such as transcription factor activator protein-1 (AP-1) and transcription factor of activated + cells (NFAT).[21] As summarized in Table 10.2 we found that *N*-acetyl declopramide was a potent modulator of NFκB, AP-1, and NFAT, whereas declopramide was less active. Interestingly, when the effect of *N*-acetyl declopramide on NFκB

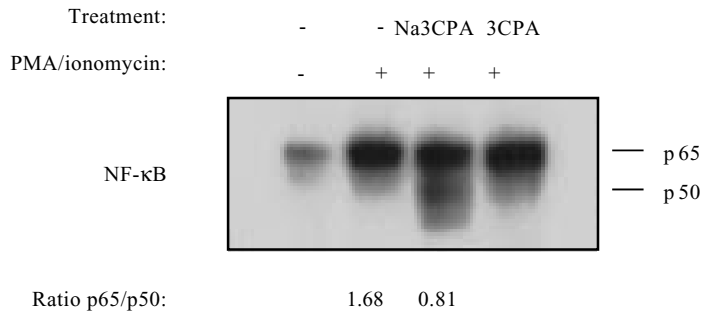


FIGURE 10.2 EMSA showing the effects of *N*-substituted benzamides on the composition of NFkB subunits in the nucleus. Jurkat cells were preincubated with 1 mM of declopramide (3CPA) or *N*-acetyl declopramide (Na3CPA) for 1 hr after which the cells were stimulated for 4 h with 50 ng/ml PMA and 1 μ*M* ionomycin. Nuclear extracts were prepared and incubated together with a ^{32}P-radiolabeled oligonucleotide containing the NFkB consenus sequence. The samples were separated on a 5% polyacrylamide TBE gel which was subsequently dried and analyzed by autoradiography. (Data from Lindgren et.al. 2001.)

and NFAT was antagonistic, the effect on AP-1 was agonistic. We have earlier shown that *N*-acetyldeclopramide may influence NFκB activation by inhibiting the breakdown of inibitor kappa B (IkB),[17] but we have now also seen that *N*-acetyl declopramide changes the composition of the nuclear NFκB complex by increasing the content of the inhibitory component p50 (Figure 10.2).[21] From these experiments we concluded that by acetylating *N*-substituted benzamides at a single position, derivatives are obtained that show low apoptotic-inducing capacity,[17] but are modulators of transcription factors involved in the regulation of inflammation and immunity.

Further efforts in this area should be aimed at developing novel compounds that show a desired profile with regard to transcription factor antagonisms or agonisms. For example, the development of a compound that specifically inhibits NFκB could be of great importance for the treatment of some inflammatory disorders, as well as be used in combination treatment against some cancers. A compound that would concomitantly inhibit NFκB and AP-1 would have an ideal profile for the treatment of rheumatoid arthritis, and an AP-1 agonist might be advantageous for the treatment of immune-compromised patients. We believe the data presented above are a good example for future drug discovery involving benzamide and nicotinamide candidates, i.e., identifying the structural features permitting several molecular targeted modes of action that could be combined into mediating the desired clinical indications. For example, we have reviewed more than ten structural subclasses of benzamides and nicotinamides that have been patented as potential drug candidates. Based on these data, it is now possible to identify structural features that permit several modes of action that can lead to multiple clinical indications. They are *N*-substitution of the aromatic carboxamide, ortho ring substitution to the carboxamide, and the presence of an aromatic amino group.

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11 Chemoprotective Effect of a Novel PARP Inhibitor

Gyorgy Rabloczky, Laszlo Jaszlits, Gyorgy Bardos, Balazs Sumegi, Kalman Tory, Ildiko Racz, Beatrix Farkas, Attila Sandor, Zoltan Berente, Erzsebet Osz, Judit Szilvassy, Istvan Sziklai, Sandor Bernath, and Peter Literati Nagy

CONTENTS

11.1 INTRODUCTION

Taxol, cisplatin, and carboplatin are the most frequently used anticancer drugs, and are highly effective against different types of cancer. However, high-dose carboplatin, cisplatin, or taxol therapy is often accompained by serious side effects affecting the kidney, bone marrow, and peripheral neurons, and this may force termination of the treatment or dose reduction. The exact mechanism of carboplatin, cisplatin, and taxol toxicity is not completely clear. In the case of cisplatin, generation of reactive oxygen species (ROS)[1,2] can be involved. Most recently, it has also been indicated that taxol may induce mitochondrial permeability transition of ROS production.[3] Therefore, poly(ADP-ribose) polymerase (PARP) inhibitors that protect the cell from oxidative damages can be useful to decrease the toxicity of these drugs. The protective effect of free radical scavangers and antioxidants in cisplatin toxicity supports the central role of oxidative damage in the pathomechanism of disease.[4] The

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mitochondria are a significant source of endogenous free radicals as a consequence of cisplatin and taxol treatment.[3-5] It is well documented that ROS induce single-strand DNA breaks, which, in turn, activate nuclear PARP (EC 2.4.2.30), and activation of PARP has been observed in cisplatin-treated cells.[6] Excessive activation of PARP depletes its substrate, NAD^+, and subsequently ATP, which eventually leads to cell death.[7] PARP inhibitors can prevent overactivation of the enzyme, and also have a protective effect on mitochondria.[8,9] They can reduce the mitochondrial ROS production,[8] and, therefore, moderate the subsequent energy crisis thereby saving the cell. Indeed, beneficial effects of PARP inhibition have been documented in various diseases where oxidative damage plays an important role in the pathomechanism.

In previous papers we provided evidences of the protective potential of a new PARP inhibitor, BGP-15, against the severe oxidative damage caused by cisplatin in the kidneys.[10-12] It has been demonstrated that BGP-15, *O*-(2-hydroxy-3-piperidinopropyl)-3-carboxylic acid amidoxime, a novel hydroximic acid derivate, protects against cisplatin-induced nephrotoxicity without compromising its antitumor activity. In these papers we emphasized the potential protective role of PARP inhibitors in neuronal damage, and hepatotoxicity.

In this chapter we discuss the newer results on cytoprotection against peripheral sensory neuropathy, ototoxicity, hematotoxicity, and hepatotoxicity obtained by the use of a novel nontoxic PARP inhibitor, BGP-15. The chemoprotective activity of BGP-15 is related to the modulation of PARP activity and the protection of mitochondria from oxidative damages.[13] It is well known that a large number of therapeutically used drugs, for example, various nonsteroidal slow-acting antirheumatic drugs,[14] and analgetics, for example, acetaminophen (AAP) display profound hepatotoxic effects in widespread use.[15] On the base of experimental results, it has been suggested that the hepatotoxic effects caused by some drugs involve an ADP-oribosylation step, which can be blocked by PARP inhibitors.[15] AAP overdose causes fulminant hepatic and renal failure.[16] In recent years it has been shown that inhibitors of PARP protect the liver of test animals from the toxic effects of AAP.[17] Thus, nicotinamide (NA), a selective inhibitor of PARP, suppresses the hepatic release of glutamate-oxalacetate transaminase (GOT) and glutamate-pyruvate transaminase (GPT) in mice suffering from AAP-induced hepatitis.[15]

Regarding its very perspective pharmacodynamic properties, safety evaluation of BGP-15 has been performed as well.

11.2 NEUROPROTECTIVE EFFECTS OF BGP-15 IN PERIPHERAL SENSORY NEUROPATHY

The use of antitumor drugs is often disrupted by the development of side effects including damage of gastrointestinal, hematological, nephrological, and neurological origin. Taxol and cisplatin are commonly used in the treatment of ovarian, breast, and lung cancer, and their use has been shown to increase the survival of patients.[18,-20]

Neurotoxicity symptoms and severe peripheral neurotoxicity limit the use of both taxol and cisplatin. Cisplatin- and taxol-induced peripheral *sensory* neuropathy

is dose-related,[21-23] which is not accompained by major changes in motor nerves or by autonomic dysfunction.[24,25] The mechanism of neurotoxicity induced by taxol has been proposed to involve the formation of abnormally large and stable microtubule structures of the peripheral nerves leading to disturbed axonal transport.[26] In contrast, cisplatin causes depolymerization of microtubules and formation of abnormally small microtubule structures[27] and disturbs axonal transport by inducing intraneuronal Ca^{2+} overload.[28]

Novel chemoprotective drugs that effectively prevent the toxic complications of anticancer chemotherapy are currently needed. Various new treatments have been tried to reduce neurotoxicity of cisplatin or taxol such as neuroprotective L-type calcium blockers,[29,30] adrenocorticotropic hormone (ACTH) analogues,[31-34] nucleophilic sulfur-thiols,[35-39] neurotrophins,[25] and reduced glutathion (GSH[40]). The only calcium antagonist examined *in vivo* that has been shown to prevent neurotoxicity is nimodipine,[32] The oral or intraperitoneal administration of nimodipine has been shown to be effective in protecting from cisplatin-induced reduction in H-related sensory nerve conduction in rats without impairing its antitumor effect. Of the thiol compounds, sodium thiosulfate and diethyldithiocarbamate are potent inhibitors of DNA platination.[41] Dimethyldithiocarbamate provides protection from nephrotoxicity, mortality, and weight loss. No animal experiment demonstrating neuroprotection with these compounds has been reported. Another thiol compound is amifostine, a "pro-drug" that is converted into its more effective metabolite (WR-2721) by the action of alkaline phosphase. Its mechanism of action is probably based on protection from free radical accumulation and prevention of nucleic acid alkylation. Several studies demonstrated the protection of WR2721 against renal and hematological toxicities, but no *in vivo* studies are available on neurotoxicity. GSH is a physiological intracellular thiol. It proved to be nephroprotective and neuroprotective without affecting cisplatin anticancer activity.[42] GSH mechanism of neuroprotective action is due to its ability to protect cells from free radical damage. ACTH and its analogues are able to reduce the effect of toxic conditions.[32] The most effective analogue of ACTH was the ACTH4-9 fragment (Org 2766) in cisplatin neuropathy.[43] It has been suggested that ACTH analogues might enhance some endogenous reparative factor within the neuron. However, no other toxicities of cisplatin are affected by the ACTH analogues.[44] Most experiments with growth factors have been performed *in vitro*. Neurotrophin-3 has been reported to reverse cisplatin neurotoxicity in a rat model evaluated by neurophysiological methods.[25] No other protective effects on non-neural cells have been reported. Nevertheless, the neurotrophin family members, including nerve growth factor (NGF) and free radical scavangers (reduced glutathion; GSH), seem to be the most promising as suggested by animal studies[45] and human clinical trials.[42]

The chemical structure of BGP-15 resembles that of nicotinamide, which is a natural inhibitor of PARP enzyme. *In vitro* enzyme kinetic study showed that BGP-15 is indeed an inhibitor of PARP, which binds to the active site of this nuclear enzyme[46]. It has been well known that ROS and nitric oxide (NO) are formed under several pathological conditions (ischemia, inflammation, toxic effects, etc.) inducing the elevation of DNA strand breaks, which activate the nuclear PARP enzyme.[10,47,48]

Cisplatin not only interferes with axonal transport,[36] but also generates ROS, superoxide anions, and hydroxyl radicals[1], and increases DNA strand breaks.[6,49-51] We have already demonstrated that BGP-15 protects against ischemia-reperfusion injuries,[13] and cisplatin-induced nephrotoxicity.[11] In the present experiments the chemoprotective effect of BGP-15 has been studied in neurotoxicity caused by cisplatin or taxol using electrophysiological methods. Neuropathy is characterized by the presence of altered sensory nerve conduction velocity (SNCV).

By using square wave stimuli, H-reflex-related sensory response and motor response was elicited, and nerve conduction velocity was determined.[52] Intraperitoneal administration of both cisplatin and taxol treatment decreased sensory nerve conduction velocity, whereas BGP-15 by itself did not have an effect. At 50 and 100 mg/kg oral doses, BGP-15 significantly improved the SNCV values (Table 11.1). Higher dose (200 mg/kg, oral) does not induce any greater effect in the nervous function of cytostatics-treated animals. The restoring effect of BGP-15 in the SNCV is different in the case of neurotoxicity caused by different anticancer drugs. The SNCV reducing effect of cisplatin is antagonized by BGP-15 in part (Table 11.2), whereas that of taxol is completely normalized. This finding supports the assumption that BGP-15 is a potent neuroprotective compound. However, the effect of BGP-15 is not quite clear, but its PARP-inhibitory capacity and, as a result, its protective potential against free radical damage may be involved in its neuroprotective efficacy. Enhanced production of ROS was observed also in the case of

TABLE 11.1
Sensory Nerve Conduction Velocity is Totally Restored by BGP-15 in Sensory Neuropathy Induced by 5 mg/kg Taxol Treatment

Groups	SNCV (m/s)	
Control (n = 10)	36.22 ± 0.5	*
Taxol (n = 7)	27.61 ± 1.1	
Taxol + BGP-15 (50 mg/kg) (n = 8)	36.28 ± 1.1	
Taxol + BGP-15 (100 mg/kg) (n = 8)	34.86 ± 0.8	
Taxol + BGP-15 (200 mg/kg) (n = 7)	35.26 ± 1.2	

Animals (male Wistar rats delivered by Charles River Hungary) were anaesthetized by intraperitoneal (i.p.) injections of ketamine (SBH-Ketamine, 125 mg/kg bw). The H-reflex-related SNCV and motor nerve conduction velocity (MNCV) were calculated by measuring the distance between the sciatic notch and the ankle sites, and the latency during the two responses. Latencies of the main peaks of the M (motor) and H (Hoffmann) responses (i.e., time from the start point of the curve) were measured for both electrode locations. Nerve conduction velocity (NCV) was calculated by the following equations: motor nerve: MCV = electrode distance/(M_s – M_t); sensory nerve: SCV = electrode distance/(H_t – H_s), where MCV and SCV are motor and sensory nerve conduction velocities, *M* is the latency of the M response (motor potential). *H* is the latency of the H-response (Hoffmann potential); *t* is the tibial, *s* is the sciatic site. Initial values of NCV were determined in each animal of every group on day 0. On the next day (day 1) several dosing regimes were started. In groups that were subjected to BGP-15, that compound was given orally daily. The control groups received physiological saline. The groups that received taxol alone or in combination with BGP-15 were exposed to taxol administration on every second day starting on day 1 and stopped on day 10. BGP-15 daily administration lasted for 25 days; second measurement was done on day [27]. Mean ± SE is given. *$p<0.05$

TABLE 11.2
Sensory Nerve Conduction Velocity is Partially Restored by BGP-15 in Sensory Neuropathy Induced by 1.5 mg/kg Cisplatin Treatment

GROUPS	SNCV (m/s)
Control (n = 8)	39.96 ± 2.5
Cisplatin (n = 7)	24.47 ± 2.1
Cisplatin + BGP-15 (100 mg/kg) (n = 7)	29.79 ± 1.7
Cisplatin + BGP-15 (200 mg/kg) (n = 6)	30.56 ± 1.1

Cisplatin was given daily i.p. for five consecutive days with or without BGP-15. The daily oral BGP-15 administration continued for 15 days. Control rats received physiological saline. The first and second measurements were performed on day 0 and day 16, respectively. (For details of method and measurement see Table 11.1.) Mean ± SE is given. *$p<0.05$; +$^{+}p<0.01$.

taxol-induced stimulation of murine esophageal cells[53,54] as well. The BGP-15 induced inactivation of overactivated PARP seems to be a viable therapeutic mechanism to limiting cellular injury in the peripheral nervous system and to improve the outcome of the pathological conditions associated with overproduction of ROS and with consequent obligatory PARP activation.

11.3 INFLUENCE OF BGP-15 ON CISPLATIN-INDUCED OTOTOXICITY

Cisplatin produces a dose-limiting, permanent, high-frequency sensory-neural hearing loss besides peripheral neuropathy, and a dose-related cumulative renal insufficiency with tubular necrosis and interstitial nephritis.[55] The potential for dose-limiting and permanent cochlear (neuro)toxicity remains despite present methods of hypertonic saline, perhydration, and mannitol diuresis prior to drug administration.[56, 57] Treatment with cisplatin significantly increased auditory brain stem response (ABR) thresholds.[58-60] BGP-15 at the dose applied did not protect from cisplatin-induced increase in ABR thresholds.

The interpeak latency periods were significantly prolonged as determined by the time elapsed between appearances of the first and fifth ABR wave peaks. The administration of cisplatin at dose of 3 mg/kg/day over 5 days produces a marked prolongation of the interpeak latency period in anesthetized guinea pigs. The effect of repetitive doses of BGP-15 on these cisplatin-induced detrimental changes was tested. The interaction between the two substances does not result in responses different from that induced by cisplatin alone in case of ABR thresholds. However, BGP-15 significantly reduced the inter-peak latency prolongation induced by cisplatin (Figure 11.1) suggesting the dominance of BGP-15 effect at least at the dose applied on sequential electrical activation of the ascending auditory nerve and brain stem pathways rather than on primary sound perception.

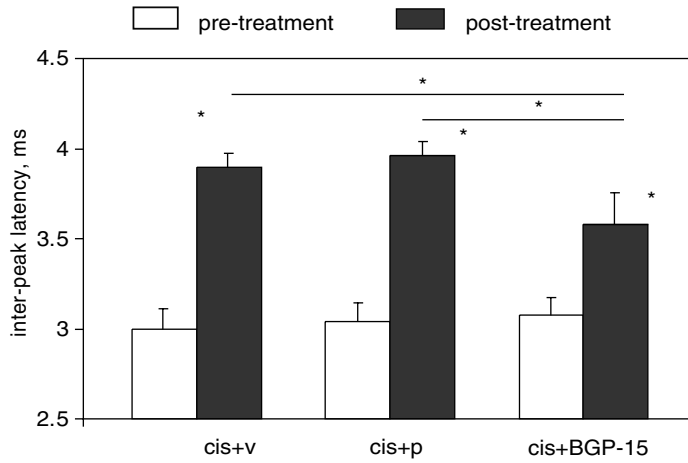

FIGURE 11.1 Effect of BGP-15 on cisplatin-induced prolongation of the interpeak latency period in guinea pigs. ABRs were analyzed in male guinea pigs. Tests were carried out either prior to or after treatment. ABR thresholds were measured in response to 100 μs clics (i.e. standard auditory signal) at a frequency of 2 kHz. The interpeak latency periods were determined (the time lag between first and fifth ABR waves) in each pattern to characterize sequential electrical activation of the ascending auditory nerve and brain stem pathways. Guinea pigs were treated with 3 mg/kg i.p. daily dose of cisplatin either with or without 100 mg/kg i.p. daily dose of BGP-15 for 6 days. ABR interpeak latency period (ms) data are expressed as means ± standard deviation (SD) of the mean (ANOVA followed by a modified t-test for repetitive measurements). Abbreviations: cis = cisplatin; BGP = BGP-15; v = vehicle; p = placebo. *$p<0.05$

11.4 CHEMOPROTECTIVE EFFECT OF BGP-15 IN CARBOPLATIN-INDUCED HEMATOTOXICITY

The most common limitation to the clinical use of anticancer drugs is their hematological toxicity. Myelosuppression becomes particularly severe in the high-dose regimens and especially with carboplatin. This newer member of platinum drugs is relatively free of nephrotoxicity and neurotoxicity, but its use is limited by profound myelosuppression.[61] It is obvious that any reduction of their myelosuppressive effects would permit further dose escalation of carboplatin and greater versatility in combination regimens based on reduced toxicity.[62] In fact, the improvement of the therapeutic ratios of platinum drugs is possible by using chemoprotectors. The hematoprotective effect of BGP-15 has been tested in mice treated with carboplatin. After a 13-day treatment with 15 mg/kg/day i.p. carboplatin, the white blood cell (WBC) and platelet counts decrease and serum glutamate pynwate transaminase (GPT) enzyme level increases significantly on the 16th days (i.e., 3 days after cessation of carboplatin treatment). If BGP-15 (in 100 mg/kg/day and 200 mg/kg/day oral doses) is administered simultaneously with the above carboplatin treatment, WBC count turns to the normal level after 200 mg/kg/day BGP-15 treatment. The critical reduction of the

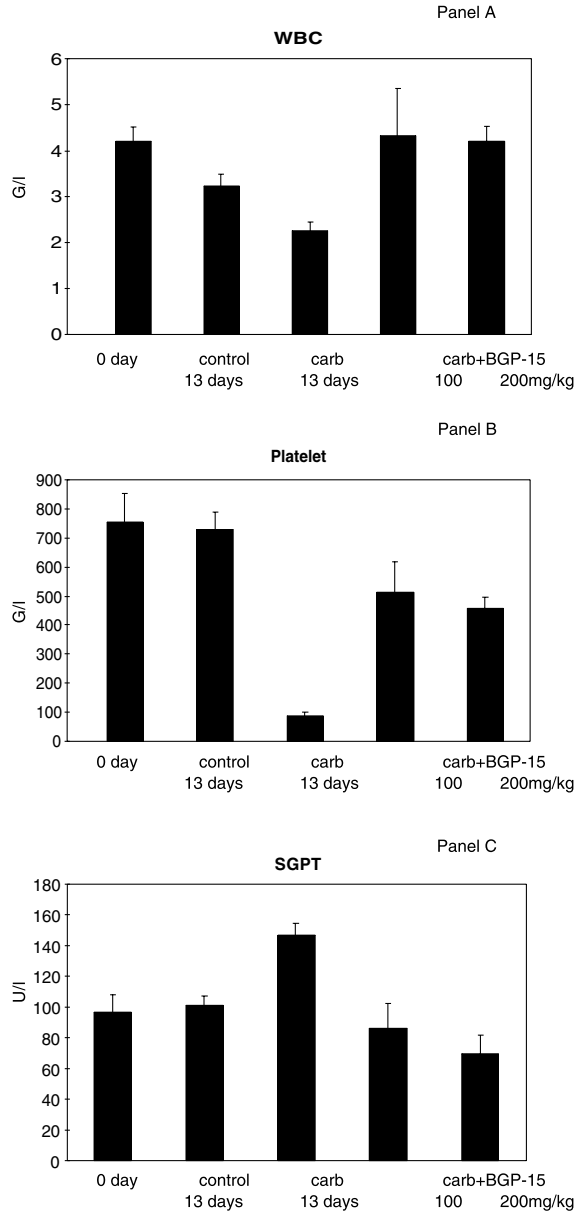

FIGURE 11.2 Effect of carboplatin with or without BGP-15 on the WBC (A), platelet count (B), and SGPT level (C) in mice. Treatment: 15 mg/kg/day i.p. carboplatin (carb) or 10 ml/kg/day i.p. physiological saline given with or without 100 or 200 mg/kg/day oral dose of BGP-15 for 13 days. Blood sampling: on day 16 (mean ± SE).

platelet count, induced by carboplatin alone, significantly reverses after both doses of BGP-15. The elevation of GPT enzyme activity, induced by carboplatin alone, returns to normal in response to either dose of BGP-15.

These experimental results show the ability of BGP-15 to ameliorate the carboplatin-induced hematotoxicity. Although the evaluation of the hemato- and/or myeloprotective potential of that novel chemoprotectant needs further support, the cytoprotective efficacy of BGP-15 appears in several normal tissues (Figure 11.2).

11.5 HEPATOPROTECTIVE INTERVENTION WITH PARP INHIBITORS

The use of analgesic drug acetaminophen (AAP) is increasing in recent years as a substitute for aspirin. Unlike aspirin, it does not irritate the stomach lining. However, its poor anti-inflammatory activity tempts many patients to overdose.[63,64] A single oral application of AAP in a dose of 500 mg/kg to male NMRI mice increases the serum glutamate oxalacetate transaminase (GOT) and GPT activities when compared to saline-treated control animals. It is well known that inhibitors of poly(ADP-ribosyl)ation protect the liver from the toxic effect of AAP.[15] The present study reports the profound hepatoprotective effects of BGP-15, an inhibitor of PARP,[13] in mice suffering from AAP-induced liver damage. We compare the effects of NAA, which is also a PARP inhibitor, on liver toxicity induced by AAP in terms of the release of GOT and GPT into the serum of mice.

We could clearly demonstrate that NAA suppresses AAP-induced hepatotoxicity at a concentration of 200 mg/kg orally (Figure 11.3). The same is true for BGP-15 at the same dose both in single (Figure 11.4), and repeated-dose experiments (Figure 11.5). AAP is a powerful inducer of cytochrom P-450. The action of the P-450 system on AAP produces a highly reactive quinone-imine that combines to the sulfhydryl groups of protein. The inactivation of proteins leads to death of liver cells. The GSH (an intracellular antioxidant defense system) protects liver proteins because it competes with the quinone-imine for the reaction with proteins. This causes the rapid depletion of intracellular GSH. A complete depletion of hepatic GSH is achieved with 500 mg/kg of AAP. GSH is a powerful consumer of superoxide, singlet oxygen, and hydroxyl radicals.[65] It seems likely that a possible application of BGP-15 may be to protect in some other drug-induced toxicity.

11.6 SAFETY PROFILE AND PHARMACOKINETIC PROPERTIES OF BGP-15

Anticipated clinical use of BGP-15 will be the oral treatment for patients with malignancies suffering from the organotoxic complications of anticancer drugs. BGP-15 is pharmacologically active in oral doses of 50, 100, and 200 mg/kg as a chemoprotectant in mice, rats, and guinea pigs (i.e., it prevented the organotoxic complications of some anticancer drugs) after systemic oral administration. In the course of the pharmacokinetic analysis, the absorption of BGP-15 from the gastrointestinal

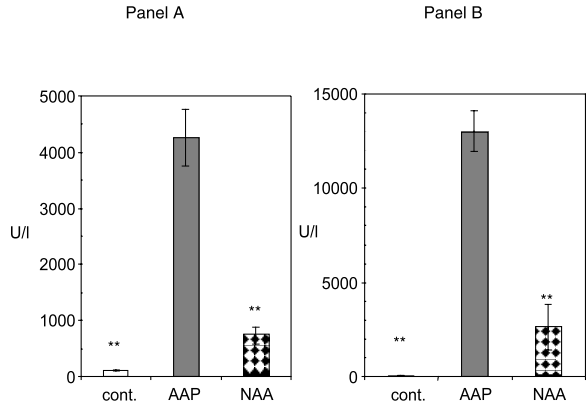

FIGURE 11.3 Effect of NAA (200 mg/kg, oral) on AAP-induced elevation of GOT (A) and GPT (B). Experiments were carried out in male NMRI mice. Animals were sacrified 16 h after treatment and the activity of GOT and GPT were determined photometrically in serum, according to Bergmeyer.[69] Data were analyzed with Mann–Whitney's nonparametric test. Results are presented as means ± standard error (SE), **p < 0.01 was considered significant. Abbreviations: U/l = unit/liter; cont. = control; AAP = acetaminophen; NA = nicotinamide.

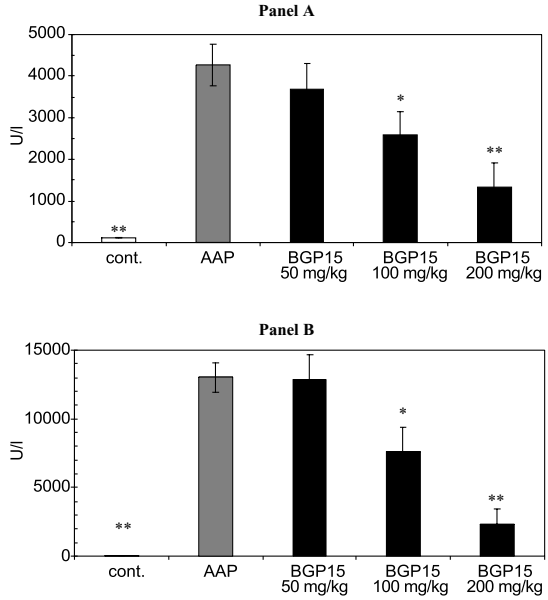

FIGURE 11.4 Effect of BGP-15 on AAP–induced elevation of GOT (A) and GPT (B). Experiments were carried out in male NMRI mice. Animals were starved for 12 h and then AAP (in a single oral dose of 500 mg/kg) and BGP-15 (in single acute oral doses of 50-100-200 mg/kg) as well as NA (in a single oral dose of 200 mg/kg) were administered. Mann–Whitney test *:p < 0.05; *p < 0.01. The dose–response curve to BGP-15 showed a dose-related effect on AAP-induced elevation of GOT and GPT after single oral doses of 50-100-200 mg/kg. Abbreviations: see Figure 11.3 legend.

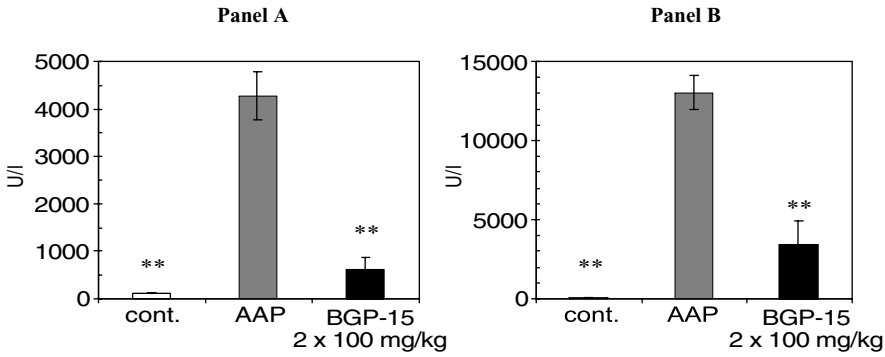

FIGURE 11.5 Effect of BGP-15 on AAP–induced elevation of GOT (A) and GPT (B) (pretreatment of BGP-15 for 7 days). Experiments were carried out in male NMRI mice. The animals were repeatedly pretreated with BGP-15 orally, twice a day (2 × 100 mg/kg) for 7 days. At day 8 the animals were starved for 12 h and AAP (in a single oral dose of 500 mg/kg) as well as the last dose of BGP-15 (200 mg/kg) were given orally. BGP-15 strongly prevented the elevation of enzyme activity induced by AAP in repeated oral administration twice a day: 2 × 100 mg/kg for 7 days. Abbreviations: see Figure 11.3 legend. Mann–Whitney test ** $p < 0.01$.

tract is rapid and complete in rat and dog. Rapid and intense penetration is observed in several tissues, except brain and spinal cord (i.e., weak passage of radioactivity through the blood–brain barrier). The oral bioavailability of BGP-15 is complete in both rat and dog. The main route of excretion is by urine. According to the acute toxicity studies the oral LD_{50} value of BGP-15 is higher than 2000 and 1000 mg/kg in mice and rats, respectively. Following IV or oral dosing, the main toxic symptoms and death occur within a 6 h period following treatment. Lethality when it occurred was dose dependent. BGP-15 was applied to rats orally by gavage for 28 days in 80, 400, 800, and 1200 mg/kg body weight dose in the subsequent subacute toxicity study. Lethality occurred in the highest dose group: two females and one male animal died during the first 2 weeks. The death of three high-dose animals could be attributed to some kind of functional disorder (respiratory failure) but histopathological changes never occurred in these animals. The no-observed-adverse-effect level (NOAEL) was determined at 400 mg/kg daily dose; toxic dose was 1200 mg/kg for both sex groups.

The 4-week subacute toxicity of BGP-15 performed by oral administration to beagle dogs (including toxicokinetic analysis) revealed that after daily dose of 50-150-300 mg/kg BGP-15 none of the dogs died prematurely. The no-effect level was ≤150 mg/kg/day dose of BGP-15 given orally by capsule. The dose-related exposure was reflected in the dose-related increase in the area under the curve (AUC). Plasma clearance was not influenced by the dose. No accumulation was noted.

In mutageneity studies, BGP-15 was found non-mutagenic in the bacterial Ames test and the mammalian erythrocyte micronucleus test. In the mammalian

chromosome aberration test, BGP-15 did not possess any clastogenic effect in the presence or absence of the metabolic activation system. It was ineffective in gene mutation assay, as well. Thus, BGP-15 in *in vitro* and *in vivo* tests did not show any mutagenic hazard.

11.7 CONCLUSION

Neurotoxicity and myelotoxicity are major dose-limiting side effects that may greatly interfere with the treatment of patients with cancer.[66] Cisplatin-induced neurotoxic side effects such as paresthesis and incoordination, even when considered mild or moderate by the oncologist not necessitating withdrawal from treatment, may greatly reduce the quality of life of the patients.[67] Similarly, taxol treatment is often accompanied by peripheral neuropathy, a major dose-limiting side effect.[68] Thus, it is greatly beneficial to prevent, or at least reduce, the peripheral neurotoxicity of cisplatin and taxol. According to Cavaletti et al.[67] the optimal neuroprotective agent should (1) not diminish the anticancer effect of the antitumor agent, (2) have a substantial protective effect against drug-induced toxicity, and (3) have a safe toxicity profile. In a previous paper it was demonstrated that BGP-15, a novel PARP inhibitor, did not reduce the anticancer potential of various antitumor drugs,[10] while protecting against nephrotoxic side effects.[11] In addition to its nephroprotective activity, BGP-15 prevented or partially blocked the development of peripheral sensory neuropathy caused by either taxol or cisplatin treatment. Moreover, results of initial investigations demonstrate the hematoprotective potential of BGP-15, as well. The cytoprotective potential of BGP-15 against drug-induced toxicities is further strengthened by observations that it prevented the APP-induced hepatotoxicity.

A clear advantage of BGP-15 over the few existing cytoprotective agents is that it is effective upon oral administration and has a good safety profile, making it a promising drug in future clinical development.

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12 Beneficial Effect of GPI 6150 Treatment in Multiple Animal Models of Diseases

Jie Zhang

CONTENTS

12.1 INTRODUCTION

Nicotinamide adenine dinucleotide (NAD^+) is a key molecule for cellular energy metabolism. Its reduced form, NADH, serves as a carrier that stores chemical energy derived from oxidation of fuel molecules such as glucose and fatty acid. In aerobic organisms, the high-potential electron in NADH is transferred through the electron transport chain in mitochondria to oxygen. In this process of oxidative phosphorylation, ATP is generated to supply most of the energy requirement of cell functions, including the synthesis of NAD^+ itself. The function of NAD^+/NADH, as a high-potential electron transporter, intrinsically couples it to cellular energy status. Such a link puts NAD^+ in a unique position suitable to reflect the overall environmental situations in terms of nutrient supply. The concentration of NAD^+ could subserve as a sensor to

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gauge whether conditions are favorable for optimal growth and propagation. However, when it is used as a coenzyme in the cycle of $NAD^+ \longleftrightarrow NADH$, the level of this pyridine nucleotide remains unchanged. Rather, the NAD^+ level is affected by the rate of its synthesis and consumption as a substrate, which is greatly influenced by the extracellular stimuli, e.g., oxidative stress.

In addition to serving as a coenzyme to carry electrons back and forth, NAD^+ is also used as a substrate for multiple enzymatic reactions.[1] A major reaction is ADP-ribosylation, in which one or more ADP-ribose units are transferred from NAD^+ to modify proteins and regulate protein functions. In this process, which is catalyzed by either mono-ADP-ribose transferase or poly(ADP-ribose) polymerase (PARP), which is also known as PARS for poly(ADP-ribose) synthetase, NAD^+ is split into nicotinamide and ADP-ribose. To regenerate NAD^+, an equivalent of four ATP molecules is needed. The PARP family now consists of PARP-1, PARP-2, PARP-3, vPARP, Tankyrase 1, and Tankyrase 2 and more members are expected.[2,3] Other NAD^+-utilizing enzymes include ADP-ribose cyclase, e.g., CD38, which synthesizes cyclic-ADP-ribose as a signal transduction molecule that triggers calcium release.[4] Recently, a family of SIR2 (silent information regulator) enzymes has been found to be histone deacetylases.[5,6] Deacetylation by SIR2 requires NAD^+ and generates 1-*O*-acetyl-ADP-ribose.[7,8]

By far, NAD^+ appears to be mainly consumed by PARP-1 to continuously synthesize poly(ADP-ribose), especially when this highly abundant nuclear enzyme is overactivated by extensive DNA damage. PARP-1 contains two zinc-fingers at its N-terminus, which bind to DNA and detect single-strand breaks. DNA breakage triggers a conformation change in PARP-1 to synthesize poly(ADP-ribose), an elaborate polymer consisting of branch patterns and more than 200 units of ADP-ribose. PARP-1 accounts for more than 90% of poly(ADP-ribose) produced, as residual polymer was detected at a level that is less than 10% in cells derived from the mice with homozygous deletion of PARP-1 gene.[9] This is consistent with PARP-1 as the high-output enzymes in the PARP family. For comparison, the Km for PARP-1 is 2.6-fold lower than that for PARP-2 and the k_{cat}/K_m ratio for PARP-1 is 18 times higher than that for PARP-2.[10] The high abundance and high activity of PARP-1 renders it most capable of all the members of PARP family of using NAD^+ to produce poly(ADP-ribose). The functions for other recently identified PARPs remain to be fully elucidated.

Extensive PARP-1 activation leads to depletion of NAD^+. *In vivo*, there is a rapid turnover of poly(ADP-ribose). It is almost immediately degraded by constitutively active poly(ADP-ribose) glycohydrolase (PARG).[11] The half-life of poly(ADP-ribose) is less than a minute.[12] Prolonged PARP-1 activation, followed by PARG degradation of the polymer, results in a significant drop of cellular NAD^+ level, which is related to the drop of ATP level. Energy depletion is believed to be the principal consequence of PARP-1 overactivation that ultimately contributes to necrotic cell death and tissue destruction.[13] Studies using both pharmacological inhibition and PARP-1 gene deletion have yielded corroborative evidence to validate PARP-1 as a target for ischemia and reperfusion injuries. In animal models of ischemia for brain, heart, muscle, kidney, retina, intestine, or multiple organ failure, various PARP inhibitors

have demonstrated efficacies in different laboratories. Similarly, when PARP-1 knockout mice are subject to various organ ischemia conditions, the damages are significantly ameliorated. In addition, PARG inhibitors have recently been found to be effective in blocking cell death elicited by hydrogen peroxide.[14,15] PARG inhibition appears to be as effective as PARP-1 inhibition to preserve the cellular energy level and prevent oxidative cell death. PARG emerges as an alternative target for intervening in the poly(ADP-ribose) turnaround pathway. Furthermore, in a rat model of transient cerebral focal ischemia by middle cerebral artery occlusion, postischemia treatment with a PARG inhibitor showed significant reduction in infarct size.[16] Taken together, the new strategy of blocking poly(ADP-ribose) metabolism by PARP-1 or PARG inhibition warrants of further drug discovery and drug development efforts to explore fully possible clinical benefits from this important biological pathway.

What is the normal physiological function of PARP-1? The apparent gross normal life of mice with PARP-1 gene deletion would imply that, for an individual organism, PARP-1 is not absolutely necessary, at least for living in the artificial laboratory environment.[17-19] Alternatively, the loss of PARP-1 can be functionally compensated by other PARPs, although at present, there is no evidence of upregulation of other PARPs in the PARP-$1^{-/-}$ mice. At a first look, it seems contradictory to all the essential functions attributed to PARP-1, such as gene expression, DNA replication, DNA rearrangement, differentiation, and mutagenesis. Yet, the fact that PARP-1 gene is highly conserved from *Dictyostelium* to humans strongly argues for an evolutionary advantage it may have conferred. Perhaps the roles of PARP-1 could be more reconcilable in the context of evolutionary benefit to the species, rather than in the life span of an individual. Based on the two fundamental properties of PARP-1, namely, as a sensor of DNA damage and as a major consumer of NAD^+, it is tempting to speculate that PARP-1 can fill the role of a "modulator" that adjusts the overall cellular energy status according to how favorable the environment is for growth and propagation (Figure 12.1). From an evolutionary perspective, nature would favorably select for species that could take the advantage of available food resources, when there is ample, to quickly expand the group through reproduction. On the other hand, when faced with an adverse environment, e.g., scarce food supply, it would be better off for the individual to slow down the metabolism such that entering into a "dormant" state would promote survival in harsh conditions and ensure future growth. Reactive oxygen species, e.g., superoxide anion and hydrogen peroxide, have become an inherent by-product since eukaryotes employed mitochondria for aerobic energy production and internally contributed to oxidative stress. Other free radicals, such as nitric oxide, have been employed as signal molecule, which would also result in nitrosative stress. Early eukaryotic organisms also must survive in a harsh atmosphere filled with agents that damage DNA, proteins, lipids, and carbonhydrate. In contrast to other biomolecules, the majority of eukaryotic DNA, the "junk" DNA, can record environmental hits without suffering a loss of biological function, since it does not encode any genes. Conceivably, the use of functionally silent "junk" DNA as a stress register preserves it from elimination from the genome by evolution. This speculation offers one explanation to justify the burden of maintaining more than 90% noncoding DNA sequence in the eukaryotic genome. PARP-1 appears uniquely fit for

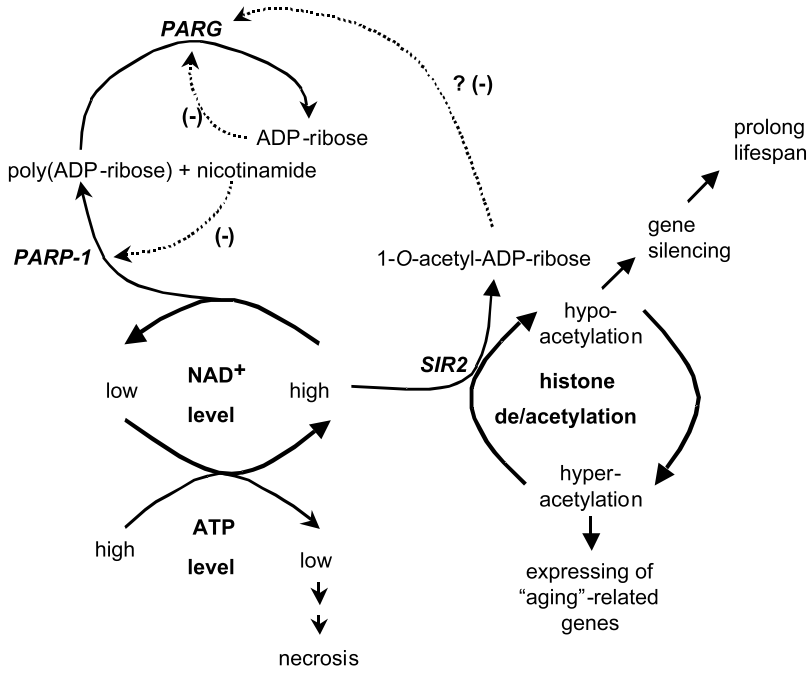

FIGURE 12.1 A proposed mechanism on how the balanced NAD^+ level may influence cell growth, necrosis, propagation, and gene silencing. See text for discussion.

adjusting intracellular energy barometer NAD^+ according to environmental cues. PARP-1 may be involved in coordinating the overall growth status by translating the extracellular conditions into fluctuating thresholds of NAD^+ levels. For limited oxidative stress, as reflected in minimal DNA damage, it is worth spending energy to fix it. When escalating stress is recorded on extensive DNA breaks, it is prudent to halt the cell cycle for thorough checking before any runaway cancerous cells potentially threaten the well-being of the individual. For overwhelming oxidative damage, necrosis might be an effective means to limit damage. In a well-controlled artificial laboratory environment, where food is abundant and oxidative stress is low, mice with PARP-1 gene deletion can apparently live a life indistinguishable from their wild-type littermates, as the evolutionary function of PARP-1 is likely to become non-selective under these conditions. However, exposure to high doses of γ irradiation reveals lowered survival rate in the PARP-1 knockout mice, which suggests the advantage PARP-1 could confer in an extreme environment.[18] So far, there is no report of increasing incidence of tumorogenesis in the three strains of PARP-1 knockout mice after many generations of breeding. Embryonic stem cells from wild-type and $PARP^{-/-}$ generated similar teratocarcinoma when injected into nude mice, although tumors derived from $PARP^{-/-}$ clones contained syncytiotrophoblastic giant cells.[20] Changes in PARP-1 knockout mice remain at the molecular level, such as different gene expression pattern, increasing sister-chromatid exchange, and shortening

telomere.[18,21-24] These changes could be due to the lack of whole PARP-1 protein, which has multiple activities, e.g., regulating transcription, in addition to the loss of the catalytic activity for poly(ADP-ribose) synthesis. One might infer that animals treated with PARP inhibitors may experience fewer side effects, especially when there is no selective pressure from the environment on growth and reproduction.

It is interesting to notice that a family of SIR2 proteins, which has been implicated in mediating cellular aging process, has recently been found to be histone deacetylase and the deacetylation process requires NAD^+ as a substrate to produce 1-*O*-acetyl-ADP-ribose.[7,8] Originally discovered in *Saccharomyces cerevisiae*, the SIR2 gene mutations cause abnormal expression of regions of the genome that are normally silent, i.e., transcriptionally inactive. Such regional gene silencing is typically associated with packaging chromatin into inaccessible structure, heterochromatin. Although SIR2 is nonessential for yeast viability, its mutation resulted in expression of genes implied for the aging process in yeast. It is required to mediate the process of extending the life span of yeast growing in low-nutrient media.[25] Another gene necessary for this "antiaging" phenomenon in yeast is NPT1, which is a key component in the synthesis of NAD^+.[25] Delineation of the biochemical reaction clearly established that the deacetylation requires NAD^+.[7,8] The recently solved NAD^+/SIR2 co-crystal structure offers direct physical proof for the involvement of NAD^+ in the process,[26] although it remains a puzzle why an energetically favorable deacetylation reaction would require the coupling of the hydrolysis of high-energy glycosidic bond in NAD^+ by SIR2, which serves as a *de facto* NADase. SIR2 catalyzes the hydrolysis of NAD^+ to yield nicotinamide. Simultaneously, it removes the acetyl group from lysine residues in histone and transfers it onto ADP-ribose to yield a novel molecule 1-*O*-acetyl-ADP-ribose.[7,8] *In vivo*, the causal relationship is yet to be established for the sequential events, whether lowering NAD^+ would retard histone deacetylation or massive deacetylation would lead to low NAD^+. In rodents, caloric restriction has been shown to be a reliable means of prolonging life span. It is well documented that limiting food intake could stall the aging process and promote longevity. There is recent evidence to suggest that the underlying biological pathway may be evolutionarily conserved. In *Caenorhabditis elegans*, overexpression of Sir2.1 increases mean life span by as much as 50%.[27] Sir2.1, a *C. elegans* homologue of SIR2, apparently acts through the insulin or insulin-like growth factor-I pathway, which is known to be involved in regulating longevity.[27] Incidentally, insulin release is reported to be regulated by cyclic ADP-ribose, which is synthesized by CD38 from NAD^+ and acts on ryanodine receptor to potential calcium release from endoplasmic reticulum.[28] Conceivably, SIR2 can link the histone acetylation/deacetylation cycle to the poly(ADP-ribose) turnaround cycle through the threshold set by the level of NAD^+. Intimate connection between the two pathways could also be established via the by-product of SIR2, 1-*O*-acetyl-ADP-ribose, which might act as an endogenous inhibitor for PARG, since ADP-ribose is a known, albeit weak, feedback inhibitor of PARG (see Figure 12.1).[29] It has been documented that there is a correlation between the long life span and high PARP-1 activity in mammalian species.[30] In humans, lymphoblasts from centenarians also have increased PARP-1 activity.[31] It would be interesting to test whether PARP-1 knockout mice would be

less responsive to calorie restriction-induced longevity especially in combination with environmental stress. Assuming SIR2 activity parallels with NAD^+ level, one would speculate that high calorie consumption may correlate with metabolic stress, which leads to PARP-1 activation, NAD^+ depletion, lowering SIR2 activity, histone hyperacetylation, shortening of life span, while low calorie diet would do the opposite. (see Figure 12.1).

Because of the fundamental biological effects of PARP activation and the enormous clinical potential of PARP inhibition, there is a tremendous interest in the pharmaceutical industry to develop specific small-molecule PARP inhibitors for possible clinical testing. Here, GPI 6150, 1,11b-dihydro-[2*H*]benzopyrano[4,3,2-*de*]isoquinolin-3-one, is used as an example to illustrate the efforts made by Guilford scientists to screen, identify, synthesize, and test novel PARP inhibitors.

12.2 SCINTILLATION PROXIMITY ASSAY FOR PARP

Critical to the speed of lead identification is the throughput of the enzyme assay. Conventional radioactive PARP assay requires the separation of the polymer product from the NAD^+ substrate, a rate-limiting step that hampers large-scale chemical library screening to identify novel small-molecule PARP inhibitors. The scintillation proximity assay (SPA) technology has been widely used to bypass separation of substrate from product. In SPA, the β-particles emitted from the substrates dissipate in the aqueous solution, while the radioisotope decays from products excite the scintillant beads that bind to the products.[32] SPA eliminates the requirement of separation between free ligand and complex, and thus greatly increases throughput. To develop a PARP-SPA, we tested whether biotinylated NAD^+ can also be utilized for poly(ADP-ribose) synthesis. The radioactive polymer with biotin-labeling is then suitable for detection by SPA (Figure12.2).[33]

Biotinylated NAD^+ was originally synthesized for the purification and identification of novel substrate protein targets for ADP-ribosylation.[34] We found that PARP-1 can utilize biotinylated NAD^+ to synthesize poly(ADP-ribose) polymer. The biotin label greatly simplifies monitoring the enzymatic activities of PARP and its homologues and may facilitate identifying and characterizing acceptor proteins of poly(ADP-ribose) polymer. A number of PARP-related enzymes have recently been cloned. They include PARP-2, PARP-3, Tankyrase 1, Tankyrase 2, and vPARP.[2,3] The expressed recombinant PARP homologues can produce bona fide poly(ADP-ribose) polymer. In the past, biotinylated NAD^+ has been shown to serve as a substrate for mono(ADP-ribose) transferase, such as dephtheria toxin. Now we have demonstrated that biotinylated NAD^+ can also be used for poly(ADP-ribosyl)ation. Based on the sequence homology in the catalytic domains among PARP-1 and its homologues, it is likely that biotinylated NAD^+ can be used by PARP-2, PARP-3, Tankyrase 1, Tankyrase 2, and vPARP for synthesis of poly(ADP-ribose). Such biotin-labeling methods offer a convenient means to determine the activities of PARP and its analogs. Furthermore, there is little known about the substrates for PARP-2, PARP-3, Tankyrase 1, Tankyrase 2, and vPARP. The biotin tag makes it possible to

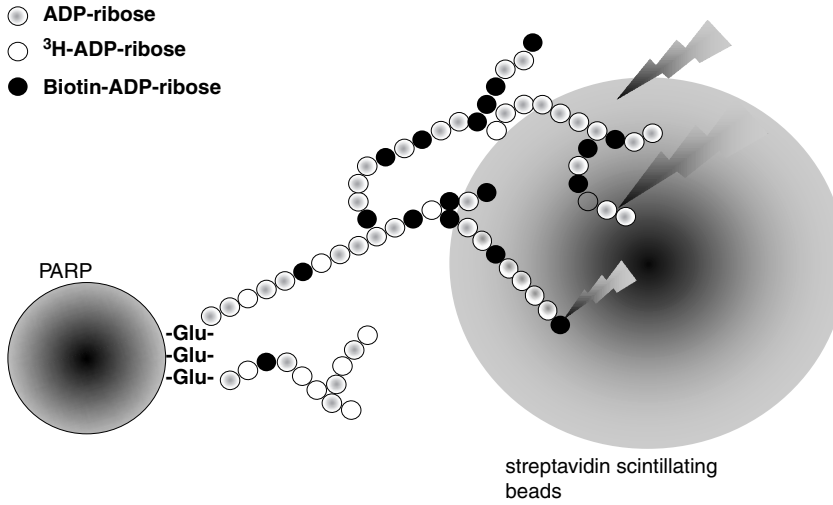

FIGURE 12.2 The principles of PARP-SPA. A mixture of NAD^+, biotin-NAD^+, and ^{3}H-NAD^+ is used by PARP to synthesize poly(ADP-ribose) that contains the labels of biotin and tritium. The polymer binds to the avidin-SPA beads through the association of biotin-ADP-ribose and the avidin. Thus, ^{3}H-ADP-ribose units are brought to close vicinity of the beads and excites the scintillant. No separation of ^{3}H-NAD and poly(ADP-ribose) is necessary. (From Cheung, A. and Zhang, J., *Anal. Biochem.,* 282, 24, 2000. With permission.)

use affinity purification to identify and characterize acceptor proteins for poly(ADP-ribose). Another derivative utility for biotinylated poly(ADP-ribose) is for monitoring *in situ* activation of PARP in histological analysis, as an alternative to radioactive labeling.[35-38]

The biotinylation of poly(ADP-ribose) enables us to develop an SPA assay for PARP. The procedure circumvents the requirement of separation between substrate and product, a rate-limiting step in large-scale screening for PARP inhibitors. By eliminating the "washing" step, the PARP-SPA reduces assay time. The 96-well or other miniature assay format can be readily adapted to automatic liquid sample handling robotics, and the automation greatly increases the throughput of compound screening. After directly comparing PARP-SPA against radioactive PARP assay, we found no difference in terms of sensitivity and inhibition profile of selected PARP inhibitors.[33]

12.3 THE SPECIFICITY OF GPI 6150

GPI 6150 (1,11b-dihydro-[2*H*]benzopyrano[4,3,2-*de*]isoquinolin-3-one, $C_{15}H_{11}NO_2$, MW 237) represents one of the earliest PARP inhibitors identified at Guilford Pharmaceuticals, Inc. It belongs to the tetraheterocyclic lactam family of PARP inhibitors, which was distinct from the previous known PARP inhibitors in the

benzamide or dihydroisoquinoline derivatives. Although we have subsequently discovered other series of PARP inhibitors with improved potency, e.g., the 5[*H*]phenanthridin-6-ones family of PARP inhibitors,[39] GPI 6150 remains to be one of the most thoroughly characterized compounds with remarkable efficacy in a variety of animal models of diseases. It is used here as an example to illustrate a continuous pursuit of specific, potent, nontoxic, and efficacious PARP inhibitors in the Guilford PARP program.

GPI 6150 is a representative compound for one of several classes of PARP inhibitors we have identified by using lead screening with computer-assisted drug design. The enzyme kinetic analysis indicated that the compound competed with NAD^+ to inhibit at the catalytic site of PARP. We found the compound could inhibit PARP activation by oxidative DNA damage in cells and consequently preserve the cellular NAD^+ level.[40] The prevention of energy depletion by this compound is believed to explain its cellular protective effect against free radical damage to cells.

There are two major concerns about the PARP inhibitors, their specificity, and possible side effects of PARP inhibition. The first concern on specificity mainly arises from the fact that most PARP inhibitors bind to the NAD^+ site in PARP. Since NAD^+ is widely used as a coenzyme in redox reactions for energy production, will PARP inhibitors interfere with cellular metabolism? We chose a variety of dehydrogenases catalyzing NAD^+ to NADH conversion and found no effect of GPI 6150, even at high concentration, on these pathways.[40] Thus, it is unlikely that GPI 6150 would interfere with NAD^+/NADH reactions in cells. This is consistent with the substantial structural difference between the PARP catalytic domain, which uses NAD^+ as a substrate, and the NAD^+ site in dehydrogenases, which uses NAD^+ as a coenzyme.[41] Other eukaryotic enzymes using NAD^+ as substrate include mono(ADP-ribosyl) transferase, CD38 (NAD^+ cyclase) and PARP isoforms.[1-4] Using diphatheria-cataylized mono-ADP-ribosylation of eukaryotic elongation factor 2 as a prototypical mono-ADP-ribosylation reaction, we found GPI 6150 is selectively more potent against poly(ADP-ribosyl)ation ($IC_{50} \sim 0.1\ \mu M$) than against mono-ADP-ribosylation ($IC_{50} \sim 9\ \mu M$). Similarly, GPI 6150 did not inhibit CD38-catalyzed conversion of NGD (nicotinamide guanidine dinucleotide) to cyclic GDP-ribose (Hon Cheung Lee, personal communication). In addition, GPI 6150 did not inhibit PARP cleavage by caspase-3 and caspase-7. Also, GPI 6150 turned out negative in a general screening that included more than 100 targets involved in major pathways of signal transduction and metabolism. Finally, within the PARP family, GPI 6150 appears to be equally potent to inhibit poly(ADP-ribosyl)ation. The values of IC_{50} against PARP-1 and PARP-2 by GPI 6150 are in the indistinguishable range. Thus, GPI 6150 could be a useful agent to inhibit poly(ADP-ribose) formation generally, regardless of which PARP isoforms produce the polymer.

Another major concern of PARP inhibitors arises from the involvement of PARP in DNA repair. From studies using PARP inhibitors or genetic manipulation to reduce PARP-1 protein level, there is accumulating evidence that PARP-1 facilitates DNA repair, especially in base excision repair.[42] In an attempt to look for possible impairment of DNA repair by GPI 6150, we found no evidence that PARP inhibition by this compound interferes with the repair and expression of exogenously damaged

plasmid DNA in cell.[40] The long-term effect of PARP inhibition remains to be established. Since the physiological functions of PARP are not well defined, it is difficult to predict the consequence of chronic PARP inhibition on normal subjects. Although early experiments seem to suggest that PARP inhibition may adversely affect DNA repair, DNA replication, gene expression, and other cellular functions, the apparent normal embryonic development, growth, reproduction, and life span of PARP-$1^{-/-}$ mice attest that PARP plays a nonvital role in these processes. Alternatively, the PARP-1 functions can be compensated for by other redundant mechanisms. Inhibition of PARP-1 catalytic activity is likely to cause fewer side effects than eliminating PARP-1 protein itself, which, in addition to poly(ADP-ribose) production may function through interacting with transcription regulators.[43-48] For example, fibroblast cells prepared from PARP-$1^{-/-}$ mice has an elevated level of tetraploidy,[49] which is presumably due to the lack of PARP-1 protein as a safeguard for genomic integrity. However, chronic inhibition of wild-type PARP-$1^{+/+}$ fibroblasts by GPI 6150 did not result in increased tetraploidy,[50] which suggested that inhibition of poly(ADP-ribose) production itself is not sufficient to yield transformation. It is possible that noncatalytic domains in the PARP play major roles in mediating transcription regulation and safeguarding chromosomal stability.

12.4 EFFICACIES OF GPI 6150 IN ANIMAL MODELS OF DISEASES

We, together with our collaborators, have demonstrated that GPI 6150 provides strong protection against necrosis in animal models of focal cerebral ischemia, traumatic brain injury, regional myocardial ischemia, bowel ischemia, and arthritis.[51-55] Recent studies using mice with deletion of gene for PARP-1 have established that PARP activation is the major component mediating death caused by lipopolysaccharide (LPS)-induced shock,[56,57] hyperglycemia induced by streptozotocin (STZ),[19,58,59] and damage to dopaminergic neurons induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP).[60] Compared to wild-type littermates, mice homozygous for PARP deletion are resistant to the deleterious effects elicited by LPS, STZ, or MPTP. We tested the effect of pharmacological inhibition of PARP by GPI 6150 in mouse models of type-I diabetes, septic shock, Parkinson's disease, and gout arthritis. The results showed the efficacy of the compound and broadened the indications for its potential clinical uses.

12.4.1 The STZ Diabetes Model

The earliest indication for a potential clinical benefit of PARP inhibition came from studies showing that PARP inhibitors can prevent destruction of β-islet cells by STZ in a rodent model of type I diabetes. In fact, nicotinamide was found to prevent hyperglycemia in STZ treated rats, before it was recognized as a PARP inhibitor, albeit a weak one.[61] The role of PARP activation in the streptozotocin/diabetes model was established when Yamamoto and Okamoto[62] demonstrated the depletion of NAD^+ pool was coupled with the loss of insulin secretion, which could be restored by PARP

inhibitors such as nicotinamide and benzamide. Definitive proof for PARP activation as a mediator of necrosis of β-islet cells was obtained when the PARP-1 knockout mice became available and shown to be completely resistant to pancreatic damage by STZ.[19,58,59]

We tested GPI 6150 in the mouse model of STZ-induced hyperglycemia. Groups of ten ICR derived male mice weighing 22 g were used. GPI 6150 or vehicle (100% DMSO) was administered intraperitoneally three times (25 mg/kg each time) at 15 min before and 2.75 and 24 h after the mice were challenged with a single dose of STZ (150 mg/kg, IV). Blood samples were obtained at 48 h after STZ challenge and the glucose level was determined. GPI 6150 treatment significantly reduced serum glucose level from vehicle-treated group of 386.1 ± 35.1 mg/dl to 290.6 ± 2.8 mg/dl ($p < 0.05$) (Figure 12.3A). For comparison the non-STZ-treated group had a serum glucose value of 154 ± 3.3 mg/dl. In another test, GPI 6150 (at dose of 10 mg/kg) and vehicle (100% PEG400) were administered orally 30 min before and 4, 12, 24, and 36 h after STZ. The oral dosing of GPI 6150 resulted in reduction of blood glucose level from 303.0 ± 22.3 mg/dl to 216.4 ± 13.0 mg/dl ($p < 0.01$) (Figure 12.3B). Thus, GPI 6150 is efficacious when given orally, which is consistent with pharmacokinetic analysis that reveals oral bioavailability of the compound (Guilford Pharmaceuticals, Inc., unpublished result).

12.4.2 The LPS Septic Shock Model

There is evidence supporting the role of PARP activation in the inflammation process either directly through tissue destruction or indirectly through regulating expression of cytokines and adhesion molecules, and PARP inhibitors have demonstrated anti-inflammatory activities.[63] One of the widely used multiple-organ failure model elicited by overwhelming immune response is the lipopolysaccharide (LPS)-induced septic shock. After LPS challenge, the survival time and rate for PARP-1 homozygous knockout mice were remarkably longer and higher than those of PARP wild type mice.[56,57] GPI 6150 was administered intraperitoneally (60 mg/kg) to a group of 10 C57/BL male mice weighing 18 to 20 g at daily dosing for three consecutive days. The first dose was given 30 min after the mice were challenged with a single dose of LPS, (from *Escherichia coli*, LD^{100} of 20 ng/animal) plus galactosamine (20 mg/animal IV). The second and third doses were given 24 h later on day 2 and 3. The vehicle control-group received 100% DMSO. Mortality was recorded every 12 h over a 3-day period. At the end of 72 h observation, the mean survival time for GPI 6150-treated group was 51.6 h, as compared to 19.2 h for the control group ($p = 0.002$ by Wilcoxon test) (Figure 12.4). Of the treated group 40% remained alive at the end of the experiment, whereas none of the untreated group lived beyond 48 h.

12.4.3 The MPTP Parkinson's Disease Model

MPTP selectively damages dopamine neurons and thus provides an animal model for Parkinson's disease. It is metabolized by monoamine oxidase B to 1-methyl-4-phenylpyridine (MPP^+), which is the active component mediating neuronal damage. We found

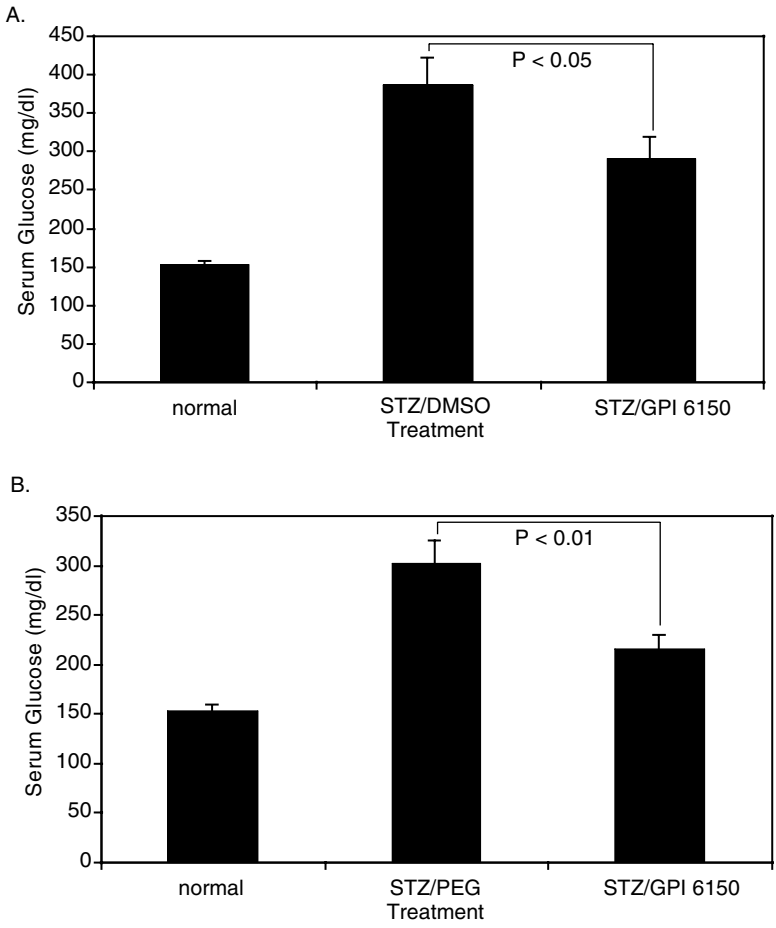

FIGURE 12.3 Intraperitoneal (A) or oral (B) administration of GPI 6150 prevents hyperglycemia in STZ-diabetes model.

that exposing PC-12 cells to either MPP^+ or MPTP immediately causes DNA damage and PARP activation, which suggests the involvement of the PARP pathway in mediating necrosis induced by the neurotoxin.[64] Subsequently, Cosi and associates[65,66] found that benzamide treatment decreases the loss of dopamine and prevents the drop of NAD^+ in mouse striatum. The pharmacological PARP inhibition data from *in vitro* and *in vivo* studies were corroborated later with experiments by Dawson and colleagues[60] who demonstrated that PARP-$1^{-/-}$ mice were spared from MPTP toxicity. Low potency and poor bioavailability of benzamide family of PARP inhibitors required the high dose of 160 to 640 mg/kg to offer neuroprotection in the MPTP

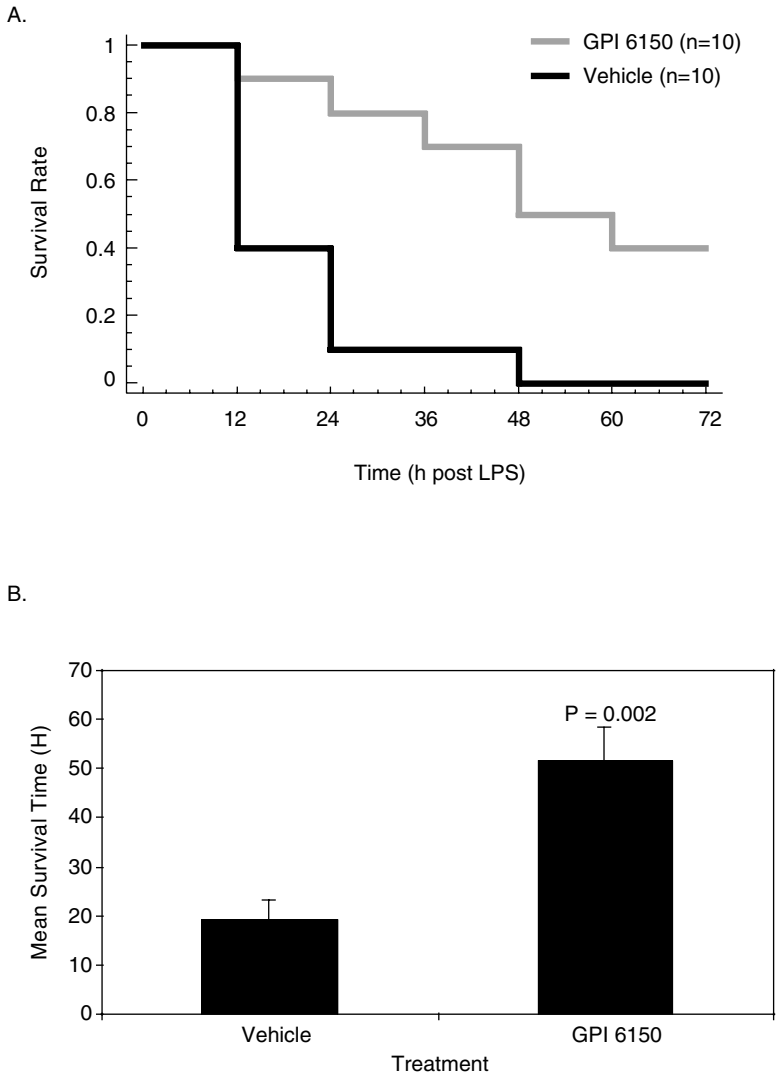

FIGURE 12.4 GPI 6150 treatment significantly reduces mortality rate (A) and prolongs mean survival time (mean ± standard error) (B) in LPS-septic shock model.

model.[66] With improved potency, specificity, and rapid penetration into brain by GPI 6150, we evaluated the compound in a concurrent MPTP-drug model.

Male CD1 mice (20 to 25 g) received five daily i.p. injections of MPTP (30 mg/kg) in saline vehicle. Mice were treated with GPI 6150 at 25 mg/kg i.p. given twice a day for 5 days. GPI 6150 was administered 15 min before and 2 h after the daily MPTP injection. Mice were sacrificed 5 days after their first MPTP injection.

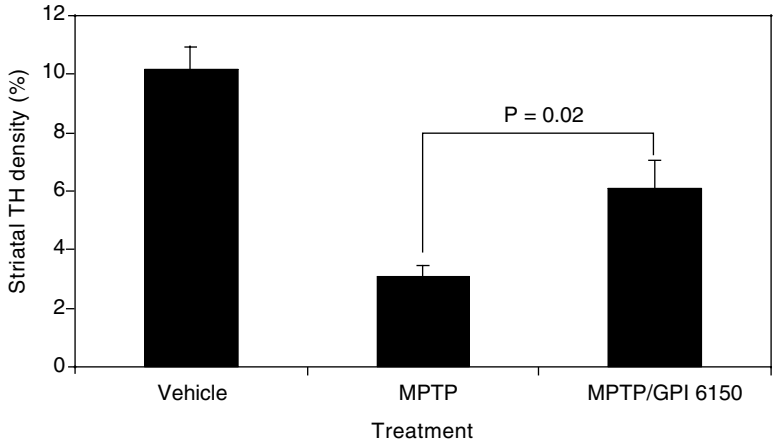

FIGURE 12.5 GPI 6150 protects dopaminergic neurons in MPTP Parkinson's disease model.

For histological analysis, mice were perfused with 10% formalin, their brains post-fixed overnight and then transferred to 30% sucrose saline. Frozen 30-mm sagittal striatal sections and 25-mm coronal nigral sections were cut; every fourth section was reacted for free-floating immunohistochemistry using a polyclonal tyrosine hydroxylase (TH) antibody (Pel Freeze, 1:2500 at 4°C for 4 nights), processed using the avidin:biotin peroxidase method (Vector Elite kit) and visualized with diaminobenzidine (Polysciences). TH fiber density in the central striatum was performed at 630× magnification. For each mouse striatum, five representative 0.1 × 0.1 mm fields in the core region were photographed using a digital video camera. The percentage of sample field covered by TH-positive processes and terminals was calculated using an image analysis program ("Simple," Compix, Inc., Pittsburgh, PA). The mean and standard deviation of striatal innervation density was calculated for each group. The magnitude of striatal deafferentation due to the MPTP lesion was assessed by dividing the observed striatal innervation values obtained in MPTP/vehicle treated cases by the mean striatal innervation density in vehicle/vehicle group and expressed as % loss.

There was a 70% loss of striatal TH innervation density following MPTP administration (Figure 12.5). GPI 6150 treatment offered protection of 42% ($p = 0.02$, Student's t-test) of innervation density that would otherwise have been lost due to the lesion by MPTP.

12.4.4 The MSU Gout Arthritis Model

Deposition of crystals of monosodium urate (MSU crystals) in the joint articular space is the etiological cause of inflammation in gout. MSU crystals have been shown to activate neutrophils to release an array of mediators that may be, in part, responsible for the local and systemic inflammatory manifestations found in crystal-induced joint disorders. The strong anti-inflammatory activity of GPI 6150 in arthritis model[55] prompted us to evaluate its effect in a murine model of experimental gout.

TABLE 12.1
GPI 6150 Reduced Neutrophil Migration to Urate Crystal in a Murine Model of Experimental Gout

Experimental Groups	Neutrophils (10^6 per mouse)
Pre-treatment, i.p. dosing	
Untreated	5.87 ± 0.28 (6)
DMSO (vehicle)	6.30 ± 0.33 (8)
GPI 6150	2.85 ± 0.34 (8)*
Post-treatment, i.p. dosing	
DMSO (vehicle)	7.99 ± 0.48 (8)
GPI 6150	5.47 ± 0.43 (8)*
Post-treatment, oral dosing	
PEG (vehicle)	6.34 ± 0.42 (8)
GPI 6150	4.84 ± 0.37 (7)*

*Data are shown as mean ± S.E. of (*n*) mice per group. Statistical differences were determined by ANOVA, plus Bonferroni test. A *P* value of < 0.05 was taken as significant.

Male outbred Swiss albino mice (20 to 22 g) were used. Peritonitis was induced by injection of 3 mg MSU crystals in 0.5 ml PBS (0.1 *M*, pH 7.4), and the recruitment of neutrophils into the cavity evaluated at the 6 h time point. GPI 6150 was solubilized at 45 mg/ml in 100% DMSO and injected into the peritoneal cavity at 45 mg/kg either 1 h before or 30 min after MSU injection. In another experiment, GPI 6150 was prepared in polyethylene glycol 400 (PEG400) at 2.25 mg/ml and the animals were dosed by oral gavage at 45 mg/kg at 15 min after MSU injection. Animals were then euthanized by CO_2 exposure and the peritoneal cavity washed with 3 ml of PBS supplemented with 3 m*M* EDTA and 25 U/ml heparin. An aliquot (100 µl) of the lavage fluid was then diluted 1:10 in Turk's solution (0.01% crystal violet in 3% acetic acid). The samples were then vortexed and 10 µl of the stained cell solution was placed in a Neubauer hematocymometer and neutrophil numbers counted using a light microscope.

Both pre- and post-treatment with GPI 6150 significantly reduced neutrophil infiltration caused by MSU crystal in peritoneal (Table 12.1). Pretreatment with GPI 6150 inhibited neutrophil recruitment by more than 50%, whereas post-treatment maintained a 32% reduction. In a more clinically relevant dosing regimen, oral treatment with GPI 6150 also achieved significant reduction (24%) of neutrophil migration.

12.5 CONCLUSIONS

Perhaps the earliest clue that implies the pathological role of PARP can be traced to study in the early 1980s that showed PARP activation mediates STZ damage to β-pancreatic cells in a diabetic model. In the mid-1980s, NAD^+ depletion was recognized as a major consequence of PARP activation after DNA damage by radiation and chemotherapeutic agents. Later, endogenously produced free radicals, such as

nitric oxide and peroxynitrite, were found to cause necrotic cell death via the PARP pathway. Soon, PARP inhibitors demonstrated efficacies in numerous models of ischemia and inflammation. Simultaneously, studies using PARP-1 knockout mice substantiated PARP-1 as a novel target for drug research. An extensive search for potent, selective small-molecule PARP inhibitors yielded multiple families of new PARP inhibitors. The new generation PARP inhibitors possesses improved solubility profiles suitable for further clinical development. Preclinical studies of these compounds have shown promising and remarkable efficacy in several disease models, especially in anticancer, neuroprotection, and inflammation areas. One can anticipate that some of the advanced PARP inhibitors are poised to enter clinical trial soon.

ACKNOWLEDGMENT

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13 Protecting Effect of PARP Inhibition on Ultraviolet Light–Induced Skin Damage

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CONTENTS

13.1 INTRODUCTION

The ultraviolet (UV) components of sunlight are now recognized as major environmental factors deleterious to human health.[1-4] Skin cancer is the most common malignancy among Caucasians.[2,4-8] The UV light in the solar spectrum is conventionally divided into UVC (200 to 290 nm; this does not reach the Earth), UVB (290 to 320 nm) and UVA, which is subdivided into short-wave UVA II (320 to 340 nm), and long-wave UVA I (340 to 400 nm). The UV rays of sunlight penetrate into the skin as a function of their wavelengths. Radiation of shorter wavelengths (UVB) is mostly absorbed in the epidermis and interacts predominantly with keratinocytes. Radiation of longer wavelengths (UVA, 320 to 400 nm) penetrates deeper, affecting the epidermal and dermal cells. Convolution of the spectra with biological damage

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action spectra shows that, despite the significantly greater incidence of UVA radiation (95% of the UV reaching the Earth), the predominant acute and chronic damages to the skin are associated with the UVB portion of the solar spectrum (in a ratio of 4:1).[9-11] However, the adverse effects of UVA and UVB are usually additive.[11,12] Photodamage, specific damage produced in the skin tissue by single or repeated (cumulative) exposure to UV light (290 to 400 nm), is considered to be the initiating step of photocarcinogenesis.[13] UV radiation-induced injury to the skin can be subdivided into acute (e.g., sunburn) and chronic (e.g., photoaging, solar keratosis, and skin cancers) photodamage.

Cellular DNA has been considered to be the principal molecular target for most of the biological effects of UV radiation.[1,7,14] At the molecular level, there is evidence that UV radiation generates DNA damage to skin cells either directly by producing cyclobutane pyrimidine dimers (CPD), (6-4) photoproducts, or indirectly by increasing the level of reactive oxygen species (ROS), which facilitate DNA oxidation. These processes are involved in many biological effects of UV exposure, including inflammation, phototoxic reactions, immune suppression, photoaging, photocarcinogenesis, etc.[12,15-20] It has been shown that UVA rays mostly act indirectly by generating ROS, which can subsequently exert a multitude of effects, such as lipid peroxidation, protein oxidation, DNA oxidation, activation of transcription factors, and generation of DNA strand breaks.[21] While UVB light can also generate ROS, it is most effective in direct interaction with DNA, with the formation of DNA photoproducts (thymine dimers), which are converted into single-strand DNA breaks via DNA repair enzymes.[2,3,12,22,23] However, by elevating the levels of ROS in skin cells, UVA rays trigger two other important pathways (induction of matrix metalloproteinases and mutations in the mitochondrial DNA) leading to photoaging. The acute effects of UV light such as sunburn and the role of UV radiation in immune suppression and in the induction and development of nonmelanoma skin cancers, squamous and basal cell carcinomas developed from keratinocytes, are overwhelmingly attributed to UVB radiation.[9,11]

High doses of UV irradiation due to excessive sunlight exposure induce extensive DNA damage that impairs the DNA repair capacity, and results in permanent DNA damage, which can be responsible for the deleterious effects of UV radiation.[24,25] These observations have led to the development of new strategies (e.g., liposome-encapsulated T4 endonuclease V, a bacterial DNA repair enzyme) to facilitate DNA repair in human cells and improve photoprotection.[17] Data suggest that enzymes and molecules, which decrease DNA damage or improve repair, can be used as photoprotective agents.

It is now evident that poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30, 113 kDa), an evolutionarily conserved nuclear enzyme, plays an essential role as a survival factor in replicating cells that have suffered limited DNA damage induced by environmental and endogenous genotoxic agents.[26] Nuclear PARP is activated by single-strand DNA breaks when the enzyme is bound to broken ends of DNA, and is inactivated when released as a result of auto-ADP-ribosylation.[27-29] PARP uses NAD^+ (β-nicotinamide adenine dinucleotide) as its substrate to synthesize poly(ADP-ribose) and ADP-ribosylates different nuclear proteins. Extensive DNA damage leads

to excessive PARP activation, which induces the depletion of NAD^+ and ATP eventually resulting in cellular dysfunction and necrotic cell death.[29-31] The disturbance of the energy balance of the cells leads to manifestation of pathological processes (e.g., ischemia–reperfusion injury, diabetes, septic shock, etc.), which can be significantly ameliorated by genetic inactivation or pharmacological inhibition of PARP.[26,29,32]

Previously, we observed the protective effect of *O*-(3-pyperidino-2-hydroxy-1-propyl) pyridine-3-carboxylic acid amidoxime dihydrochloride (BGP-15), a new PARP inhibitor, against ischemia–reperfusion-induced oxidative injury in a Langendorff-perfused heart model system. Our data suggested that BGP-15 moderated ischemia–reperfusion-induced oxidative damage, inhibited PARP, and improved the recovery of high energy phosphate intermediates.[33,34]

Cellular DNA is considered to be the principal molecular target for most of the biological effects of UV radiation, which generates DNA damage, and its repair results in the formation of single strand DNA breaks. When overactivated, nuclear PARP worsens the oxidative cell damage and impairs energy metabolism, raising the possibility that moderation of PARP activation following DNA damage may protect skin cells from UV radiation. The topical effects of the novel PARP inhibitor *O*-(3-pyperidino-2-hydroxy-1-propyl) pyridine-3-carboxylic acid amidoxime monohydrochloride (BGP-15M) on acute and chronic natural and artificial UV light-induced skin damage and its potential protective effects in a hairless mouse model were investigated.

13.2 PHOTOPROTECTIVE PROPERTY OF TOPICALLY APPLIED PARP INHIBITOR (BGP-15M) — CLINICAL AND HISTOLOGICAL INVESTIGATIONS

The photoprotective ability of BGP-15M-containing cream (concentration: 5 to 20%) was tested in groups of hairless mice exposed to a single erythematogenic 2 MED (minimal erythema dose) UVB radiation. The MED of UV irradiation was determined 24 h prior to start of each experiment.[35] Signs of acute UV damage, erythema and edema formation in unprotected skin areas were assessed visually, compared to the controls, and evaluated by using the Draize score system. The results are summarized in Figure 13.1. Under the given circumstances, the BGP-15M cream at concentrations >5% furnished complete protection against acute UV damage to the skin. The photoprotective concentration of this novel PARP inhibitor on topical application was ≥10%.

BGP-15M cream tested at concentrations ≥10% for its effects on solar exposure (4 MED) prevented the skin from the establishment of the clinical and histological signs of photodamage (Figures 13.2 and 13.3). The novel PARP inhibitor BGP-15M proved to be photoprotective against solar and artificial UV radiation–induced clinical symptoms or histological abnormalities of sunburn.

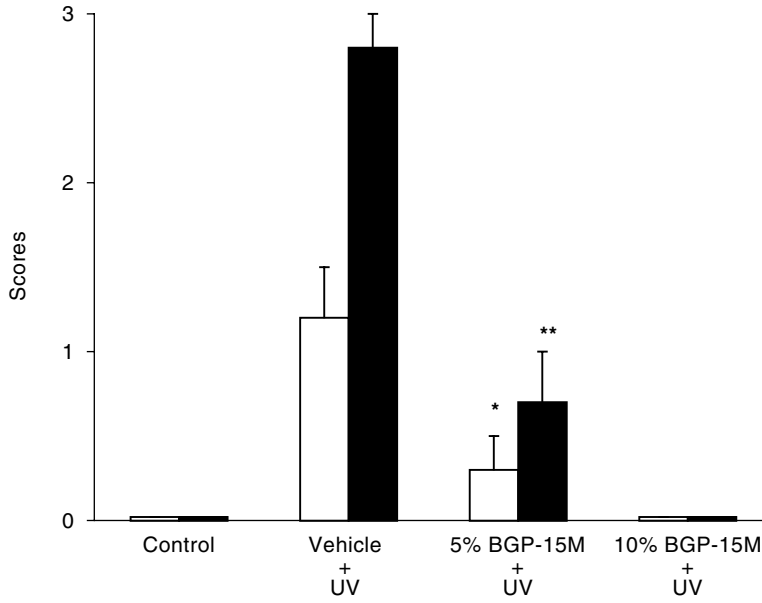

FIGURE 13.1 BGP-15M, a PARP inhibitor, protects against the clinical signs of sunburn. Test areas (1 cm^2 uncovered skin) of hairless (CRL:hr/hr BR) mice (age: 6 to 8 weeks, weighing 21 to 25 g) were treated with either 5 to 20% BGP-15M-containing creams or vehicle (2 mg cream/cm^2 skin surface) 15 min prior to erythematogenic 2 MED UVB exposure. Skin from untreated-unexposed animals served as controls. Clinical signs (erythema: filled bars; edema: open bars) were evaluated 24 h after UVB exposure using the Draize score (0: none to 4: severe). Values are means ± SEM for 6 animals/group. Values different from vehicle-pretreated, UV-exposed values at a significance level of $*P < 0.01$ or $**P < 0.001$.

13.3 EFFECT OF BGP-15M ON ACUTE DNA DAMAGE AND ADP-RIBOSYLATION IN UV-EXPOSED SKIN

Because skin cancer is an increasing public health and economic problem, and epidemiological as well as experimental data demonstrate that UV radiation is a major cause of all skin cancer (more than 1,000,000 cutaneous malignancies are diagnosed in the United States alone each year), research has made progress in understanding the mechanism of photomolecular events within photodamage of the skin.[2,36-40]

The DNA in skin cells is constantly subjected to environmental stress, e.g., solar radiation. Even normal cellular processes are often accompanied by the formation of ROS which in turn can damage the DNA.[36,41,42] In addition, UV radiation can directly injure the genome, thereby inducing or altering the repair system via activation or overactivation of PARP.[14,15,42] The UV-induced direct DNA damage can be investigated by determining the production of cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts.[43-46] It has been reported that the induction of CPD in epidermal and dermal cells following exposure of skin to UV radiation is dose and time

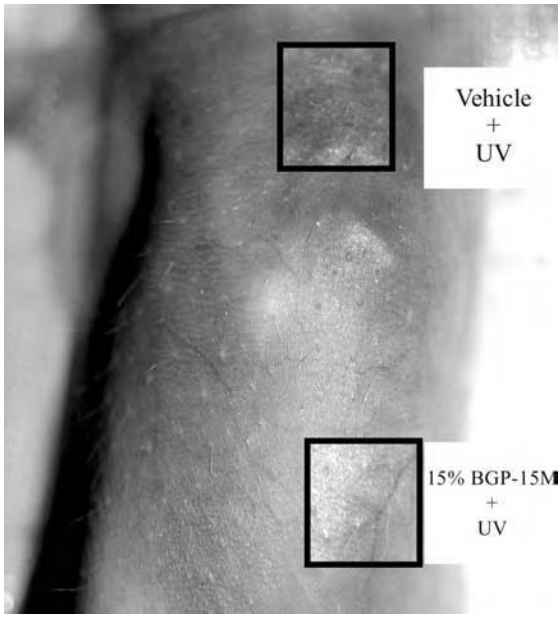

FIGURE 13.2 Effect of BGP-15M cream on acute photodamage in hairless mouse. The proximal skin surface area was pretreated with vehicle and the distal area with 10% BGP-15M cream (2 mg cream/cm^2 skin surface) 15 min prior to solar exposure (4 MED). Clinical investigation was made 24 h after UV exposure.

dependent.[2,46] At an early time point (after 30 min) CPD$^+$ cells could be observed and they remained elevated for 48 h. The count of CPD$^+$ cells proved to be lower in the dermis as compared to that in the epidermis.[2,23,47] The amount of CPD formation could be influenced by factors such as individual variations of capacity to repair photodamaged DNA, reaction to UV light, body sights, skin color, phototype, thickness of the skin, etc.[2,6,14] To study epidermal DNA photolesions in previously unexposed skin, which showed very low constitutive levels of melanin, UVB and UVA radiation were used to induce thymine dimers in melanocytes. The levels detected were comparable with those found in adjacent basal cell keratinocytes.[44] Besides direct absorption of UV-generating lesions as CPD, DNA may be damaged by indirect mechanisms, i.e., ROS causing DNA oxidation and DNA strand breaks that produce oxidative lesions as 8-oxo-2′-deoxyguanosine is being formed (8-oxodG).[23,43,48-51] Cook et al.[43] reported about the induction of cyclobutane thymine dimers and 8-oxodG by UVA radiation used in PUVA (Psoralen + UVA) therapy as a result of oxidative damage, which are potential causes of mutations seen in PUVA-derived squamous cell carcinoma (SCC).[43,52,53] Besides keratinocytes, melanocytes, the dominant cell types of the epidermis represent about 5 to 10% of basal cell population. Melanin precursors and melanin polymers are considered photoreactive.[54-57] Photooxidation leading to the formation of H_2O_2 can reach the nucleus and induce strand breaks or oxidative damage via Fenton reaction.[36,58] The biosynthesis of melanin is confined to the melanosome, which is isolated from other cellular components; however, photooxidative stress may damage its membrane and promote the leakage of reactive chemicals, and therefore damaged melanosomes could be associated with cytotoxic

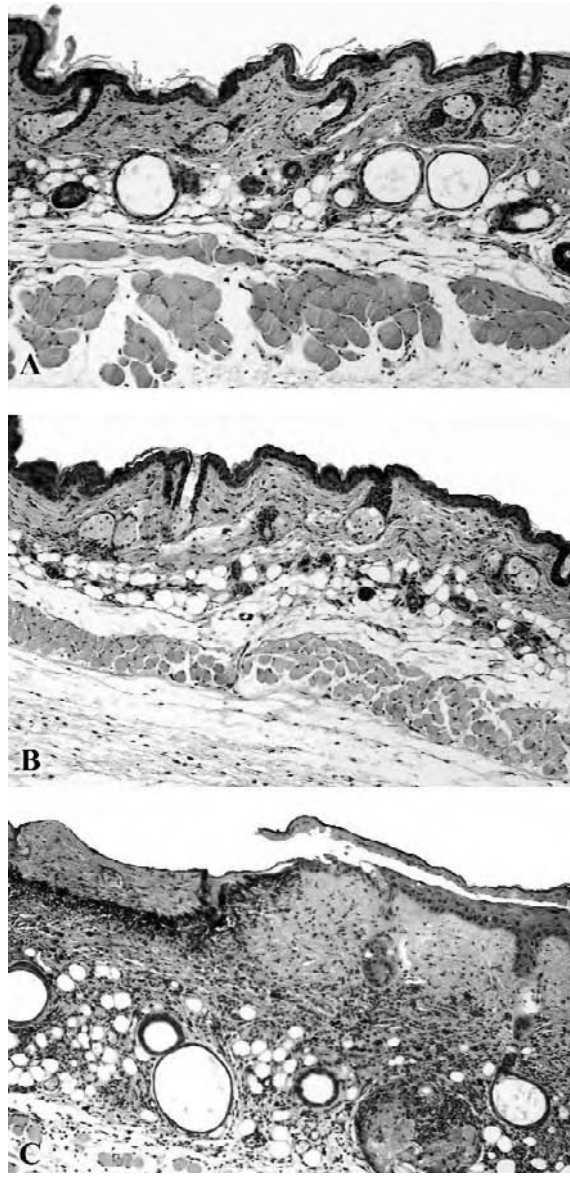

FIGURE 13.3 Effect of BGP-15M cream on histopathological signs of sunburn. Groups of mice were exposed to solar radiation (4 MED); 24 h later skin samples were taken for histological and immunohistological examinations. (A) Untreated-unexposed skin with normal morphology: thin orthokeratotic stratum corneum; two-three-layered epidermis; few cellular components and abundant fibers in the dermis. (B) 10% BGP-15M-pretreated, UV-exposed skin: without morphological changes. (C) Vehicle-pretreated UV-exposed skin: full-thickness epidermal necrosis with fibrin precipitates (left); partial necrosis of dermis with marked subepidermal edema (right); bandlike, dense, inflammatory infiltrate of neutrophils in the middermis (H&E, 100×).

phenomena.[36,41] The pool of reactive intermediates involved in melanogenesis could behave as an endogenous photosensitizer and generate oxidative stress when irradiated by UV, inducing a high level of strand breaks in melanocytes.[36] Caucasian human melanocytes synthesize both eumelanin and pheomelanin, through photoinstability of pheomelanin, which is considered to be a source of free radicals.[59] In contrast, Negroid individuals synthesize only eumelanin, which is capable of scavenging the superoxide anion and H_2O_2.[59,60] It should be noted that melanocytes are less resistent to H_2O_2-induced cytotoxicity than keratinocytes or fibroblasts.[36,60] Constitutional alteration in the scavanger system (different pattern of antioxidants and of membrane fatty acids of melanocytes) can be present in a person with increased sensitivity to the effect of UV light.[61,62]

To evaluate the potential protective effect of the novel PARP inhibitor BGP-15M applied topically to protect against natural UV radiation-induced DNA damage, a hairless mouse model was used. Single-strand DNA breaks were determined by the alkaline fluorescence analysis of DNA unwinding, as described by Birnboim and Jevcak.[33,63] DNA samples were prepared from the native (untreated and unexposed), 15% BGP-15M-containing cream-treated, 15% BGP-15M-containing cream, or vehicle-pretreated and sun-exposed (4 MED UV) skin of mice. Under our experimental conditions, most of the DNA in unexposed-untreated (native) skin was undamaged (Figure 13.4). Although the high dose of UV radiation involved in the exposure to sunlight generated a large amount ($P < 0.001$) of single-strand DNA breaks (undamaged DNA < 30%) in the vehicle-pretreated hairless mouse skin, it could be decreased ($P < 0.01$) by topical treatment with ≥ 10% BGP-15M prior to erythematogenic 4 MED UV exposure (Figure 13.4). Topically applied BGP-15M has the ability to protect the DNA, the main target of UV radiation, from most of this injury. It is likely that ROS are generated by mitochondrial respiration and it is possible that BGP-15M decreases the intracellular ROS level, which would explain why BGP-15M treatment decreased the number of single-strand DNA breaks in the skin cells.

Boukamp et al.[51] suggested that factor, as elevated temperature measured in skin areas of sunburn may contribute to photoinjury by generating DNA damage via the same indirect oxidative stress pathway. Ray et al.[64] found that mitochondrial (mt) DNA may be a significant target of direct effect of UV, and reported a five- to tenfold increase in the level of thymine dimers in purified mtDNA from UV-exposed HaCaT keratinocytes. PARP activation is generally considered to be a consequence of the oxidative cell damage mediated by single-strand DNA break formation.[27,31,65] In addition, there are data suggesting that PARP inhibitors partially protect mitochondrial respiration from externally added oxidants, but these inhibitors failed to protect the genome from single-strand DNA breaks.[66,67]

In the hairless mouse model ADP-ribosylation of nuclear proteins was determined by Western blot analysis.[33] ADP-ribosylated proteins were detected with anti-ADP-ribose monoclonal antibody, visualized by the enhanced chemiluminescence (ECL) method, and the signal intensities were quantitated. In skin samples exposed to erythematogenic 4 MED UV radiation, the poly(ADP-ribosyl)ation of PARP proved to be excessive (Figures 13.5 and 13.6). This process could be significantly reduced by using BGP-15M topically in a concentration of ≥10%

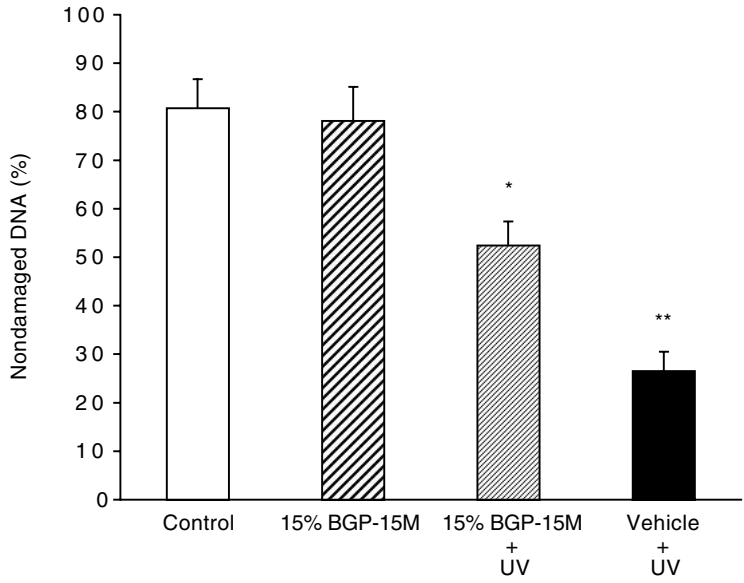

FIGURE 13.4 Effect of BGP-15M on UV exposure-induced single-strand DNA break formation in the skin. Single-strand DNA break formation was determined in skin samples derived from untreated-unexposed (control), 15% BGP-15M-treated-unexposed, 15% BGP-15M- or vehicle-treated 4 MED UV-exposed animals, by alkaline unwinding assay.[33,63] Values are means ± SEM for five animals/group. *Values different from vehicle-UV-exposed values at a significance level of $P < 0.01$. **Values different from the control values at a significance level of $P < 0.001$.

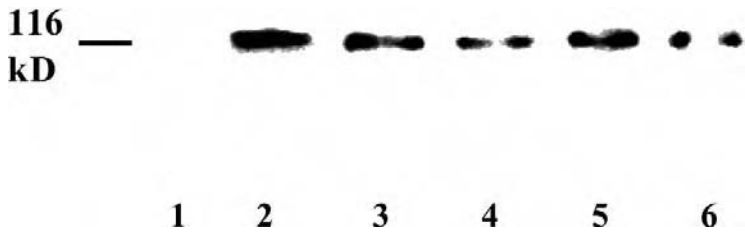

FIGURE 13.5 Western blot signal intensities of PARP. ADP-ribosylation of PARP was determined in skin specimens taken immediately after erythematogenic UV exposure by Western blot analyses.[33] ADP-ribosylation levels of unexposed-untreated (native) skin (lane 1); ADP-ribosylated PARP (positive control) (lane 2); vehicle-pretreated, UV-irradiated skin (lanes 3 and 5); 10% BGP-15M-pretreated, UV-irradiated skin (lane 4); 20% BGP-15M-pretreated, UV-irradiated skin (lane 6).

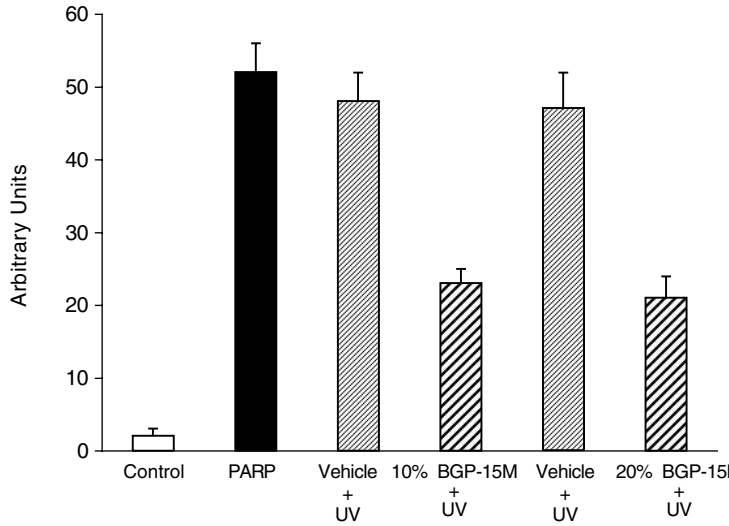

FIGURE 13.6 Effect of topically applied BGP-15M on ADP-ribosylation of PARP in the skin. ADP-ribosylation of PARP was determined in skin specimens taken from hairless mice by Western blot analysis.[33] Western blot signal intensities were quantified with the Image Tool (Version 1.27) image processing program and expressed in arbitrary units ± SD. Samples: unexposed-untreated (control); PARP (positive control); vehicle-pretreated, UV-irradiated; 10% BGP-15M-pretreated, UV-irradiated; 20% BGP-15M-pretreated, UV-irradiated skin.

(Figures 13.5 and 13.6). Immunohistological analysis of the epidermal cells from the vehicle-pretreated, UV-exposed skin indicated an intensive nuclear staining when anti-ADP-ribose antibody was used. In accordance with the results of Western blot analysis of the BGP-15M-pretreated samples, the nuclear staining of the epidermal cells was decreased (data not shown). This finding was in agreement with our previous observations that *in vitro* BGP-15 inhibited isolated PARP with IC_{50} = 120 μ*M* at 1 m*M* NAD^+ in Langendorff-perfused hearts.[33]

13.4 ROLE OF BGP-15M IN UV-INDUCED SUNBURN CELL FORMATION

Since a correlation exists between the UV dose delivered and the number of sunburn cells (SCs) in the epidermis, the increase in the number of SCs was simply regarded as a marker of the severity of solar damage.[68] SCs are characterized as single-standing keratinocytes undergoing apoptosis following UV radiation in UV-exposed skin.[15,69] They demonstrate the typical morphological signs characteristic of apoptotic cells: a shrunken eosinophilic cytoplasm with a condensed, pyknotic nucleus; shrinkage and membrane blebbing are followed by chromatin condensation and genomic DNA fragmentation.[69,70] SCs are detectable as early as 8 h after UV exposure, reach

the maximal amount after 24 to 48 h, and disappear after 60 to 72 h.[70] Different molecular pathways and their interactions are thought to be involved in the UV-mediated apoptosis of keratinocytes, including DNA damage, the activation of tumor suppressor gene p53, the triggering of cell death receptors either directly by UV or by autocrine release of death ligands, mitochondrial damage, and cytochrome *c* release.[15,71-74] It has been recognized that p53 is critically involved in the formation of SCs, since mice lacking functional p53 (p53 knockout mice) revealed significantly fewer SCs upon UV exposure than did UV-exposed wild-type mice.[75] As the formation of SCs is a consequence of UV-induced DNA damage, it is assumed that there is a link between SC formation and p53 (a DNA damage checkpoint protein).[15,76] UV-damaged keratinocytes that have failed to repair their DNA damage will die as SCs, thereby escaping the risk of becoming malignant.[77] Demonstration of the importance of the formation of SCs indicated that DNA damage is crucial in mediating UV-induced apoptosis.[71]

The effect of BGP-15M on UV-induced apoptosis by determining the SC formation in H&E-stained specimens derived from vehicle- and BGP-15M cream-pretreated 2 MED UVB-exposed skin samples, as well as from native control skin revealed the following findings: in UVB-exposed samples pretreated with ≥5% BGP-15M cream, the mean number of SCs formed was significantly decreased ($P < 0.01$) as compared with the number observed in vehicle-pretreated, UV-exposed ones (Figure 13.7). The reduced formation of SCs in the BGP-15M-pretreated UV-exposed mouse skin confirms the photoprotective ability of this PARP inhibitor.

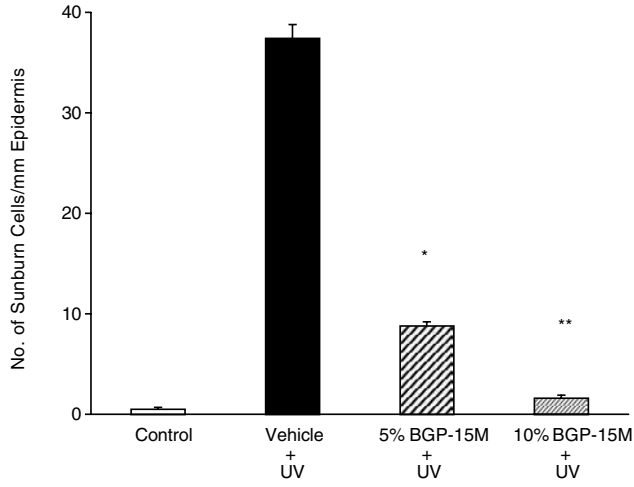

FIGURE 13.7 Effect of BGP-15M cream on sunburn cell formation in UV-irradiated skin. Sunburn cells (apoptotic keratinocytes) were investigated in H&E-stained specimens derived from untreated-unexposed control skin; vehicle-pretreated, UV-exposed skin; 5% BGP-15M cream-pretreated, UV-exposed skin; 10% BGP-15M cream-pretreated, UV-exposed skin. Samples were counted at 400× magnification in the interfollicular epidermis located above a 0.25-mm-long part of the basement membrane using 80 fields/group. Values are means ± SEM for six animals/group. Values different from vehicle-pretreated, UV-exposed values at a significance level of $*P < 0.01$ or $**P < 0.001$.

13.5 EFFECT OF BGP-15M ON UV-INDUCED IMMUNE SUPPRESSION IN THE SKIN

There is a strong correlation between UV-induced DNA damage and immune suppression in the skin.[78-81] Pyrimidine dimers, cis-urocanic acid, and ROS can play important roles in the UV-induced immune suppression of the skin by suppressing the induction of T-cell-mediated responses such as contact hypersensitivity and delayed-type hypersensitivity as a promoter of photocarcinogenesis.[23,78-81] A cascade of mediators, i.e., prostaglandin E2, IL-10, tumor necrosis factor α (TNFα), etc. are thought to be involved in the development of UV-induced immune suppression.[78,82] IL-10 is known to be an important suppressor of both T-lymphocyte and antigen-presenting cell effector functions, affecting the ability of Langerhans cells to present antigen, allowing them to stimulate T-helper type 2 (Th-2) cells, but inducing anergy in Th-1 cells.[83] IL-10 acts on macrophages to downregulate MHC (main histocompatibility complex) Class II expression, resulting in the inhibition of cytokine synthesis by activated T cells and natural killer cells.[84] Many studies have demonstrated that IL-10 is upregulated by UVB radiation in keratinocytes and plays a central role as an immunoregulator of UVB-induced reduction in delayed-type hypersensitivity reactions.[85-88] A correlation has been found between the restoration of immunity and the reduction in the levels of this cytokine.[86] IL-10 production by the keratinocytes was therefore utilized to test the effects of BGP-15M on UV-induced immune suppression. IL-10 production in UV-irradiated mouse skin was determined by immunohistological staining with anti-IL-10 antibody. The cytoplasmic staining of the epidermal keratinocytes in the UV-exposed vehicle-pretreated skin was intensive (Figure 13.8C) as compared to the untreated-unexposed control (Figure 13.8A). Cytoplasmic staining for IL-10 was only very rarely observed among the epidermal cells in samples derived from ≥10% BGP-15M-pretreated, UV-irradiated samples (Figure 13.8B). Topically applied BGP-15M (≥10%) treatment prior to solar exposure reduced the level of keratinocytes stained with anti-IL-10 antibody to close to the control value (Figure 13.8). This suggests that the novel PARP inhibitor may abolish the effect of UV-induced keratinocyte-derived IL-10, which systemically suppresses delayed-type hypersensitivity responses to alloantigen by the downregulation of antigen-presenting cell functions and by triggering the formation of antigen-specific suppressor T cells.

13.6 EFFECT OF BGP-15M ON PHOTOCARCINOGENESIS IN HAIRLESS MOUSE SKIN

Accumulation of UV photodamage in the skin resulting from chronic exposure to sunlight over the lifetime of an individual causes photoaging of human skin. Photoaging is characterized by clinical, histological, and biochemical changes that differ from alterations in chronologically aged but sun-protected skin.[89] The intrinsic or chronological aging, which occurs in all types of skin, is often masked by photoaging. Photoaging is much more common in patients with skin types I and II, while it is less prominent in dark-skinned individuals.[61] The most common clinical

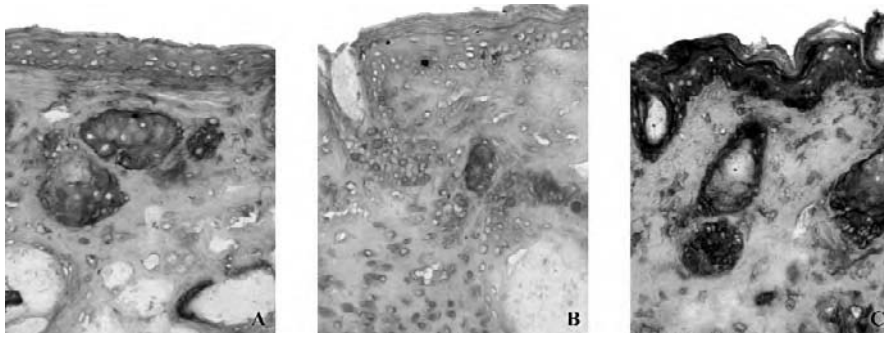

FIGURE 13.8 Effect of BGP-15M cream on IL-10 protein expression of epidermal keratinocytes in UV-exposed skin. UV-induced immune suppression in mouse skin was examined via the IL-10 protein expression by the epidermal keratinocytes. Immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections was carried out with anti-IL-10 polyclonal antibody (Sigma Chem. Co.) according to the streptavidin-biotin-peroxidase technique.[99] (A) Untreated-unexposed control skin; (B) 10% BGP-15M-pretreated, UV-irradiated skin; (C) Vehicle-pretreated, UV-exposed skin (200×).

manifestations of photoaging among Caucasians are fine wrinkling, freckles, telangiectasia, lentigines, guttate hypermelanosis, amelanotic pseudoscars, and in darker skinned individuals it appears as a hypertrophic pattern, lentigines, diffuse hyperpigmentation, thickened pebbly skin, etc.[12,89] All cells of the skin are altered.[90] Damaged keratinocytes lead to actinic keratosis which are irregular, brown-red plaques, mostly affecting the face. Histologically, they are *in situ* squamous cell carcinomas.[75,89-91] Damage to melanocytes may produce changes such as hyper- and hypopigmentation, lentigo maligna, and malignant melanoma. Solar elastosis (terms "sailors' skin," "farmers' skin") refers to a combined injury to collagen elastin and ground substance producing yellow papules or plaques.[90,92] The proliferation of elastin in the dermis can be induced by both UVB and UVA radiation.[90,92] Damage of endothelial cells is manifested as telangiectases and purpura in human skin.[90]

The influence of the topically applied BGP-15M on the effects of chronic exposure to UVB radiation in hairless mice was studied by clinical, histological, and immunohistological investigations during the 32-week (5 × 1 MED UVB/week) period of UV exposure to determine the development of signs of photoaging and photocarcinogenesis. Age-matched groups of animals were pretreated with 15% BGP-15M cream or vehicle prior to UVB exposure in the uncovered area of skin surface of the back to detect macro- and micromorphological changes. They were compared to the untreated-unexposed control group of animals undergoing chronological aging process without photoaging. Animals were scored for skin lesions and the appearance and number of skin tumors were detected. The first tumor appeared 18 weeks after starting UV exposure in vehicle-pretreated animals (Figure 13.9). By week 23 all animals in this group ($n = 12$) had one or more skin tumors (≥1 mm in diameter) (Figure 13.9). At the end of the UV treatment (week 32), all lesions were examined histologically. Histopathological changes in elastic fiber network were recognized by Orcein-Giemsa staining; thickening of the epidermis and carcinogenesis were

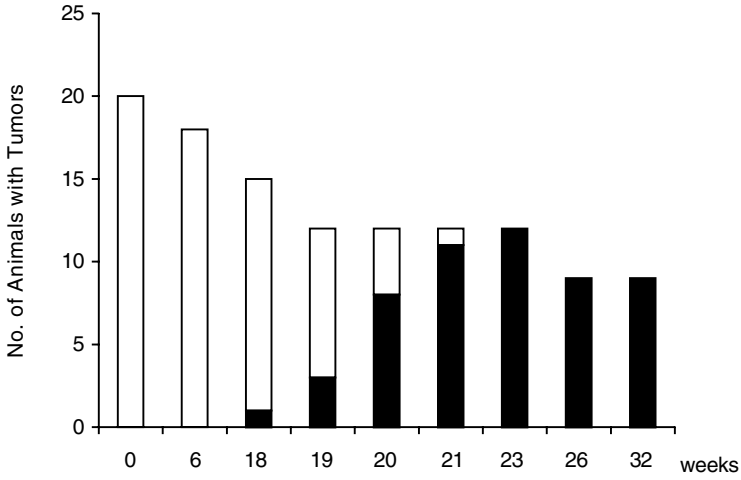

FIGURE 13.9 Timetable of photocarcinogenesis in UV-irradiated hairless mouse skin. Groups of hairless mice ($n = 20$) were treated with 15% BGP-15M cream or vehicle prior exposure to 1 MED UVB 5×/week for 32 weeks. Clinical signs of photoaging and photocarcinogenesis were observed daily. After 6, 18, 19, and 26 weeks of UV exposure 3 animals from each group were sacrificed, and, at the end of the treatment (after week 32) the remaining 8 animals were sacrificed for histological and immunohistochemical analysis. Tumor formation ≥ 1 mm were detected in all groups. No clinical or histological signs of skin tumor were observed in 15% BGP-15M-cream-pretreated UVB-exposed animals. Number of animals: open

followed by H&E staining and immunohistological reaction with p53 antibody. Figure 13.10C demonstrates one of the cases with multiplex tumors from the group of vehicle-pretreated UV-exposed animals with an intense p53 expression verified in the invasive SCC and in tumor cells forming *in situ* carcinoma. In BGP-15M-pretreated UV-exposed animals, no pathological changes could be observed except a 1.5- to 2-fold thickening of the epidermis with hypergranulosis (Figure 13.10B). Pretreatment with BGP-15M protected against the manifestation of an *in situ* or invasive carcinoma during the 32-week study period.

Accumulation of p53 protein has been observed in many different types of human and animal cells both *in vivo* and *in vitro* after treatment with a variety of DNA-damaging agents, including UV irradiation.[51,93-95] The wild-type form of p53 has been shown to mediate cell cycle arrest by increasing p21Cip1/WAF1 and Gadd45 following DNA damage, to participate in DNA repair, and to induce apoptosis.[15,75,76,94] The proportion of epidermal cells expressing the wild-type form of p53 after UVB exposure is highly variable among the different mouse strains.[3] In chronic UV exposure experiments by the time when increasing p53 expression was observed in the basal and suprabasal keratinocytes, most of these cells had contained mutant conformation of p53.[96]

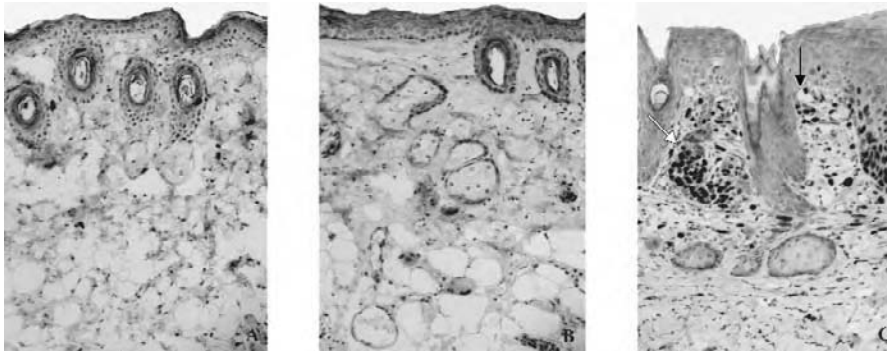

FIGURE 13.10 Immunohistological and histopathological changes in the skin of hairless mice after 32-week UVB-exposure. (A) Untreated-unexposed skin (age-matched control of UV-exposed animals) with normal skin morphology: thin orthokeratotic stratum corneum; epidermis composed of two to three layers of keratinocytes; without p53 expression; few cellular components and abundant fibers in the dermis. (B) 15% BGP-15M-pretreated, UV-exposed skin: thickening (1.5-fold) of the epidermis with hypergranulosis; no positive staining for p53; no changes in the dermis. (C) Vehicle-pretreated UV-exposed skin: prominent parakeratotic hyperkeratosis; marked thickening of the epidermis; invasion of the dermis by epidermal masses; the atypical cells localized in the epidermis and invading to the dermis strongly express p53. The black arrow shows acantholytic part of the tumor characteristic for carcinomas developed from solar keratosis. The white arrow signed the invasion in the dermis (ABC-peroxidase method, NovaRed substrate,[100] 150×).

The mutation spectrum analysis on a large series of UVB light-induced squamous cell carcinomas of hairless mouse showed that p53 expressed in these carcinomas mutated mainly at the sites known as UVB mutation hot spots at codons of p53 gene. This spectrum is not found in other p53-expressing murine tumors and has equivalents in human skin carcinomas (codon 272).[75,93,97] It has been reported that not only UVB but also long-wave UVA (>340 nm) radiation induced skin tumors in hairless Skh:hr1 mice after daily exposure.[97] In these tumors the incidence of p53 alteration was low as compared to UVB-induced tumors. Positive staining for the p53 protein was observed in only 50% of the tumors and the pattern of staining was more irregular and less dense as compared to UVB-exposed samples. Mutations were mainly located at codon 267.[97]

UV-induced damage plays a crucial role in the p53 tumor suppressor gene inactivation during the skin carcinogenesis process, and may be a useful marker in monitoring the development of human and murine skin tumors.

13.7 CONCLUSIONS

The most harmful biological effects of UV radiation are related to DNA damage induced in the skin cells, and are considered to be the initiating step of UV-induced pathological processes. DNA can be directly injured by UV light, or it can be

damaged by UV-induced ROS production. The repair of damaged DNA results in the formation of single-strand DNA breaks, which activate poly(ADP-ribose) polymerase, a nuclear enzyme resulting in elevated oxidative cell damage. These observations raised the possibility that moderation of DNA damage and subsequent PARP activation may protect skin cells from UV radiation. Our data indicate that the BGP-15M-containing cream decreases the UV-induced acute photodamage to the skin and sunburn cell formation, probably because BGP-15M treatment can decrease UV-induced DNA damage and PARP activation. These effects of BGP-15M are related to its ability to decrease the intracellular ROS production, which is the prominent cause of DNA damage, and of inhibiting the ADP-ribosylation of PARP. The decrease of UV-induced IL-10 expression is also in accordance with its moderation of UV-induced PARP activation (see Figures 13.5 and 13.6) and the decrease of UV-induced ROS production (see Figure 13.4). It has been known that both the inhibition of PARP and the decrease of ROS level can moderate the expression of cytokines.[98]

The most important factors in UV-induced chronic skin damage are the accumulation of mutations and deletions in tumor suppressor genes (e.g., p53) of skin cells leading to uncontrolled cell proliferation and the development of different types of tumors. Therefore, we expected that by the same mechanism as was seen in acute experiments, BGP-15M caused a reduction in UV-induced ROS production and DNA damage in chronic experiments. Furthermore, by protecting DNA, BGP-15M decreases the level of p53 mutations and tumor formations in UV-irradiated skin. Our chronic data clearly support this hypothesis, indicating the feasibility of the development of novel skin protecting agents, which can protect the skin against acute and chronic UV radiation-induced photodamage by directly interfering in the UV-induced ROS-mediated cellular processes.

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14 Role of Poly(ADP-Ribose) Polymerase Activation in the Pathogenesis of Diabetes Mellitus and Diabetic Vascular Dysfunction

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CONTENTS

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14.1 ISLET CELL DESTRUCTION AND AUTOIMMUNE DIABETES

14.1.1 Role of Autoimmune Processes and Free Radicals and Oxidants in the Pathogenesis of Islet Cell Death

Insulin-dependent diabetes mellitus (type I) is an autoimmune disease occurring predominantly in children and young adults resulting in destruction of the pancreatic ß-cells but not the other islet endocrine cells. Type I diabetes is characterized by prolonged periods of hyperglycemia, via reduced uptake of glucose and relative increase in glucagon secretion and gluconeogenesis. There is an increase in fat catabolism with production of ketone bodies and a fall in blood pH. If diabetic subjects do not receive insulin therapy, the metabolic effects can result in coma and death. The destruction of the islet ß-cells is caused by an autoimmunological attack involving an initial hyperexpression of class I major histocompatability complex (MHC) molecules by all of the islet endocrine cells, which is followed by ß-cell exclusive expression of MHC class II molecules. Expression of the MHC proteins induces an insulitis whereby the islet is infiltrated by mononuclear cells including lymphocytes, macrophages, and plasma cells. The actual trigger for the process of ß-cell destruction is poorly characterized but is either an external factor (viral, chemical) or an internal stimulus (cytokines, free radical), which damages a proportion of the ß-cells leading to release of specific ß-cell proteins (insulin, GAD 65, etc.), which can be taken up by antigen presenting cells and processed to antigenic peptides. The antigen-presenting cell in association with the MHC class II molecules expressed on the ß-cells carries this antigenic peptide to T-helper cells. The latter recognize the complex and become activated, resulting in transcription of cytokine genes including interferon-γ which can feed back onto the antigen-presenting cells to increase expression of interleukin-1B (IL-1ß) and tumor necrosis factor-alpha (TNF-α). The T-helper cells also activate B lymphocytes that produce islet cell autoantibodies, which is followed by cytotoxicity by killer cell activation. In the serum of a large proportion of individuals with an increased risk of developing type I diabetes, (auto-) antibodies against specific ß-cell antigens such as insulin,[1] proinsulin,[2] and glutamic acid decarboxylase[3] have been detected. Cytotoxic T lymphocytes are also activated via the T-helper cells and are attracted to the increased MHC class II expression on the ß-cells. The products of immune cell activation including cytokines and free radicals are the direct participants in inducing ß-cell death. The final event in the autoimmune process is the removal of cell debris by macrophages.

The elucidation of these events in the pathogenesis of type I diabetes was made possible by the development of rodent models of type I diabetes. Initial

models utilized ß-cell toxins such as streptozotocin, which was found to be able to induce diabetes either directly or via an immune cell-mediated mechanism depending on whether a single large dose[4] or multiple low doses[5,6] were administered. Results from these initial studies were subsequently extended and refined by using the BioBreeding (BB) rat[7] and the non-obese diabetic (NOD) mouse,[8] two spontaneous genetic models of diabetes. The development of diabetes in these spontaneous models was found to share a number of features with the pathogenesis of the human disease, with the appearance of autoantibodies against the ß-cell proteins[9] and the development of an immune cell infiltration of the islet (insulitis).[6,10]

Destruction of the ß-cell in diabetes has been attributed to the production of various immune cell mediators such as cytokines and free radicals produced in the islet itself. Major mediators of ß-cell death appear to be nitric oxide (NO) and various related free radical and oxidant species. High concentrations of NO are produced in the islet directly from the infiltrating macrophages[11] and indirectly from induction of NO synthase (NOS) in various cell types of the islet following exposure to immune cell-produced cytokines such as IL-1ß, TNF-α, and interferon-γ. Islet cells that respond to cytokines in this way include the islet capillary endothelial cells[12] and the ß-cell themselves,[13] while the glucagon-producing (alpha) cells do not express iNOS in response to cytokines.[14] The induction of NOS and production of free radical species have been implicated in the development of diabetes in many of the animal models, with iNOS-deficient mice being protected from streptozotocin-induced diabetes[15] and iNOS detected in the pancreas of the NOD mouse[16] along with evidence of peroxynitrite formation (a reactive oxidant produced from the rapid reaction of NO and superoxide).[17] NOS inhibitors have been demonstrated to prevent diabetes in the streptozotocin,[18] NOD mouse,[18] and BB rat[19] models of type I diabetes.

Isolated rat, mouse, and human islets of Langerhans have frequently been used to identify the events involved in ß-cell dysfunction and death in diabetes. Exposure of islets to appropriate combinations of proinflammatory cytokines for 24 to 48 h results in inhibition of insulin secretion,[13,20,21] which is mediated by NOS induction and subsequent free radical formation.[13,20] Cytokines also inhibit islet DNA synthesis[22] and glucose oxidation through the inhibition of the mitochondrial enzyme aconitase.[23] Cytokines were also found to decrease islet ATP,[23] cAMP[23], and NAD^+ levels,[24-26] and to inhibit insulin biosynthesis.[23] Cytokine combinations also cause DNA damage in isolated islet cells from both rat and human, an effect that is mediated by NO and related radicals and oxidants.[25,27,28] The final outcome of exposure of islets to cytokines is cell death: necrosis[29] and/or apoptosis.[30]

Many of the effects induced in islet cells by cytokine treatment have been duplicated using chemically generated free radicals, such as NO, oxygen free radicals, and peroxynitrite. Inhibition of insulin secretion,[31,32] induction of DNA damage by free radicals,[25,27,28,33] and islet cell lysis[34] and apoptosis[30] have all been observed in rat and human islets exposed to NO, reactive oxygen species, or peroxynitrite *in vitro*. The consequences to the ß-cell of this cellular dysfunction are multiple and involve a variety of oxidant-mediated protein and lipid modifications. Importantly, Okamoto and co-workers[35] proposed that the primary DNA damage and subsequent decrease in cellular NAD^+ levels are linked by activation of the NAD^+-consuming nuclear enzyme

poly(ADP-ribose) polymerase (PARP, EC 2.4.2.30). They proposed that the decrease in NAD^+ levels is responsible for the loss of cellular ATP and leads to the inhibition of proinsulin biosynthesis, ultimately resulting in loss of ß-cell viability and cell death.[35] Recent work has identified several minor isoforms of PARP; the isoform commonly termed "PARP" throughout this chapter refers to the major isoform of the enzyme (also termed PARP-1).

14.1.2 Role of PARP: Pharmacological Studies

The development of the hypothesis that primary DNA damage in the ß-cell leads to activation of PARP with subsequent depletion of intracellular NAD^+ leading to inhibition of proinsulin synthesis, ß-cell necrosis, and diabetes has led to the testing of various pharmacological inhibitors of PARP in all animal models of diabetes. The most common inhibitor used is nicotinamide, the amide derivative of nicotinic acid. Nicotinamide, more commonly known as vitamin B3/niacin, was first isolated from rice bran in 1911 and found to be a water-soluble vitamin with a recommended daily allowance of 0.3 mg/kg/day. Niacin consists of two distinct but chemically related components, nicotinamide and nicotinic acid. Nicotinamide was first isolated in 1935 from horse erythrocytes with a structure consisting of a pyridine ring with an amide group in position three. Niacin is a component of NAD^+, a coenzyme involved in many cellular oxidation–reduction reactions and is a PARP inhibitor of rather low potency (1 to 30 m*M* in cell-based assays).[36] Interestingly, nicotinamide is also the by-product of poly(ADP-ribose) synthesis from NAD^+, and thus it serves as a feedback inhibitor of the enzyme. Other PARP inhibitors have also been used including benzamide analogues such as 3-aminobenzamide and, more recently, isoquinolines, benzopyrones, and phenanthridinones. All of these latter compounds are more potent and specific PARP inhibitors than nicotinamide. PARP inhibitors have been used *in vivo* in all of the animal models of type I diabetes, and have frequently been accompanied in parallel with *in vitro* studies to investigate the role of PARP in the response of ß-cells to inflammatory mediators such as cytokines and the free radical species produced from nitrogen and oxygen.

14.1.2.1 *In Vivo* Studies

Many *in vivo* studies utilized chemically induced animal models of diabetes where diabetes is induced in the rat by a single intravenous or intraperitoneal injection of streptozotocin at 50 to 200 mg/kg. One of the earliest studies with nicotinamide using this model was in 1967 by Schein and colleagues[37] who found that intraperitoneal injection of 500 mg/kg nicotinamide preserved ß-cell function and prevented development of diabetes in rats treated with a single high dose of streptozotocin. This protection from hyperglycemia was coupled with a preservation of islet NAD^+ levels and prevention of ß-cell destruction. Inhibitors of PARP including nicotinamide, picolinamide, and 3-aminobenzamide injected intravenously to rats were found to prevent both alloxan and streptozotocin-induced depletion of NAD^+ and inhibition of proinsulin synthesis, provided the inhibitors were given within 5 min of administration of the diabetogenic agents.[35,38-40] Further studies with 3-aminobenzamide indicated that

this compound dose-dependently prevented the development of diabetes in streptozotocin-treated rats.[41] The extent of protection by PARP inhibitors against streptozotocin- or alloxan–induced diabetes in rats is highly dependent on the potency of the inhibitor and time of application. Nicotinamide is a relatively weak inhibitor of PARP and is most effective at preventing streptozotocin-induced diabetes when administered simultaneously or a short time after streptozotocin.[42] In contrast 3-aminobenzamide, a somewhat more potent inhibitor of PARP, is maximally effective when administered within 45 to 60 minutes of the streptozotocin but can partially protect from diabetes development even when administered 120 min after streptozotocin.[42] Treatment of rats with streptozotocin results in a massive decrease in pancreas insulin content. Treatment with either nicotinamide or 3-aminobenzamide restores insulin content to 60% of the levels seen in control animals.[43] Interestingly, immunohistological examination of the pancreas showed a near-complete preservation of the morphology and normalization of the insulin- and glucagon-containing cells in the islets of PARP inhibitor–treated rats suggesting that islet morphology but not necessarily the complete islet function is preserved by PARP inhibition.[43] In general, administration of nicotinamide at doses between 75 and 1000 mg/kg results in a dose-dependent reduction of streptozotocin-induced hyperglycemia and diabetes; 3-aminobenzamide is a more potent inhibitor with concentrations of between 50 and 300 mg/kg being effective.

PARP inhibitors have been far less potent in protecting against the MLDS model of diabetes, which is usually induced in mice rather than rats. The induction of diabetes by injecting a low dose of streptozotocin (30 to 50 mg/kg) daily for 5 days involves not only the direct destruction of ß-cells by streptozotocin but indirect destruction by induction of an autoimmune response against the ß-cell. Nicotinamide (200 or 500 mg/kg) protected MLDS-treated mice from hyperglycemia[5,44] but had only a weak effect on the development of insulitis.[45] The more potent inhibitors of PARP such as the benzopyrone, 5-iodo-6-amino-1,2-benzopyrone (INH_2BP), or the phenanthridinone derivative, PJ-34,[46] (Figure 14.1) exert much more dramatic protective effects than nicotinamide: doses of 30 to 60 mg/kg/day are able to reverse both the MLDS-induced hyperglycemia and loss of pancreas insulin content. These observations suggest that PARP inhibition greatly protects pancreatic ß-cells from both streptozotocin- and immune cell-mediated destruction.

Nicotinamide has also been used to influence the onset of diabetes in the two genetic models of type I diabetes with varying degrees of success. In the NOD model, recent studies provided evidence for the presence of peroxynitrite,[17] a potent NO-derived oxidant, which is able to induce pancreatic ß-cell dysfunction and death *in vitro*, and which is a potent activator of PARP due to its ability to induce DNA single-strand breakage (see below). By using specific antibodies and immunohistochemical methods, it was found that cells positive for nitrotyrosine, an indicator of peroxynitrite generation, were significantly more frequent in islets from acutely diabetic NOD mice (22 ± 6%) than in islets from normoglycemic NOD mice (7 ± 1%) and control BALB/c mice (2 ± 1%). The nitrotyrosine positive cells in islets were identified as macrophages and also ß-cells. Most of the ß-cells in islets from acutely diabetic NOD mice were nitrotyrosine positive (73 ± 8%), whereas significantly

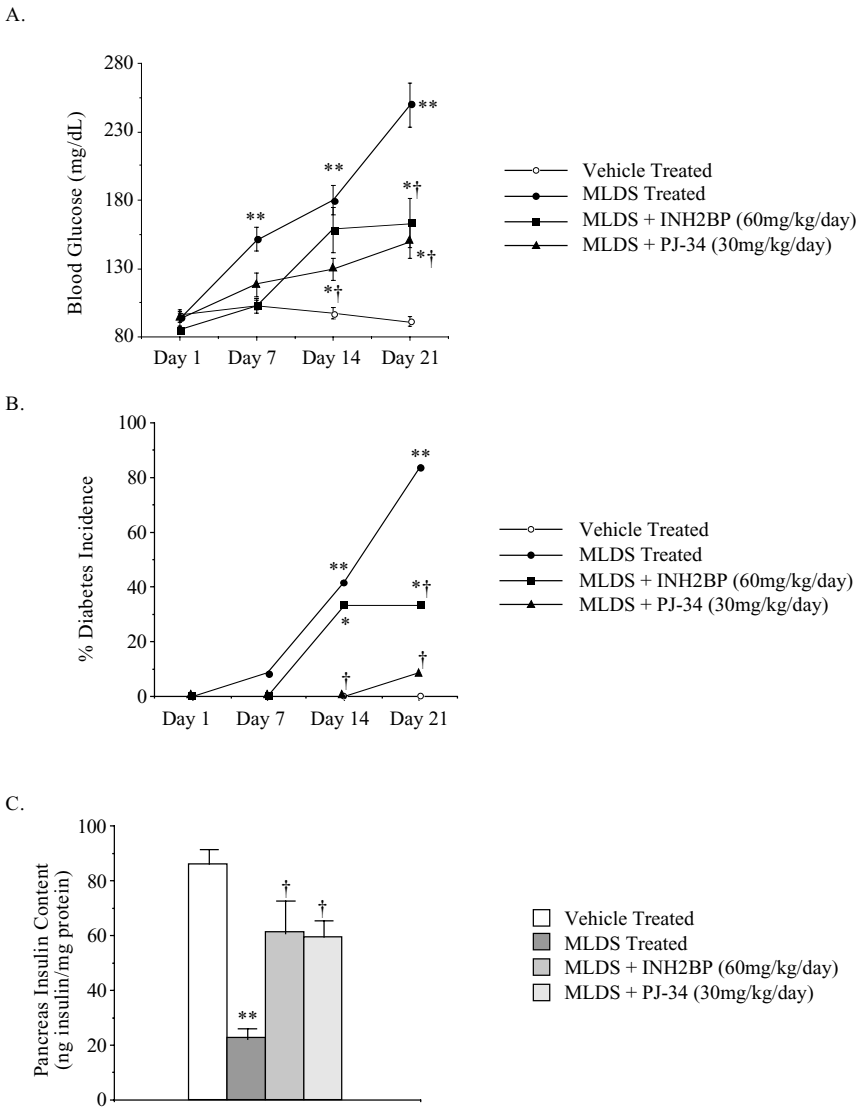

FIGURE 14.1 Both the benzopyrone PARP inhibitor INH_2BP and the phenanthridinone PARP inhibitor PJ-34 protect mice from MLDS-induced diabetes. Mice treated on 5 consecutive days with streptozotocin (40 mg/kg, i.p.) show progressive development of hyperglycemia (A) and increased incidence of diabetes (B) with a dramatically decreased pancreas insulin content (C) 21 days after the first streptozotocin injection. INH_2BP and, more potently, PJ-34 reduce both the hyperglycemia and incidence of diabetes and prevent ß-cell destruction, as evidenced from the measurement of pancreatic insulin content. Results are mean ± SEM. Diabetes is defined as a blood glucose level > 200 mg/dl by *Students* t-test * $p < 0.05$, ** $p < 0.001$ vs. untreated mice, † $p < 0.05$ vs. MLDS-treated mice.

fewer ß-cells were nitrotyrosine positive in islets from normoglycemic NOD mice (18 ± 4%) and BALB/c mice (5 ± 1%). Also, the percentage of ß-cells in islets from NOD mice (normoglycemic and diabetic) correlated inversely with the frequency of nitrotyrosine positive ß-cells.[17] In the NOD mouse, nicotinamide prevents the development of both the spontaneous,[47,48] and cyclophosphamide accelerated[49,50] models of type I diabetes. Unlike in the MLDS model of diabetes, in the NOD model nicotinamide is able to suppress insulitis by a significant degree,[48] as well as reducing MHC class protein expression in the pancreas of NOD mice.[51] These observations indicate a role for PARP in immune cell infiltration in inflammatory conditions, an effect also observed in other diseases.[52] In fact, treatment of NOD mice with nicotinamide for only 5 weeks at a very early age is able to suppress diabetes over the following 25 weeks,[53] indicating that PARP plays a fundamental role in the early stages of diabetes development. In recent studies, chronic treatment of NOD mice with the potent PARP inhibitor PJ 34 both protected against the cyclophosphamide-accelerated induction of diabetes and, when PJ 34 treatment was started from 5 weeks of age, markedly suppressed the spontaneous development of diabetes (Figure 14.2).

These protective effects of PARP inhibitors in the NOD model of diabetes are possibly related to inhibiting ß-cell necrosis and preventing immune cell activation by proteins released from necrotizing islet cells. ß-Cell death by apoptosis has been implicated in many of the models of type I diabetes,[50,54-56] and nicotinamide has been recently shown to reduce ß-cell apoptosis in the cyclophosphamide-accelerated model of diabetes in the NOD mouse.[50] Interestingly, the diet of the NOD mouse has been shown to influence the efficacy of nicotinamide treatment, with sucrose supplementation increasing the ability of nicotinamide to prevent diabetes.[57] The development of diabetes in the BB rat has proved to be more resistant to PARP inhibition than that in the NOD mouse. To date, only nicotinamide has been used to attempt suppressing diabetes in this model. Despite a large dose administered each day (500 mg/kg/day), there was no effect on the disease incidence or the hyperglycemia in the BB rat,[58] whereas in another study a slight tendency for a decrease — from 60 to 42% incidence — was observed.[59] Although disappointing, this result may reflect a potency issue rather than a hypothesis problem. With more potent and specific inhibitors of PARP now available, the BB rat model should be revisited to define the role of PARP activation in the pathogenesis of diabetes in this particular animal model.

14.1.2.2 *In Vitro* Studies

With the role of PARP in the development of diabetes confirmed in animal models, its role in ß-cell death has been subject to extensive investigations *in vitro*. Islets of Langerhans isolated from rat, mouse, or humans are all functionally inhibited and ultimately destroyed by inflammatory cell mediators such as cytokines and free radicals as well as chemical ß-cell toxins such as streptozotocin and alloxan. The role of PARP in these processes has been investigated in a multitude of studies, using various pharmacological enzyme inhibitors.

Application of streptozotocin to isolated mouse and rat islets results in the formation of DNA stand breaks,[60] activation of PARP,[60] and a decrease in ß-cell NAD^+[61]

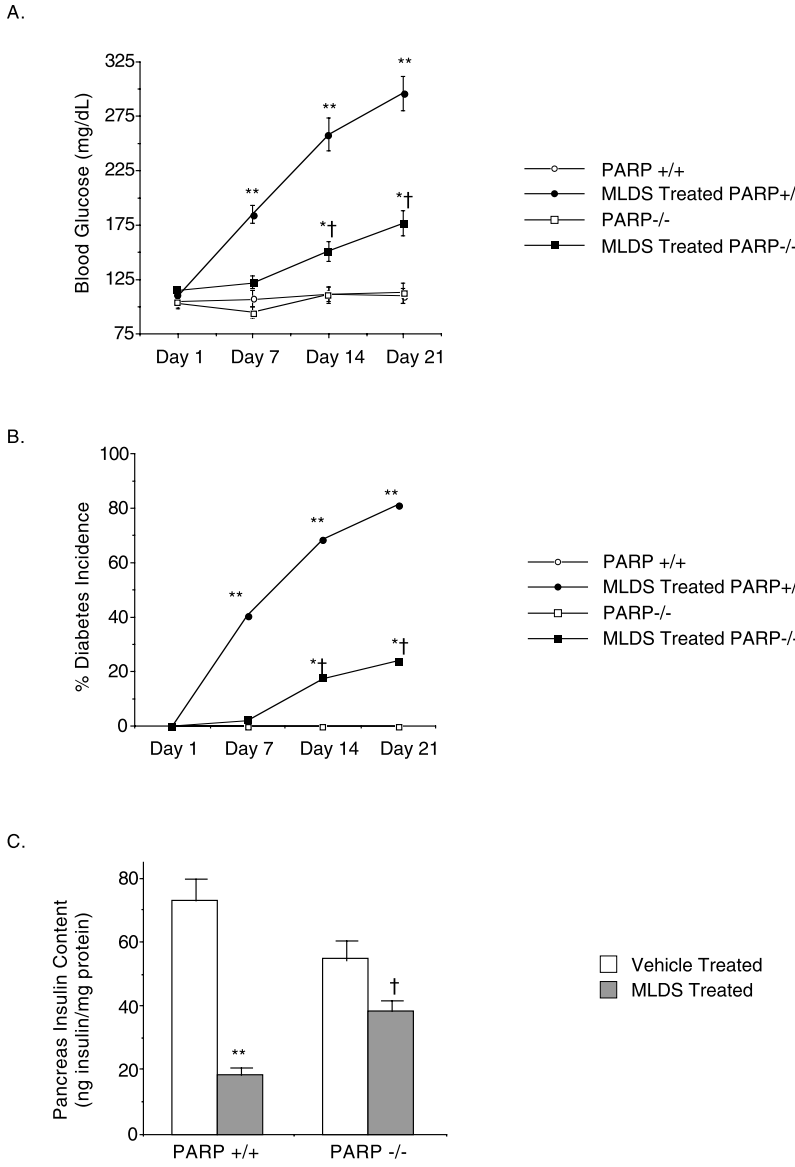

FIGURE 14.2 PARP-deficient mice are resistant to MLDS-induced diabetes. MLDS was induced as outlined in Figure 14.1. The $PARP^{+/+}$ mice show a progressive development of hyperglycemia (A) and increased incidence of diabetes (B), with decreased pancreatic insulin content (C) 21 days after the first streptozotocin injection. These effects were markedly attenuated in the MLDS-treated $PARP^{-/-}$ mice. Results are mean ± SEM; by *Students* t-test * $p < 0.05$, ** $p < 0.001$ vs. untreated mice of the same phenotype, † $p < 0.05$ vs. MLDS-treated $PARP^{+/+}$ mice.

and proinsulin content,[62] along with an inhibition of insulin secretion.[41,42] Application of nicotinamide or 3-aminobenzamide, although not preventing the DNA damage induced by streptozotocin, protected the functionality of the islets by preventing the decrease in NAD^+ and proinsulin as well as by partially reversing the inhibition of insulin secretion.[41,42,61] The protection from streptozotocin by PARP inhibitors is at its peak when they are applied simultaneously with the streptozotocin. Delaying the administration of the inhibitor to 20 or 30 min still partially protected the islets from the deleterious effects of streptozotocin. The reversibility of the streptozotocin-induced damage in ß-cells by PARP inhibitors is a function of the degree of preservation of intracellular NAD^+ pools. Streptozotocin chemically induces necrosis in isolated human islets,[63] an effect that is also reversed by nicotinamide,[63] suggesting a role for PARP activation in human islet death as well. Curiously, despite protecting rats from alloxan-induced diabetes *in vivo*, nicotinamide is unable to protect *in vitro* rat islets from alloxan-mediated toxicity on insulin secretion or glucose-stimulated proinsulin biosynthesis, but is able to partially protect against the inhibition of glucose oxidation and maintains cellular NAD^+ levels.[64] The mechanism of action of alloxan is a little different from that of streptozotocin: whereas streptozotocin causes DNA damage directly by alkylation, alloxan produces oxygen free radicals as well as causing DNA strand breaks and activation of PARP. In addition, alloxan also exerts numerous PARP-independent effects, for example, on mitochondria or the plasma membrane.[64]

The role of PARP in the deleterious effects of cytokines on isolated islets is not as clear as with the chemically induced damage. This finding is possibly due to the use of nicotinamide for these studies, as, at the doses used, nicotinamide also acts as a protein synthesis inhibitor and free radical scavenger (in addition to being a PARP inhibitor). Nicotinamide has been shown to have either no effect[26,65] or a partial protective effect[66] against cytokine-inhibited insulin release or stimulation of NO formation from isolated islets. Use of a more potent PARP inhibitor, INH_2BP, does reverse cytokine-mediated inhibition of glucose stimulated insulin secretion,[67] an effect found to be independent of inhibition of NO formation.[67] Nicotinamide was able to reverse the inhibitory effects of IL-1ß on accumulated insulin release and NO production,[68] but only when applied at millimolar concentrations. Similar effects were seen on human islets exposed to a combination of proinflammatory cytokines where millimolar (10 or 20 m*M*) concentrations of nicotinamide were able to partially counteract the effects of cytokines on increased NO formation and inhibition of insulin secretion.[69] Exposure of human islets to cytokine combinations also causes islet cell destruction as determined by islet DNA and insulin contents, which decrease dramatically,[70] again effects that are reversed by nicotinamide.[70] NO has been shown to be the major mediator of cytokine-induced damage and the induction of NOS in islet cells by cytokines totally dependent on NF-κB activation.[71] Reports have implicated PARP in promoting activation of NF-κB,[72] but this promotion appears to be due to the physical presence of the enzyme (through molecular scaffolding functions and physical association) rather than its catalytic activity; thus, the activation of NF-kB is less affected by pharmacological inhibitors than by genetically disrupting the PARP gene.[46,72,73] Examination of islet protein expression following exposure to IL-1ß shows

a marked change with both increases and decreases in various proteins. Simultaneous treatment with nicotinamide prevented the IL-1ß-mediated increased expression of 16 of those proteins.[74] This observation is in line with emerging data demonstrating that PARP plays a pivotal role in regulating transcription of various cellular proteins (see also below). Once identified, these proteins may provide further information about the role of PARP in protecting islet cells. Nicotinamide also prevented cytokine-mediated lysis of mouse islet cells,[75] but was unable to protect rat, mouse, or human islets from cytokine-mediated apoptosis.[63] This observation is in accord with the evidence that the cell death pathway triggered by PARP activation and NAD^+ depletion is necrosis, rather than apoptosis.

Exposure of isolated islets to NO, reactive oxygen species, or hydrogen peroxide, either generated chemically or from activated macrophages, results in inhibition of insulin secretion, decrease in NAD^+ and proinsulin levels, along with eventual islet cell lysis, effects associated with activation of PARP. Although nicotinamide failed to prevent the formation of primary DNA damage in radical exposed cells, the presence of the compound effectively inhibited the activation of PARP, as assessed by the complete lack of poly(ADP-ribose) formation and by the preservation of intracellular NAD^+ concentrations. Nicotinamide and 3-aminobenzamide both improved the survival of rat islet cells exposed to chemically generated NO[24,76] or to ROS enzymatically generated by xanthine oxidase.[34,77,78] Nicotinamide also protected human islets from hydrogen peroxide-induced necrosis.[63] Nicotinamide was even able to protect rat islet cells co-cultured with syngeneic activated macrophages from cell lysis via an NO-dependent mechanism.[79,80] It was also found in the same study that nicotinamide was able to inhibit NOS mRNA induction in activated macrophages,[81] an effect of the compound that may have also contributed to the ß-cell protection. Interestingly, in a recent study Kretowski and colleagues[82] isolated peripheral blood from patients with newly diagnosed diabetes. After incubation with nicotinamide (100 μ*M*), the blood cells produced significantly less IL-12 and TNF-α. This observation suggests that nicotinamide is able to influence monocyte/macrophage function in peripheral human blood, thus providing another potential mechanism of protection against the development of diabetes *in vivo*.

It is noteworthy that, according to some studies, long-term exposure to high concentrations of nicotinamide can have deleterious effects on isolated islets or insulin-containing cells. Exposure of mouse islets to nicotinamide for 1 week partially inhibited glucose-stimulated insulin release and decreased the glucose oxidation rate and the ATP contents.[83] Moreover, nicotinamide itself can induce apoptosis in insulin-producing cells, an effect associated with cleavage of PARP.[84] Both nicotinamide (20 to 50 m*M*) and 3-aminobenzamide (10 m*M*) induced apoptosis in both fetal rat islets and the insulin-containing cell line RINm5F. This effect was coupled with the appearance of the 85-kDa proteolytic PARP fragment indicative of caspase-3 activation. Thus, it was proposed that inhibition of ADP-ribosylation and consequently automodification of PARP released the enzyme from DNA strand breaks leads to activation of programmed cell death.[84] Although many of the above-referenced studies concluded that these deleterious effects are due to untoward actions of long-term PARP inhibition, this conclusion is questionable in light of the following facts: (1) the effects cannot be reproduced with novel, more potent

PARP inhibitors; and (2) spontaneous islet cell death cannot be seen in the PARP-deficient mice. In light of these latter findings, it is likely that a PARP-independent, direct cytotoxic effect of nicotinamide may explain the findings described in the above-referenced experiments.

The combined results from the *in vivo* and *in vitro* studies utilizing PARP inhibitors indicate that protection from diabetes development, as observed following administration of PARP inhibitors in rodents, is mediated by direct protection against the necrotic damage of the ß-cells, as well as may be related to suppression of proinflammatory mediator production. The particularly high concentrations of the PARP inhibitors used *in vitro* to produce the protective effects, in the millimolar range, give rise to many more pharmacological effects than just PARP inhibition. Nicotinamide at 5 to 50 m*M* concentrations, used *in vitro* to protect the islets, is able to inhibit other enzymes besides PARP,[85] as well as to interfere with transcriptional[81] and translational processes.[86,87] Although the more potent PARP inhibitors which are active in the micromolar or nanomolar range exert similar protective effects as nicotinamide and 3-aminobenzamide against cytokine-mediated inhibition of insulin secretion and islet cell destruction, in order to truly define the role of PARP in diabetes the engineering of a mouse deficient in the PARP gene was required.

14.1.3 Role of PARP: Studies Using PARP-Deficient Experimental Systems

The development of PARP-deficient mouse lines has allowed the direct examination of the role of PARP in type I diabetes. Although the use of PARP inhibitors has implicated this enzyme in development of diabetes and ß-cell destruction, many of the inhibitors used have been shown to have additional actions besides inhibition of PARP, including hydroxyl radical[88] and hydrogen peroxide[89] scavenging activities along with inhibition of iNOS expression and activity.[73] In 1995, Kolb and colleagues[90] isolated islets from the newly engineered PARP deficient mouse[91] and exposed them to NO or reactive oxygen species.[13,24,77] The islets isolated from $PARP^{-/-}$ mice had no NAD^+ depletion and were resistant to both NO and reactive oxygen species toxicity, providing evidence that PARP activation is responsible for most of the loss of NAD^+ following such treatment. However, $PARP^{-/-}$ islets were not completely resistant to lysis induced by NO and reactive oxygen species, especially at higher concentrations. Similarly, application of a PARP inhibitor, 3-aminobenzamide, failed to protect against the islet lysis induced by extremely high levels of NO or reactive oxygen species, indicating the presence of an alternative pathway of cell death independent of PARP activation and NAD^+ depletion which is activated by oxidants and free radicals in ß-cells.[88]

In 1999, three independent groups[92-94] reported in short succession the massive resistance of $PARP^{-/-}$ mice to streptozotocin-induced diabetes. In all three studies $PARP^{-/-}$ mice were injected with a single dose of streptozotocin ranging from 160 to 200 mg/kg, and in all cases the control mice developed a severe hyperglycemia within a week of the injection. This action was coupled with a marked decrease in pancreatic insulin levels and morphological analysis showing significant atrophy of the islets caused by a marked loss of ß-cells. In contrast, the $PARP^{-/-}$ mice had normal blood

glucose levels, with no evidence of islet atrophy, ß-cell loss, or altered cellular arrangement. In fact, the islets exhibited a histological appearance that was similar to that of the vehicle-treated nondiabetic mice. Snyder and colleagues[94] analyzed the pancreas for both DNA stand breaks and PARP activity following streptozotocin treatment, and found an inverse relationship: islets from the $PARP^{-/-}$ mice showed a dramatically increased number of DNA stand breaks, as compared with $PARP^{+/+}$ mice. However, $PARP^{-/-}$ mice had no detectable PARP activity whereas the pancreas of $PARP^{+/+}$ mice had a 160% increase in PARP activity following streptozotocin treatment. Islets isolated from the $PARP^{-/-}$ mice were also protected from *in vitro* application of streptozotocin, showing a marked resistance to streptozotocin-induced cell lysis and a complete protection against NAD^+ depletion.[92] The complete protection of mice lacking the PARP gene from streptozotocin-induced diabetes is remarkable, and confirms the role of PARP as an essential downstream executor in the development of diabetes in this animal model. The involvement of PARP in the destruction of islet cells following application of a single high dose of streptozotocin can be related to normal development of type I diabetes in humans, despite that streptozotocin has a direct destructive effect on the pancreatic ß-cell with no other cell type immune or otherwise required. Both DNA damage and PARP activation have consistently been reported to occur in islet cells exposed to inflammatory stress,[25,27] such as that observed in the primary disease.

The susceptibility of $PARP^{-/-}$ mice to the MLDS model of diabetes has been investigated recently by our group.[67] As noted above, this model has a significant immune cell component in the ß-cell destruction and is characterized by a progressive hyperglycemia and an insulitis similar to that observed in recent onset type I diabetics. As shown in Figure 14.3, $PARP^{-/-}$ mice are significantly less susceptible to MLDS-induced diabetes, with a lower disease incidence and decreased hyperglycemia than the wild-type animals. This resistance against diabetes is coupled with a higher insulin content and therefore increased ß-cell mass in MLDS-treated $PARP^{-/-}$ mice, as compared with treated $PARP^{+/+}$ mice. However, it is notable that no complete protection was observed when these mice were treated with a single high dose of streptozotocin, indicating that PARP-independent parallel pathways activated via the immune system can also lead to ß-cell destruction. This finding is consistent with the *in vitro* data,[90] demonstrating the presence of a PARP-independent pathway of islet cell death in response to NO and reactive oxygen species.

Overall, the studies with the PARP-deficient mice have provided strong evidence for the depletion of NAD^+ resulting from PARP activation as the dominant metabolic event in the destruction of the ß-cell following DNA damage. PARP inhibition improves the resistance of the ß-cell toward the deleterious effects of proinflammatory mediators such as cytokines and free radicals and presents a clear therapeutic target for the prevention of type I diabetes.

14.1.4 Role of PARP: Clinical Studies

The concept of protecting ß-cells from inflammatory damage was introduced into human clinical trials early on. The compound selected was nicotinamide, most likely due to two obvious reasons: (1) The abundance of efficacy data with this compound,

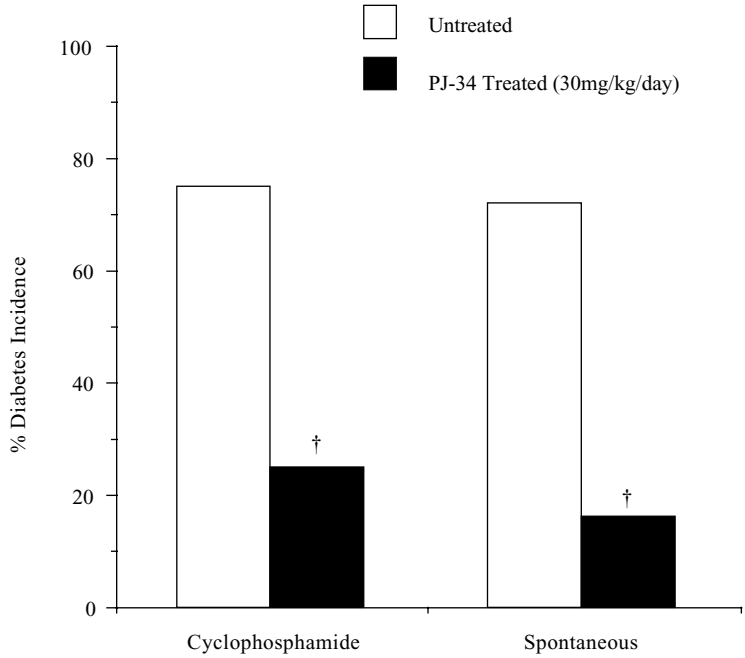

FIGURE 14.3 The PARP inhibitor PJ 34 reduces either cyclophosphamide-accelerated or spontaneous diabetes in the NOD mouse. Treatment of 10-week-old NOD mice with cyclophosphamide (250 mg/kg, i.p.) induced an accelerated onset of diabetes over 14 days. PJ 34 reduced the incidence of diabetes on day 14 as compared to untreated mice. Spontaneous development of diabetes occurs in the NOD mouse from the age of 12 weeks until 30 weeks. At 20 weeks of age there was significantly lower incidence of diabetes in the PJ-34-treated mice as compared to vehicle treated. Results are expressed as cumulative incidence of diabetes, where † $p < 0.05$ vs. vehicle-treated mice (χ^2 test).

emerging from the late 1980s and thereafter, and (2) the established safety profile and the minimal risk that it imposes on humans taking the vitamin. Two approaches to evaluating the use of PARP inhibitors in the human disease were taken: one was to evaluate the therapeutic potential in humans after the onset of type I diabetes and the second was to evaluate the prophylactic potential of PARP inhibition.

14.1.4.1 Therapeutic Potential

Newly diagnosed patients were treated with nicotinamide at doses, which ranged from 20 mg/kg up to 3 g/kg for several periods of time within 5 years of developing diabetes. A meta-analysis of ten randomized, controlled clinical trials indicated that nicotinamide had a protective effect on ß-cell activity:[95] over a year of observation it was found that the patients on nicotinamide had a better baseline C-peptide level, which indicated a better preserved insulin secretory capacity.[85] Despite

these promising findings, there was no difference between the nicotinamide and control groups in their insulin requirement for metabolic control.

14.1.4.2 Prevention of Diabetes

The prevention of diabetes by applying a PARP inhibitor like nicotinamide requires a screening test to assess likelihood for a child to develop the disease. Initial studies were preformed in children who had islet cell antibodies, which indicated there was an ongoing inflammatory reaction against ß-cells. A small-scale study by Elliot and Chase[96] in 1991 showed a promising reduction in diabetes incidence among children treated with nicotinamide. The larger-scale trial that followed also showed a significant reduction in incidence of diabetes, suggesting that inhibition of PARP does indeed prevent type I diabetes from developing in humans.[97] Various other small-scale trials of nicotinamide have also been carried out throughout the world. In 1989, a relatively short study where newly diagnosed patients were treated over a 45-day period with 1 g/day of nicotinamide found an enhanced basal and stimulated C-peptide level indicative of insulin secretory function in the treated group. However, there was no difference in the number of clinical remissions or in their insulin requirement.[98] In the same year, results from another study using 200 mg/kg/day nicotinamide appeared where patients with recent-onset diabetes were treated for 1 year. Again, similar to the previous study, they too found an increased C-peptide response following stimulation plus a reduced insulin requirement in the treated patients,[99] which probably resulted by an increasing residual islet ß-cell function. However, not all the studies with nicotinamide have been successful. Chase and colleagues[100] reported that treatment of newly diagnosed patients with 100 mg/day of age up to a maximum of 1.5 g/day nicotinamide failed to have an effect on either C-peptide levels or in remission from the disease. Pozzilli and colleagues[101] using nicotinamide in combination with steroid also reported no protective effects on ß-cell function in newly diagnosed patients. In a second study by the same group,[102] published in the same year, nicotinamide was combined with cyclosporin and again there were no significant protective effects in patients treated with either nicotinamide alone or in combination therapy. In 1999, again the same group reported on a third study with nicotinamide,[103] using two different doses of nicotinamide (25 or 50 mg/kg/day), and again found no protective effects of nicotinamide in these patients on either C-peptide levels or insulin requirements. Finally, a study where within 72 h of being diagnosed patients were started on either insulin or a combination of insulin and nicotinamide (2.1 g/day) has also failed to demonstrate any beneficial effect of nicotinamide.[104]

Following these smaller-scale studies, two placebo-controlled, double-blind trials have been initiated to evaluate the potential of nicotinamide to prevent diabetes. In the "Deutsche Nikotinamid Interventions Studie" (DENIS) 3- to 12-year-old, islet cell antibody-positive children of patients with type I diabetes were treated with nicotinamide.[105] Three years after starting the trial the study was terminated when analysis of the data failed to detect a reduction of the cumulative diabetes incidence in the nicotinamide-treated group.[106] This negative outcome does not preclude the possibility that nicotinamide has a slight protective effect, as the individuals studied

had a very high risk of developing diabetes, which has been assumed to be associated with a rapid progression of the disease. The second large study of nicotinamide in diabetes is still ongoing, and is called "European Nicotinamide Diabetes Intervention Trial" (ENDIT). The study has spread beyond the borders of Europe and includes insulin cell antibody–positive first-degree relatives (5 to 40 years old) of patients with type I diabetes.[107] This study is designed to detect even weak effects of nicotinamide treatment on the reduction of the risk of developing type I diabetes.

One must note that even if PARP is a valid target to prevent type I diabetes, nicotinamide may not be a potent enough pharmacological agent to prevent disease development. The development of more potent and specific inhibitors of PARP may mean that true prevention of type I diabetes in humans occurs only when these inhibitors are available for human use. Currently — while awaiting results from the ENDIT trial — it is interesting to note that apart from the very early studies carried out in children, no study since 1993 using nicotinamide has been successful in either reducing insulin requirements or increasing residual ß-cell function of newly diagnosed type I patients with diabetes. Even if the larger-scale randomized nicotinamide trials turn out to be unsuccessful, more potent PARP inhibitors should be tested in similar patient population, because they are likely to hold a higher potential for success.

It is interesting to note that nicotinamide has been shown to have a beneficial effect in patients with non-insulin-dependent (type II) diabetes, who had no obvious signs of autoimmunity and had no insulin cell antibodies. Patients who where hyperglycemic despite being maximally treated with sulfonylureas were treated with 0.5 g nicotinamide a day over a 6-month period. It was found that those patients on nicotinamide had an increase in C-peptide release and had improved glycosylated hemoglobin and blood glucose levels, indicating that nicotinamide was able to help in the metabolic control of these patients.[108] It is possible that some of these beneficial effects in Type II diabetics are due to the antioxidant effect of nicotinamide, rather than its ability to inhibit PARP.

14.2 DIABETIC VASCULAR DYSFUNCTION

The quality of life and life expectations of patients with diabetes are determined by the complications of diabetes rather than the primary disease. Among these complications vasculopathies affecting both the micro- and macrocirculation (evidenced clinically, among others, by accelerated atherosclerosis, dysfunction of the eye, kidney, diminished blood flow to extremities, and increased risk of developing a variety of cardiovascular diseases) are probably the most dominant factors. Diabetes is associated with a three- to five-fold increase in death from myocardial infarction and similar figures pertain to stroke. The major cause of mortality in diabetes is macrovascular disease affecting the cardiac and cerebrovascular circulation, which appears to have a complex pathogenesis.[109-113] The processes involved in atherothrombotic disease are complex and include variation in lipid metabolism, vascular responses, cell–cell interactions, and in the fluid and cellular phases of coagulation and fibrinolysis. The complex interactions between all of these processes are crucially altered by the metabolic milieu that characterizes diabetes

mellitus, tipping the delicate balance toward atheroma formation, platelet aggregation, and thrombus formation.[109-113]

In contrast to macrovascular alterations, diabetes-associated microvascular disease has been strongly related to glycemic control. Based on the availability of the most-sophisticated insulin-treatment protocols, one might think that the glucose metabolism of patients with diabetes is perfectly controlled. However, hyperglycemic episodes do occur even in the most-balanced forms of diabetes mellitus and are closely associated with the development of vascular failure. It is generally believed that vascular complications can be prevented by better controlling blood glucose levels. Nevertheless, it is a fact of life that a significant portion of diabetic patients will eventually develop some degree of vascular failure which is characterized, for example, by endothelial dysfunction.

14.2.1 Role of Reactive Oxygen and Nitrogen Intermediates in Diabetes-Associated Vascular Dysfunction

The vascular tone is regulated by various neurohumoral mediators and mechanical forces acting upon the innermost layer of blood vessels, the endothelium. The main pathway of vasoregulation involves the activation of eNOS resulting in NO production.[114,115] NO then diffuses to the smooth muscles, activates soluble guanylyl cyclase, and thereby triggers the cGMP-mediated relaxation of the smooth muscle. The activity of the endothelium extends far beyond the control of vascular tone and reactivity, and the release of NO and other vasodilating mediators clearly reflects only one aspect of the homeostatic and protective role of the endothelium. Nevertheless, endothelium-dependent vasodilation is frequently used as a reproducible and accessible parameter to probe endothelial function in various pathophysiological conditions[114-116]: endothelial dysfunction, in many diseases, precedes and predicts as well as predisposes for the subsequent, more severe vascular alterations.

Endothelial damage has been documented in various forms of diabetes, both in experimental animals and in humans. Hyperglycemia-induced mechanisms that may induce vascular dysfunction in specific sites of diabetic microvascular damage include increased polyol pathway flux, altered cellular redox state, increased formation of diacylglycerol and the subsequent activation of specific protein kinase C isoforms, and accelerated nonenzymatic formation of advanced glycation end products (AGEs). Each of these mechanisms contributes to the known pathophysiological features of diabetic complications by a number of mechanisms, including the upregulation of cytokines and growth factors.[117,118] As several of these mechanisms may be responsible for the potentially damaging overproduction of reactive oxygen and nitrogen intermediates and the associated endothelial injury, reactive oxygen and nitrogen species production may represent a final common pathway in the pathogenesis of diabetic vascular dysfunction. Indeed, recent studies have established that oxygen- and nitrogen-derived oxidants and free radicals play a significant role in diabetes-associated endothelial dysfunction.[119-122]

Strong evidence indicates that oxidative stress plays an important role in the etiology of diabetic complications.[119,120] Many biochemical pathways strictly associated

with hyperglycemia (glucose auto-oxidation, polyol pathway, prostanoid synthesis, protein glycation) can increase reactive oxygen species production. Furthermore, exposure of endothelial cells to high glucose leads to augmented production of superoxide anion. Many of the adverse effects of high glucose on endothelial functions, such as reduced endothelial-dependent relaxation and delayed cell replication, are reversed by antioxidants.[123,124] In further support for the role of oxidative stress in diabetic vasculopathy, diabetic patients have been reported to have different susceptibility to microvascular and macrovascular complications, and this susceptibility showed correlation with the endogenous antioxidant status.[119]

The question arises from what cellular sources reactive oxygen species are generated. It is a well-established fact that even under normal conditions, superoxide is constantly generated by the mitochondrial electron transport chain.[125,126] This continuous "leak" of superoxide can be markedly enhanced under conditions when mitochondrial respiration is damaged. Interestingly, reactive oxygen and nitrogen species themselves can inhibit components of the electron transport chain thereby further augmenting reactive species production.[127]

Much attention has been directed toward advanced glycation end products (AGEs) formed by covalent bonding of aldoses and proteins.[128,129] This heterogeneous class of nonenzymatically glycated proteins or lipids is found in the plasma. As a consequence of hyperglycemia, AGE formation and deposition are much enhanced in diabetes, in which their presence has been linked to secondary complications, especially microvascular disease.[128,129] As endothelial cells, similarly to mononuclear phagocytes, express receptors for AGEs, and as AGEs are known to generate reactive oxygen species, AGE-derived oxidative stress and proinflammatory mediator production are focused primarily on endothelial cells.[129] There is also increasing evidence for superoxide generation by ubiquitous NADH/NADPH oxidases for the development of vascular (particularly endothelial) dysfunction.[120]

NO produced from ecNOS (present in the endothelial cells) is responsible for many physiological effects, such as the maintenance of a basal vascular tone. The function of ecNOS can be evidenced by endothelium-dependent relaxant responses (i.e., the ability of isolated blood vessels to relax in response to vasoactive substances that induce the activation of ecNOS and the release of NO from the vascular endothelium).[114,115] Such vasoactive mediators are acetylcholine, bradykinin, and ATP, for example. On the other hand, vascular studies utilizing direct NO donor compounds, such as the nitrovasodilators, SNAP, or sodium nitroprusside, can evaluate the ability of the vascular smooth muscle to react to NO. There is now overwhelming evidence demonstrating the development of endothelial dysfunction (the reduced ability of blood vessels to relax in response to endothelium-dependent relaxants) in animal models of diabetes,[130-134] as well as in human blood vessels from patients with diabetes.[135]

Many forms of vascular dysfunction are associated with profound alterations in the biosynthesis of NO from L-arginine. Recent studies have also implicated the production of nitrogen-derived free radicals and oxidants in the process of diabetes-associated endothelial dysfunction.[121,122] Given the key role of NO in endothelium-dependent vasorelaxation, it may be surprising that NO overproduction has also been

implicated in the pathomechanism of diabetes-associated vasculopathy. For example, chronic treatment *in vivo* with NOX-101, a water-soluble NO scavenger, prevented endothelial dysfunction in diabetes in Sprague-Dawley rats in a model of streptozotocin-induced diabetes.[136] This latter finding suggests the possibility that diabetes-induced endothelial dysfunction is related to an autocrine, NO-mediated cytotoxicity in endothelial cells. To resolve the controversy about the dual role played by NO in the regulation of vascular function, it is worthwhile to remember that in diabetic blood vessels NO is produced in an oxidatively stressed cellular environment. From a chemical point of view, neither NO nor superoxide can be considered a strong oxidant toward most types of biomolecules. Thus, it is most likely that diabetic endothelial dysfunction is mediated by secondary radicals or oxidants rather than by NO per se. Several lines of evidence suggest that the powerful oxidant peroxynitrite can be formed in the diabetic vessels and may be a key mediator of endothelial dysfunction.[121] Recent clinical data demonstrated a significantly more intense nitrotyrosine staining in vascular endothelium and villous stroma of placentas of patients with diabetes, when compared to normal controls.[122] The localization of the nitrotyrosine staining indicates that endothelium-derived NO, in the presence of increased oxidant stress, converts to the cytotoxic oxidant peroxynitrite.[122] Thus, paradoxially, although endothelium-dependent relaxant function is dependent on NO, partial inhibition of NO production can actually prevent the impairment of the endothelial function in diabetic blood vessels.[136]

14.2.2 PARP, as a Mediator of Diabetic Endothelial Dysfunction *in Vitro*

Endothelial cells incubated in high-glucose medium generate oxyradicals and mitochondria have been identified as a principal site of oxyradical generation.[137a] In our recent study we found that high glucose-induced oxidative stress leads to DNA single-strand breakage and PARP activation in murine and human endothelial cells.[46] The involvement of oxyradicals and NO-derived reactive species in PARP activation suggests that peroxynitrite may be one of the final mediators responsible for single-strand breakage, and subsequent PARP activation. This notion is also supported by the finding that tyrosine nitration, a marker of peroxynitrite generation, was detected in the endothelial cells exposed to high glucose and appeared to localize in the mitochondria[46] (Figure 14.4). This finding is consistent with the mitochondrial electron chain being the primary source of oxyradical generation in endothelial cells placed in high glucose.[137a] PARP activation in response to high glucose was attenuated by superoxide dismutase or by MnTBAP, a cell-permeable water-soluble superoxide dismutase mimic, as well as by carboxy-PTIO, an NO-scavenger compound, indicating that the activation of PARP is related to intravascular generation of both reactive oxygen and nitrogen species.[46] Similarly, pharmacological inhibition of cellular NO synthesis by N^G-methyl-*L*-arginine or N^G-nitro-*L*-arginine methyl ester (but not aminoguanidine, an inhibitor of iNOS) reduced the degree of PARP activation in endothelial cells exposed to high glucose[46] (Figure 14.4).

As mentioned above, PARP activation is known to trigger a futile intracellular energetic cycle, leading to depletion of intracellular NAD^+ and high-energy

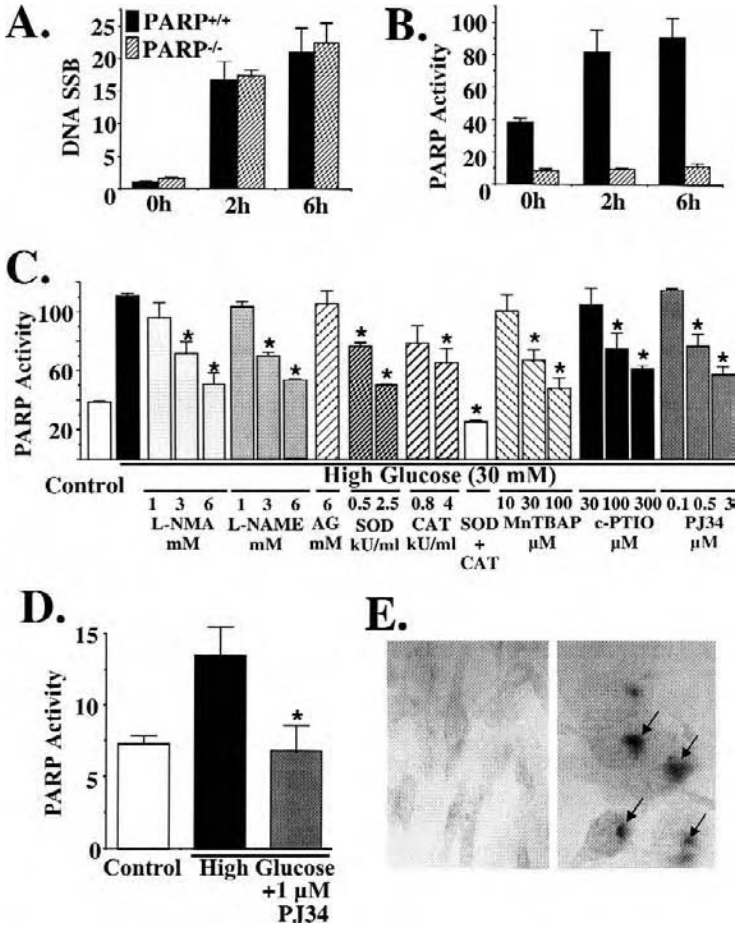

FIGURE 14.4 Reactive nitrogen species generation, ssDNA breakage, and PARP activation in endothelial cells placed in high glucose. (A) Time course of ssDNA breakage (SSB, percent of total DNA) in wild-type (■) and PARP-deficient endothelial cells (▨). (B) Time course of PARP activation in wild-type (■) and PARP-deficient endothelial cells (▨) exposed to 30 m*M* glucose, when compared to activity in normal culture medium, expressed as pmol of NAD^+ consumed/min/mg protein. (C) The effect of L-NMA, L-NAME, aminoguanidine (AG), superoxide dismutase (SOD), catalase (CAT), the combination of 2500 U/ml SOD and 4000 U/ml CAT, the SOD mimetic [manganese(III) tetrakis(benzoic acid)porphyrin chloride] (MnTBAP), the NO scavenger 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (c-PTIO), and PJ-34 on high glucose–induced PARP activation. (D) The activation of PARP in response to 30 m*M* glucose, when compared with activity in normal culture medium, in human umbilical vein endothelial cells (HUVEC), and the effect of PJ 34. * $P < 0.05$ indicates significant inhibition of PARP activation by the interventions ($n = 6$ to 8 per group). (E) Tyrosine nitration in endothelial cells maintained in normal culture medium (left) and in cells exposed to high glucose (right). Similar immunohistochemical profiles were seen in n = 4 to 5 independent experiments. (From Soriano, G. F. et al., *Nature Med.*, 7, 108, 2001. With permission of Nature America, Inc.)

phosphate levels. To test the relevance of this mechanism to the high glucose-induced cell dysfunction, we have decided to measure cellular pyridine nucleotide concentrations in the endothelial cells exposed to high glucose. There was a severe suppression of cellular high energy phosphate levels as well as a suppression of NAD^+ and NADPH levels in endothelial cells exposed to high glucose for 1 to 2 days. These effects were prevented by PARP inhibition or by $PARP^{-/-}$ phenotype.[46] Since eNOS is an NADPH-dependent enzyme, it is conceivable that the cellular depletion of NADPH in endothelial cells exposed to high glucose is directly responsible for the suppression of eNOS activity and the reduction in the endothelium-dependent relaxant ability of diabetic vessels. In patients with diabetes the effects of hyperglycemia may be exacerbated by increased aldose reductase activity leading to further depletion of NADPH and simultaneous generation of reactive oxidants.

14.2.3 PARP, as a Mediator of Diabetic Endothelial Dysfunction *in Vivo*

Based on these cellular studies, we hypothesized that in diabetes, hyperglycemia may induce PARP activation, which may contribute to development of endothelial dysfunction. To test this hypothesis we induced diabetes in mice and rats by streptozotocin treatment. The complete loss of pancreatic insulin content triggered a severe hyperglycemia throughout the 8 weeks of the experiments[46] (Figure 14.5). Inhibition of PARP activation was achieved by chronic treatment with the potent, water soluble phenantridinone derivative PARP inhibitor PJ 34, starting 1 week after the initiation of diabetes (Figure 14.5). Increases in the immunostaining for nitrotyrosine, elevated frequency of DNA strand breaks, and marked PARP activation were observed in the diabetic blood vessels[46] (Figure 14.6). The activation of PARP in the blood vessels was already apparent 2 weeks after the onset of diabetes, and thus it slightly preceded the occurrence of the endothelial dysfunction, which developed between week 2 and week 4 of diabetes in the murine model.[46] *Ex vivo* experiments demonstrated the loss of endothelial function, as measured by the relaxant responsiveness of precontracted vascular rings to acetylcholine. Delayed treatment with the PARP inhibitor — starting at 1 week after streptozotocin — ameliorated vascular poly(ADP-ribose) accumulation and restored normal vascular function without altering systemic glucose levels, plasma glycated hemoglobin levels, or pancreatic insulin content[46] (Figures 14.5 and 14.6). The endothelium-independent relaxant responsiveness of the blood vessels in response to the NO-releasing compound sodium nitroprusside remained unchanged, indicating that the ability of the endothelium to release NO, rather than the ability of the smooth muscle to relax to NO is impaired in diabetes (Figure 14.5). Chronic treatment with the PARP inhibitor did not alter blood glucose, glycated hemoglobin or vascular responses in control (nondiabetic) animals.[46] Taken together, these data indicate that PARP-dependent mechanisms of diabetic vascular dysfunction are operative *in vivo*, and PARP inhibition may be a suitable novel experimental therapeutic approach to improve the patency of diabetic blood vessels. Emerging work also demonstrates that the development of diabetic cardiac dysfunction (diabetic cardiomopathy) is also PARP-dependent.[137b] Furthermore, recent work shows that the development of diabetic cardiovascular dysfunction cannot only be

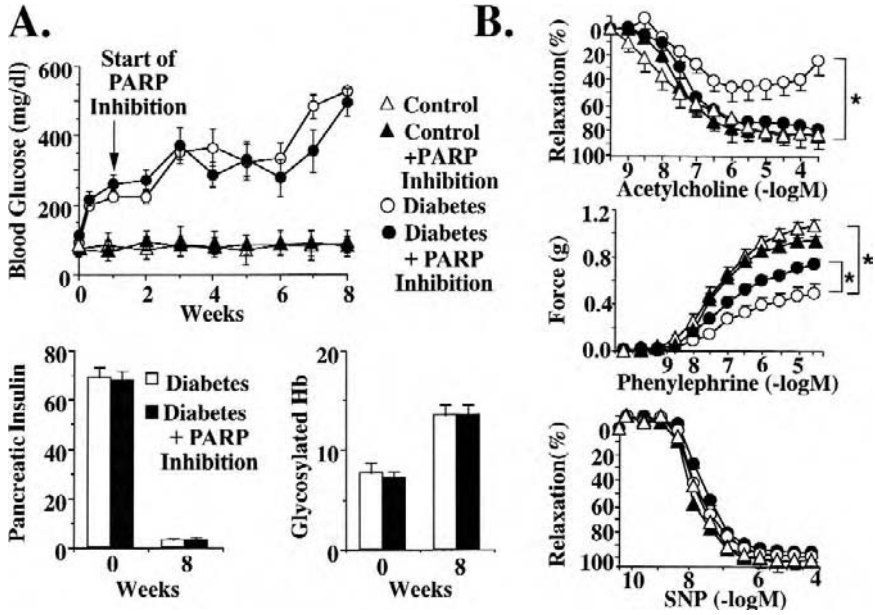

FIGURE 14.5 Reversal of diabetes-induced endothelial dysfunction by pharmacological inhibition of PARP. Symbols used for the respective groups: animals that received no streptozotocin injection (r), nondiabetic control animals at 8 weeks treated with PJ 34 between week 1 and 8 (p), diabetic animals at 8 weeks treated with vehicle (■), diabetic animals at 8 weeks treated with PJ 34 between week 1 and 8 (■). (A) Blood glucose levels, pancreatic insulin content (ng insulin/mg pancreatic protein), and blood glycated hemoglobin (Hb) (expressed as % of total Hb) at 0 to 8 weeks in nondiabetic, control male BALB/c mice, and at 0 to 8 weeks after streptozotocin treatment (diabetic) in male BALB/c mice. PARP inhibitor treatment, starting at 1 week after streptozotocin and continuing until the end of week 8, is indicated by the arrow. Pancreatic insulin content and glycated hemoglobin levels are shown at 8 weeks in vehicle-treated and streptozotocin-treated animals, in the presence or absence of PJ 34 treatment. (B) Acetylcholine-induced, endothelium-dependent relaxations. Phenylephrine-induced contractions, and SNP-induced endothelium-independent relaxations. *$P < 0.05$ for vehicle-treated diabetic vs. PJ 34 treated diabetic mice ($n = 8$ per group). (From Soriano, G. F. et al., *Nat. Med.*, 7, 108, 2001. With permission of Nature America, Inc.)

prevented by PARP inhibitors, but the inhibitors can rapidly resore normal vascular function in established diabetic animals.[137c]

Most of the work focusing on with the role of free radicals, oxidants, and PARP in the pathogenesis of endothelial dysfunction focused on the acetylcholine-induced, endothelium-dependent relaxations. Most investigators assume that a particular intervention that improves endothelium-dependent vasodilatation is likely to confer commensurate benefit on other aspects of endothelial function. However, this assumption has rarely been confirmed by experimental studies. Therefore, it will be imperative to evaluate whether the beneficial effect of PARP inhibition on endothelium-dependent vasodilatation also manifests in improvements in other functions of the

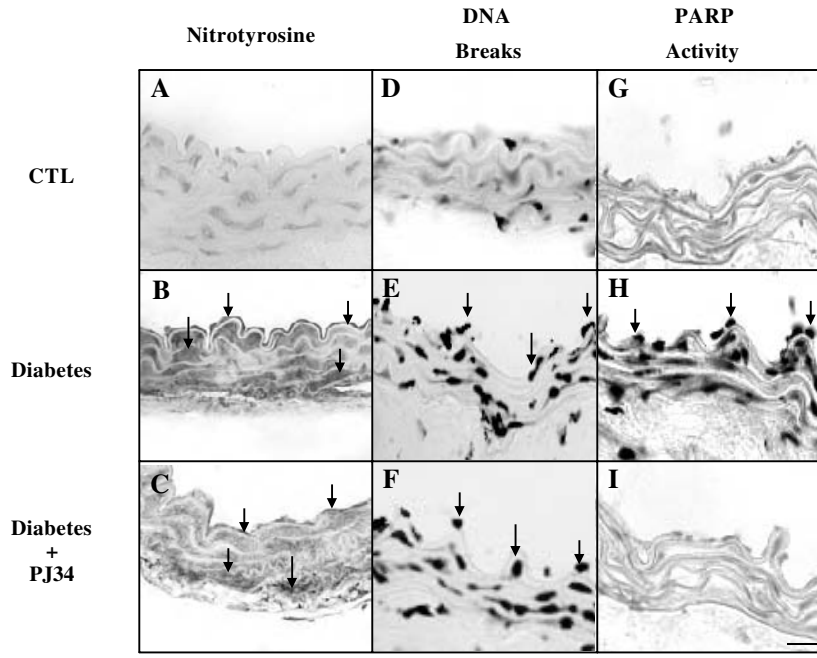

FIGURE 14.6 Reactive nitrogen species generation, ssDNA breakage, and PARP activation in diabetic blood vessels. (A to C), Immunohistochemical staining for nitrotyrosine in control rings (A), in rings from diabetic mice treated with vehicle at 8 weeks (B), and in rings from diabetic mice treated with PJ 34 (C). (D to F), Terminal deoxyribonucleotidyl transferase-mediated dUTP nick-end labeling, an indicator of DNA-strand breakage, in control rings (D), in rings from diabetic mice treated with vehicle at 8 week (E), and in rings from diabetic mice treated with PJ 34 (F). (G to I) Enzyme histochemical detection of PARP activity in control rings (G), in rings from diabetic mice treated with vehicle at 8 weeks (H), and in rings from diabetic mice treated with PJ 34 (I). Similar histochemical profiles were seen in $n = 4$ to 5 vascular rings per group. (From Soriano, G. F. et al., *Nat. Med.,* 7, 108, 2001. With permission of Nature America, Inc.)

vascular endothelium (permeability, hemostatic, antiatherogenic, regulation of cell trafficking, etc.).

Although most of the studies on the role of PARP in the pathogenesis of diabetic endothelial dysfunction, as discussed above, originated in macrovessels, there is circumstantial evidence that similar processes are operative for the pathogenesis of diabetic microvascular injury (retinopathy, nephropathy). For example, our group has recently provided evidence for PARP activation in the diabetic retina. We have examined PARP activity in retinas of experimentally diabetic rats based on immunohistochemical detection of poly(ADP-ribosyl)ated proteins. Streptozotocin-diabetic rats were given insulin in amounts that prevented weight loss, but nevertheless kept the animals very hyperglycemic (fasting blood sugar > 3 × normal). Diabetic animals and nondiabetic controls were killed after 12 to 14 weeks of study,

and cryosections of retinal tissue prepared. Little or no PARP immunoreactivity was detected in any retinal cells of nondiabetic animals. In contrast, positive staining was detected in retinas of diabetic animals, primarily in vascular cells and also blood elements. Immunostain was found in the cytoplasm of the vascular cells as well as in the nucleus.[138] In preliminary studies, some diabetic animals received diet supplemented with aminoguanidine (2.5 g/kg diet). In these animals, PARP immunoreactivity was reduced, when compared to diabetic controls.[138] Based on the current findings, and previous work demonstrating that PARP inhibition can improve the function of oxidant-challenged endothelial and epithelial cells, we propose that inhibition of PARP might offer a new therapeutic target to help inhibit the development of diabetic retinopathy. In addition, a study more than a decade ago demonstrated that the presence of glomerular depositions (mesangial distribution) of IgG, as evaluated with immunofluorescence technique, was significantly reduced in streptozotocin-diabetic rats treated with nicotinamide for 6 months.[139] Normal, age-matched controls showed no such staining.[139] With nicotinamide there is a possibility that the compound acts as an antioxidant (see above) or affects various metabolic pathways relevant for the pathogenesis of the disease.[140] Further studies, utilizing potent and specific inhibitors of PARP, are needed to further delineate the role of PARP in the pathogenesis of diabetic retinopathy and nephropathy. Recent data also indicate the pathegenic role of PARP in diabetic neuropathy. PARP-deficient mice are resistant to the depression in peripheral nerve conductance velocity in a galactosamine model (Obrosova, I. and Szabó, C., unpublished observations, 2001.

14.3 ISLET REGENERATION

The total ß-cell mass reflects the balance between the renewal and loss of these cells. While factors damaging ß-cells have attracted much attention, relatively little attention has been paid to investigate the regulation of β-cell proliferation. One of the reasons for this is that ß-cells were once viewed as finally differentiated cells incapable of proliferation, a feature that represents a predisposing factor for diabetes. However, in the last two decades an increasing body of evidence has indicated that ß-cell renewal is possible under certain physiological and pathophysiological conditions.[141] These conditions include pregnancy, hyperglycemia, pancreatectomy, or disruption of insulin receptor signaling. The fact that ß-cells can be induced to proliferate provides the rationale for experimental therapy aiming at preventing the disease by enhancing the regenerative capacity of islet cells. Indeed, animal experiments showed that rats subjected to subtotal (90%) pancreatectomy, can be rescued from diabetes by interventions enhancing the proliferation of ß-cells.[142] Somewhat surprisingly, the agents used to achieve this goal were nicotinamide and 3-aminobenzamide (3-AB), inhibitors of PARP. Administration of nicotinamide or 3-AB 1 to 3 months after partial pancreatectomy resulted in markedly reduced urinary and plasma glucose levels.[142] Moreover, the remaining pancreas of rats treated with nicotinamide or 3-AB were markedly enlarged (being about twofold larger than those of normal animals). These enlarged pancreata consisted mainly of ß-cells the mitotic indices of which significantly exceeded those of normal ß-cells.[142,143] Similar results have been reported

in a canine model of massive pancreatectomy.[144] Following these pioneering observations made in Professor Okamoto's laboratory at Kanazawa University and later at Tohoku University Graduate School in Japan, which have implicated PARP in the regulation of ß-cell regeneration, systematic work in the same laboratory revealed the molecular details of the mechanism by which PARP regulates islet cell regeneration. By screening the regenerating islet-derived cDNA library they identified a novel gene they named Reg, which was expressed in regenerating islets or hyperplastic islets of aurothioglucose-treated mice but not in normal islets, insulinomas, and regenerating liver.[145] Later, Reg expression was also found after acute pancreatitis, in pancreatic islets of nonobese diabetic mice during active diabetogenesis, in islets of BB rats during the remission phase of the disease.[146] Furthermore, pancreatic ductal cells,[147] which are thought to be progenitor cells of ß-cells have also been reported to express Reg. The rat Reg gene encodes a 165 amino acid protein (166 amino acid in humans) with a 21 amino acid (22 in humans) signal peptide.[146] Reg protein functions as a ß-cell growth factor as administration of recombinant Reg protein to non-obese diabetic mice induced the expansion of ß-cell mass.[148] Reg protein therapy also proved effective in amelioration of diabetes in partially pancreatectomized rats.[146] Transgenic mice overexpressing the Reg gene (Reg-TG) under the control of rat insulin II promoter (RIP) have also been generated but, somewhat surprisingly, showed no increase in islet cell mass.[149] Contrary to expectations, morphological analysis of pancreata from Reg-TG mice revealed increased apoptotic cell death, a decreased number of insulin-producing ß-cells and impaired glucose-induced insulin secretion.[149] The mechanism of increased ß-cell death in Reg-TG islets is unknown at present. It cannot be excluded that the high rate of apoptosis in Reg transgenic ß-cells is due to overstimulation as cells contained a sevenfold excess of Reg compared to wild-type cells. Nonetheless, compensatory islet regeneration and proliferation of ductal cells have been observed in the Reg-TG mice,[149] indicating that the primary effect of Reg may be the stimulation of ductal epithelial cell proliferation and differentiation into ß-cells. Lessons from Reg-TG mice indicate that there is much to be learned about the role of Reg in pancreas development. Further investigation is also required to elucidate the potentially tumorigenic effect of Reg in light of findings that several tumors arose in the Reg-TG mice.[149]

Reg is now part of a multigene family that is further subdivided into type I, II, and III Reg gene groups.[146] Moreover, the cDNA of Reg receptor encoding a 919 amino acid cell surface protein has also been identified.[150] Transfection of RINm5F cells with the Reg cDNA resulted in a significant increase of Reg-induced cell proliferation.[150] The receptor showed a ubiquitous expression pattern with highest expressions found in the brain, adrenal gland, spleen, and normal pancreatic islets.[150] The functional role of Reg receptor in these extrapancreatic tissues is unknown at present.

The question arises how PARP regulates the Reg-dependent growth of ß-cells. Very recently the Okamoto group has demonstrated that PARP can bind to the Reg gene promoter and takes part in the active transcriptional DNA–protein complex formed in response to IL-6+ dexamethasone stimulation.[151] Although the presence of PARP seems to be required for the formation of the active transcriptional complex,

auto-poly(ADP-ribosyl)ation of PARP had inhibitory effect on the Reg promoter activity.[151] Based on these findings, the following model was proposed to explain the role of PARP in the regulation of Reg gene expression. PARP binds to a *cis*-element of the promoter and stabilizes the transcriptional complex. Activation of PARP leads to auto-poly(ADP-ribosyl)ation of the enzyme, and poly(ADP-ribosyl)ated PARP detaches from DNA and thereby inhibits the formation of active complex. Inhibition of PARP by nicotinamide, 3-AB, or other inhibitors stabilize the transcriptional complex and enhance transcription. These findings are in line with earlier observations from Miyamoto et al. reporting the transcriptional repressor activity of poly(ADP-ribosyl)ation in nuclear receptor-mediated transcription.[152]

Recently, using differential display, an array of genes has been identified, the expression of which was either increased or decreased in PARP-deficient fibroblasts as compared with their normal counterparts.[153] Among these genes were the genes encoding for insulin-like growth factor (IGF) binding proteins 2, 4, 5, and 6, which were up- or downregulated in the absence of PARP.[153] IGFs participate in the growth and function of almost every organ, however, our knowledge on the role of IGFs in the regulation of ß-cell growth is very limited. It is conceivable that interventions such as inhibition of PARP may affect the production or availability of IGSs may also have an impact on ß-cell proliferation. This may also be true to other peptides and proteins such as hepatocyte growth factor, PTH-related protein, placental lactogen, betacellulin and glucagon-like peptide 1, which are known to augment ß-cell proliferation.[154]

The implications of these findings are that by enhancing Reg expression or expression of other ß-cell stimulatory agents, PARP inhibitors may enhance ß-cell regeneration and may thereby exert additional beneficial effects in diabetic subjects.

14.4 ISLET TRANSPLANTATION

A potential treatment option for patients with diabetes may be the replacement of destroyed ß-cells by transplants of allogenic or xenogenic insulin-producing islet cells. A large number of animal experiments using rodents showed the feasibility of this approach to the prevention or treatment of diabetes.[155] However, many of these approaches did not translate well to preclinical experiments using large animals such as dogs and nonhuman primates. Several clinical trials have also been carried out to evaluate the usefulness of islet cell transplantation in humans. The fact that in those cases, where islets were successfully engrafted, metabolic parameters normalized and insulin independence could be maintained for as long as 6 years indicates that islet cell transplantation represents a promising treatment option for patients with diabetes.[155] The field of islet cell transplantation has recently gained momentum from a recent clinical trial often referred to as the "Edmonton study" reporting remarkable success of an islet cell transplantation regimen.[156] In this study, seven patients with insulin-dependent diabetes underwent intrahepatic transplantation of large number of allogenic islets and were treated with a glucocorticoid-free immunosuppressive cocktail. The hope is that the encouraging success of this trial (insulin independence and excellent metabolic control could be achieved in seven of seven patients) will

lead to an expansion of clinical and basic science research aiming at the elaboration of more refined islet cell transplantation protocols. This study has also underscored the importance of critical parameters determining the outcome and also limiting the applicability of islet cell transplantation. These parameters include (1) the number of transplanted islets, (2) primary nonfunction, (3) recurrence of autoimmunity, and (4) the adverse effects of immunosuppressive therapy.

Based on data available in the literature, PARP appears to be involved in the regulation of many of these processes, which need to be overcome for a successful islet cell transplantation. A key factor of a successful islet cell transplantation is to obtain and transplant sufficient numbers of islets. In the Edmonton study two to four pancreases per patient were needed to obtain sufficient number of islets to achieve insulin independence.[156] This obviates the need for alternative sources of insulin-producing cells such as ß-cells differentiated *in vitro* from stem cells or xenogenic islets. Alternatively, stimulation of proliferation of the transplanted ß-cells would also lower the number of islets needed to achieve insulin independence. A mouse study showed that administration of the PARP inhibitor nicotinamide caused a more than 50% increase in the replication of transplanted islets in addition to the threefold stimulation observed in the endogenous pancreatic cells.[157] Furthermore, proliferation and differentiation of porcine fetal islet cells — the most-promising source of xenogenic ß-cells — are enhanced by nicotinamide.[158,159] Moreover, nonspecific inflammation implicated in the pathogenesis of primary nonfunction could also be prevented by nicotinamide treatment in a mouse model of syngeneic islet cell transplantation.[160] There are good reasons to believe that autoimmune insulitis as well as graft rejection could be effectively treated with PARP inhibitors. In a variety of models of inflammation PARP inhibition has been shown to inhibit inflammatory cell migration by reducing the expression of cytokines, chemokines, and adhesion molecules.[161] Further, autoimmune inflammation is also expected to be inhibited by PARP inhibitors as PARP is involved in the regulation of MHC-II expression, cell proliferation, and differentiation.[161] Moreover, iNOS expression and NO production by macrophages have been shown to contribute to damage of transplanted ß-cells,[162] and iNOS expression can be effectively inhibited by PARP inhibitors.[86,87] Animal experiments in mice provide support for this idea as nicotinamide treatment has been shown to protect islet allografts from damage and rejection.[163] In addition, in a recent pilot study, the potent PARP inhibitor PJ 34 was found to improve markedly the survival or syngeneic islet grafts in diabetic NOD mice. PJ 34 at 10 mg/kg/day delayed autoimmune diabetes recurrence in diabetic NOD mice that received syngeneic islet transplants under the renal capsule. All syngeneic islet grafts were rejected by day 18 after transplantation in vehicle-treated mice, whereas 40% of islet grafts survived to 100 days and these grafts remained intact and the mice were normoglycemic even at 60 days after PJ 34 was discontinued.[164]

In summary, PARP inhibitors can be prime candidates for adjuvant therapy of islet cell transplantation as these agents may block primary nonfunction, and autoimmune inflammation and may enhance the proliferation and differentiation of transplanted and endogenous ß-cells.

14.5 REGULATION OF PROINFLAMMATORY SIGNALING PATHWAYS BY PARP: IMPLICATIONS FOR DIABETES

The role of PARP in diabetes and various other experimental models of disease was initially believed to be mainly or exclusively related to its effects on intracellular energetics and resultant cellular dysfunction. In the last few years, however, a new series of *in vitro* and *in vivo* investigations revealed that inhibition of PARP activation has unexpected actions in regulating the expression, activation, and nuclear translocation of key proinflammatory genes and proteins. The absence of PARP or its pharmacological inhibition has been shown to suppress the activation of MAP kinase,[165] AP-1 complex,[166] and NF-κB.[46,72,167] Consequently, PARP inhibition or PARP deficiency interferes with the expression of proinflammatory genes, such as iNOS and ICAM-1, that are dependent upon these signaling pathways.[46,72,89,167-170] These observations have been revealed in *in vitro* systems, after examining a broad range of cell types, and also *in vivo*, in various experimental models of inflammation. For example, PARP inhibition blocks ICAM-1 expression in cultured endothelial cells stimulated *in vitro* by a combination of proinflammatory cytokines.[169] PARP inhibition also suppresses the expression of the chemokine MIP-1α in immunostimulated macrophages, keratinocytes, and fibroblasts *in vitro*.[171,172] Experimental evidence indeed demonstrates that PARP inhibition suppresses mononuclear cell infiltration in various experimental models of inflammation.[52,89,173-176]

The regulation by PARP of gene expression may involve the poly(ADP-ribosyl)ation of transcription factors or by the physical association with DNA strand breaks which may interfere with gene transcription. PARP may also alter the activation of proinflammatory pathways via its influence on the expression of AP-1, a heterodimer composed of *c-fos* and *jun* factors. High levels of transcriptional activation of human ICAM-1, C3, and *c-fos* require AP-1 binding to 5′ flanking regulatory regions.[177] In cultured cells, PARP inhibition blocks oxidant-induced *c-fos* mRNA expression and AP-1 activation.[166] Since the *c-fos* promoter contains an AP-1 consensus site, *c-fos* activation could trigger a positive-feedback cycle of gene expression. Superoxide anion has also been reported to induce the post-translational poly(ADP-ribosyl)ation of *c-fos*.[177]

PARP inhibition attenuates LPS and interferon–γ–induced iNOS expression in macrophages and other cell types.[72,81,89,168] As mentioned above, studies utilizing various PARP inhibitors in immunostimulated islet cells have demonstrated that nicotinamide or 3-AB can suppress the expression of the inducible isoform of NOS in the islets, and reduces reactive nitrogen species generation.[46,72, 178] In addition, as mentioned above, nicotinamide suppresses IL-12 and TNF production in peripheral blood of patients with type 1 diabetes.[82] Cytokine-induced MHC class II but not class I molecule expression in mouse islet cells is also inhibited by PARP inhibitors.[51] Similar results were observed in endothelial cells and fibroblasts as well.[179] A variety of anti-inflammatory effects have been noted *in vitro* in murine macrophages and *in vivo* in rodents. PARP inhibition has been shown, for example, to suppress endotoxin-induced expression of TNF-α, IL-6, and iNOS and to elevate the expression of the anti-inflammatory cytokine IL-10.[46,72,165-170] These effects are associated with the

suppression of LPS-mediated induction of NF-κB activation[46,72,167-170] and of MAP kinase activity.[165] Since MAP kinase plays a major role in the pleiotropic transduction of intracellular inflammatory cascades, the anti-inflammatory effects of PARP inhibition may be accounted for at this level of gene regulation. One may also expect that PARP-dependent regulation of NF-κB activation has a pleiotropic effect on the expression of proinflammatory genes, given the broad role that NF-κB plays in the transcriptional activation of cytokine and chemokine genes. A microchip analysis study recently completed has investigated the changes in the expression of 15,000 genes in wild-type and PARP-deficient fibroblasts. The study has demonstrated that under baseline conditions (i.e., in the absence of specific proinflammatory stimuli), there is a significant alteration in the expression of a whole host of genes.[180] It is likely that even more significant differences will be found in immunostimulated cells. However, systematic survey experiments comparing gene expression in immunostimulated wild-type and PARP-deficient cells using the microchip method have, to our knowledge, not yet been performed.

Overall, one can conclude that the inhibition of iNOS and proinflammatory gene expression by PARP inhibition is an effect that can be consistently demonstrated, and is relevant for both the primary disease process (islet injury) as well as for some of the secondary disease processes (vascular injury). The modulation of gene expression is likely to contribute to the beneficial effects seen with PARP inhibitors in various forms of diabetes.

14.6. SUMMARY AND IMPLICATIONS

The following working hypothesis summarizes our current understanding of the role of PARP activation in the development of inflammatory injury (Figure 14.7). The initial, autoimmune islet attack induces the production of proinflammatory mediators and free radicals and oxidants. The proinflammatory mediators enhance the recruitment of mononuclear cells, which produce more of these mediators, which yields a positive feedforward cycle. The oxidants and free radicals induce pancreatic islet cell injury and death. PARP inhibitors suppress these processes by inhibiting the actual process of islet cell injury, as well as by downregulating inflammatory mediator production. Protection from cell necrosis or shift to apoptosis in the presence of PARP inhibitors will prevent the release of cell content into the extracellular space thereby reducing feedback cycles of inflammation.

Once diabetes is established, high circulating glucose stimulates vascular free radical formation from the mitochondrial electron transport chain, from aldose reductase, NADH/NADPH oxidase, and a variety of other cellular sources. Also, high glucose may induce the upregulation of the endothelial and inducible isoforms of NO synthase, leading to enhanced NO production. At later stages of diabetes, recruitment and activation of neutrophils may also occur, which enhances vascular injury via a variety of mechanisms including neutrophil NADPH oxidase. As a consequence, peroxynitrite, hydrogen peroxide, and hydroxyl radical are formed in the vascular endothelial cells from the interaction of superoxide and NO

and by iron-catalyzed oxidation of superoxide. The resulting DNA injury and PARP activation trigger endothelial dysfunction in diabetes.

Both for the primary process of islet injury and for the secondary process of vascular dysfunction, one must consider a variety of positive-feedforward cycles. For example, oxidant stress induces AP-1 formation and generates DNA single-strand breaks. DNA strand breaks then activate PARP, which in turn potentiates NF-κB activation and AP-1 expression, resulting in greater expression of the AP-1- and NF-κB-dependent genes, such as iNOS, ICAM-1, MIP-1α, and TNF-α. Increased endothelial expression of ICAM-1 may, in turn, recruit more activated leukocytes to the inflamed islet or into the diabetic blood vessels, producing greater oxidant stress. The cycle is thus renewed as the increase in oxidant stress triggers more DNA strand breakage. The proposed cycle of inflammatory activation will be augmented by PARP-dependent MAP kinase activation and NF-κB translocation, and by consequent free radical and oxidant formation and mononuclear cell recruitment. Thus, PARP occupies a critical position in a positive-feedforward loop of diabetic islet and vascular injury. NAD^+ depletion induced by PARP activation is likely to accelerate this positive-feedback cycle by preventing the energy-dependent reduction of oxidized glutathione, the main intracellular antioxidant and most abundant thiol in eukaryotic cells. NAD^+ is the precursor for NADP, a co-factor that plays a critical role in bioreductive synthetic pathways and the maintenance of reduced glutathione pools. Depletion of reduced glutathione, as a consequence of intracellular energetic failure or overwhelming oxidant exposure, leaves further oxidant stress unopposed, resulting in greater DNA strand breakage. PARP activation and mitochondrial injury also form a self-amplifying circle, whereby PARP-dependent metabolic processes enhance mitochondrial free radical generation and permeability transition. Pharmacological inhibition of PARP, or PARP deficiency is able to suppress these deleterious mitochondrial processes. PARP-dependent depletion of NADPH directly contributes to the suppression of endothelium-dependent relaxations in diabetic blood vessels.

Since PARP occupies a critical position in a self-amplifying cycle of oxidant-driven damage, it is appropriate then to ask whether PARP inhibition is a candidate for clinical treatment of type I diabetes, or diabetic vascular dysfunction (or other secondary, systemic, or cardiovascular consequences of diabetes mellitus). As far as the human type I diabetes is concerned, the nicotinamide trials yielded conflicting results (see section 14.1.4), and it is likely that additional studies, utilizing more potent PARP inhibitors will be necessary. As far as the diabetic vascular dysfunction goes, although the activation of PARP has yet to be demonstrated in human diabetic vascular tissue, both the proximal triggers of DNA single-strand breakage (e.g., peroxynitrite),[181] and the existence of increased DNA single-strand breakage,[182,183] have been demonstrated in human clinical samples. Furthermore, DNA single-strand breakage and PARP-dependent endothelial cell injury have been demonstrated in human umbilical vein endothelial cells incubated with high glucose.[46] Finally, PARP-dependent morphological and functional injury has been demonstrated in a variety of human cell types exposed to pro-oxidant conditions.[184-188] Therefore, it is conceivable that PARP-dependent processes are being triggered in human diabetic

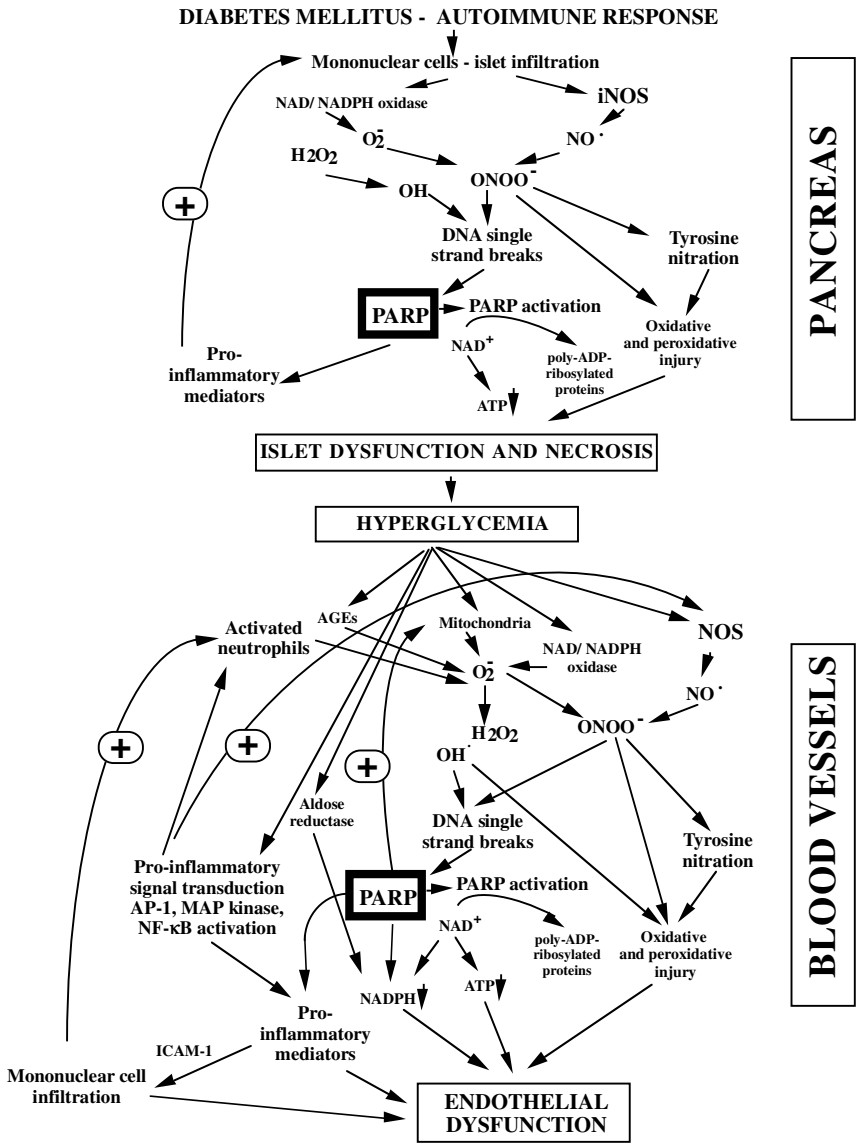
DIABETES MELLITUS - AUTOIMMUNE RESPONSE
Mononuclear cells - islet infiltration
NAD/ NADPH oxidase
iNOS
O2-
NO·
H2O2
ONOO-
OH·
DNA single strand breaks
Tyrosine nitration
PARP
PARP activation
NAD+
poly-ADP-ribosylated proteins
Oxidative and peroxidative injury
Pro-inflammatory mediators
ATP
PANCREAS
ISLET DYSFUNCTION AND NECROSIS
HYPERGLYCEMIA
AGEs
Activated neutrophils
Mitochondria
NAD/ NADPH oxidase
NOS
NO·
O2-
H2O2
ONOO-
OH·
Aldose reductase
DNA single strand breaks
Tyrosine nitration
Pro-inflammatory signal transduction AP-1, MAP kinase, NF-κB activation
PARP
PARP activation
NAD+
poly-ADP-ribosylated proteins
Oxidative and peroxidative injury
NADPH
ATP
Pro-inflammatory mediators
ICAM-1
Mononuclear cell infiltration
ENDOTHELIAL DYSFUNCTION
BLOOD VESSELS

FIGURE 14.7 Proposed scheme of PARP-dependent and PARP-independent cytotoxic pathways involving nitric oxide ($NO^{\bullet}$), hydroxyl radical ($OH^{\bullet}$), and peroxynitrite ($ONOO^{-}$) in diabetic islet cell death and hyperglycemia-associated endothelial dysfunction. Autoimmune stimuli trigger the infiltration of mononuclear cells into the pancreas. Free radicals and oxidants, produced both by the mononuclear cells and by the pancreatic ß-cells themselves, lead to DNA single-strand breakage and PARP activation in ß-cells. Depletion of the cellular NAD^{+} leads to inhibition of cellular ATP-generating pathways leading to cellular dysfunction, eventually culminating in ß-cell death via the necrotic route. A substantial loss of ß-cells leads to decreased glucose tolerance, followed by persistent hyperglycemia. High circulating glucose interacts with the vascular endothelium where it triggers the release of oxidant mediators from the mitochondrial electron transport chain, from NADH/NADPH oxidase, and from other sources. High glucose may also upregulate eNOS expression (eNOS) or trigger the expression of the inducible NO synthase (iNOS) in the endothelium. NO, in turn, combines with superoxide to yield peroxynitrite. Hydroxyl radical (produced from superoxide via the iron-catalyzed Haber–Weiss reaction) and peroxynitrite or peroxynitrous acid induce the development of DNA single strand breakage, with consequent activation of PARP. Depletion of the cellular NAD^{+} leads to inhibition of cellular ATP-generating pathways leading to cellular dysfunction. The PARP-triggered depletion of cellular NADPH directly impairs the endothelium-dependent relaxations. The effects of elevated glucose are also exacerbated by increased aldose reductase activity leading to depletion of NADPH and generation of reactive oxidants. NO alone does not induce DNA single-strand breakage, but may combine with superoxide (produced from the mitochondrial chain or from other cellular sources) to yield peroxynitrite. Under conditions of low cellular L-arginine, NOS may produce both superoxide and NO, which then can combine to form peroxynitrite. PARP activation, via a not-yet-characterized fashion, promotes the activation of NF-κB, AP-1, MAP kinases, and the expression of proinflammatory mediators, adhesion molecules, and iNOS. PARP-independent, parallel pathways of cellular metabolic inhibition can be activated by NO, hydroxyl radical, superoxide and by peroxynitrite. By promoting neutrophil recruitment and oxidant generation, positive feedback cycles are triggered both at the level of the ß-cell and the level of the endothelium. PARP activation and mitochondrial oxidant production form another positive-feedback cycle. Positive feedback circles are marked with a "+" symbol in a circle.

blood vessels *in vivo*. In fact, recent preliminary data indicate increased nuclear poly(ADP-ribose) formation in the dermal microvasculature of diabetics. This change is associated with impaired endothelium-dependent relaxant response.[189]

A variety of PARP inhibitors are in preclinical development, many with potency that greatly exceeds the prototypic agents used in experimental proof-of-concept studies. In general, PARP inhibitors may be particularly useful in the treatment of acute disorders, where issues of potential toxicity related to chronic administration are unimportant. Although the exact physiological role of PARP is still unclear and remains a matter of dispute, it is logical to suppose it plays an important role since it is one of the most abundant proteins in the nucleus. PARP has been implicated in many physiological housekeeping functions, such as gene repair, transcription, and cell cycling. Until such time as its true physiologic functions are more precisely defined, there should exist a considerable caution in the long-term administration of PARP inhibitors to humans. PARP inhibition has also been associated with an increase in sister-chromatid

exchange,[190-192] a potentially concerning finding that raises the risk of malignant transformation. PARP activation clearly leads to cell death, and some have argued that its physiological role is to eliminate genetically damaged cells, thereby reducing oncogenic potential. Indeed, PARP inhibition has been shown to facilitate the rapid ligation of DNA excision-repair patches[193] and to suppress malignant transformation in cells with DNA damage induced by irradiation and chemical carcinogens.[194] Whether chronic PARP inhibition predisposes to malignant transformation is open to question. The PARP-deficient mice have not been reported to present increased frequency of spontaneous malignancies although, to our knowledge, this issue has not yet been systematically investigated. A recent study has demonstrated increased frequency of hemangiomas and hemangiosarcomas in $PARP^{-/-}$ mice chronically fed with high doses of *N*-nitrosobis(2-hydroxypropyl)amine for 20 weeks, when compared to wild-type mice receiving the same carcinogen treatment.[195] Whether this observation has any relevance for the pharmaceutical development of PARP inhibitors remains to be seen. No spontaneous hemangiomas or hemangiosarcomas have been reported in the $PARP^{-/-}$ mice in the same study. Crossing the PARP-deficient mice with P53-deficient mice yielded conflicting results.[196,197] In the human studies with chronic administration of nicotinamide, no adverse effects have been reported. With respect to nicotinamide, the preclinical trials yielded conflicting results: in one study, it was reported that, about 1 year after the combined administration to rats of alloxan or streptozotocin with PARP inhibitors, diabetes did not develop but islet β-cell tumors were found frequently.[40] However, no follow-up studies were published on this subject, and in subsequent studies, nicotinamide failed to modify the incidence of diethylnitrosamine-induced carcinogenesis in rats.[198,199] In another study, nicotinamide was actually reported to suppress the renal tumorigenic effect of streptozotocin in rats.[200] The influence of nicotinamide on genomic stability and carcinogenesis has recently been overviewed by Hageman and Stierum.[200] *In vitro* studies show that PARP-1 function is impaired and genomic stability decreased when cells are either depleted of NAD^+ or incubated with high concentrations of nicotinamide. This may suggest that neither a massive inhibition nor an overactivation of PARP is beneficial for genomic stability. *In vitro* as well as *in vivo* studies indicate that nicotinamide deficiency increases genomic instability especially in combination with genotoxic and oxidative stress. Nicotinamide deficiency may also increase the risk for certain tumors. Preliminary data suggest that nicotinamide supplementation may protect against ultraviolet-induced tumors of the skin in mice, but data on similar preventive effects in humans are not available. Nicotinamide has been shown *in vitro* to have an antioxidant activity comparable with that of ascorbic acid. Data on nicotinamide status and genomic stability *in vivo* in humans are limited and yield ambiguous results. According to one study, 14 weeks of nicotinamide supplementation slightly increased the frequency of sister-chromatid exchanges in lymphocytes from human volunteers.[202] As a consequence of oral nicotinamide supplementation, therefore, inhibition of PARP and increased genomic instability may occur. Additional studies are needed to define an optimal level of nicotinamide supplementation in relation to genomic stability and tumorigenesis. One must keep in mind, nevertheless (as mentioned above), that nicotinamide has many pharmacological actions other than PARP

inhibition, and in fact its potency as a PARP inhibitor is rather poor. Also, a clear distinction must also be made between chronic pharmacological PARP inhibition (as in chronic drug therapy) vs. chronic genetic PARP deficiency (as in knockout animals): the latter condition will also affect cellular processes due to the absence of protein–protein interactions in which PARP is known to participate. For example, although the presence of a genomically unstable tetraploid population in $PARP^{-/-}$ fibroblasts and its loss after stable transfection with PARP cDNA is well documented,[191] the development of tetraploidity was not reproduced with chronic incubation with GPI 6150, a potent pharmacological inhibitor of PARP, in the same experimental system.[203] Nevertheless, the issues discussed above must be adequately addressed prior to considering the development of PARP inhibitors for the chronic therapy of diabetes mellitus or its complications in humans.

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15 Therapeutic Targeting of the PARPs: Future Challenges, Future Opportunities

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The preceding chapters of this monograph have demonstrated that the inhibition of poly(ADP-ribose) polymerase-1 (PARP-1) during acute genotoxic stress could potentially alleviate human suffering associated with many diseases. Although many lessons have been learned, the realization of the goal of targeting PARP-1 for therapeutic benefit has not yet been reached and much remains to be accomplished. In this closing chapter, we speculate on both future challenges and future opportunities that may lie ahead for this emerging area of therapeutics.

A better understanding of the role of PARP-1 in cell death is likely to advance the development of therapeutically useful PARP-1 inhibitors

Although many studies of the effects of inhibiting PARP-1 in animal cells laid the early groundwork for targeting the enzyme, efforts to develop new therapies were greatly accelerated by the remarkable resistance of mice with the PARP-$1^{-/-}$ genotype to the cell-killing effects of high levels of genotoxic stress. As described in preceding chapters, DNA strand breaks are the manifestation of genotoxic stress that result in PARP-1 activation. Although many of the downstream events following PARP-1 activation are understood, much remains to be learned about how its activation alters cell metabolism following a particular genotoxic stress in a specific tissue. To target specific disease states, a better understanding of cell death pathways involving PARP-1 likely will be important. Figure 15.1 shows an overview of some of the multiple ways in which PARP-1 activation could modulate life and death decisions within tissues.

PARP-1 is one of several proteins that are "DNA strand break sensors."[1,2] Through complex interactions with other such sensors, including p53 and DNA protein kinase (DNA PK), PARP-1 initiates signaling pathways that can result in cell cycle arrest, stimulation of DNA base excision repair, and stimulation of the nuclear

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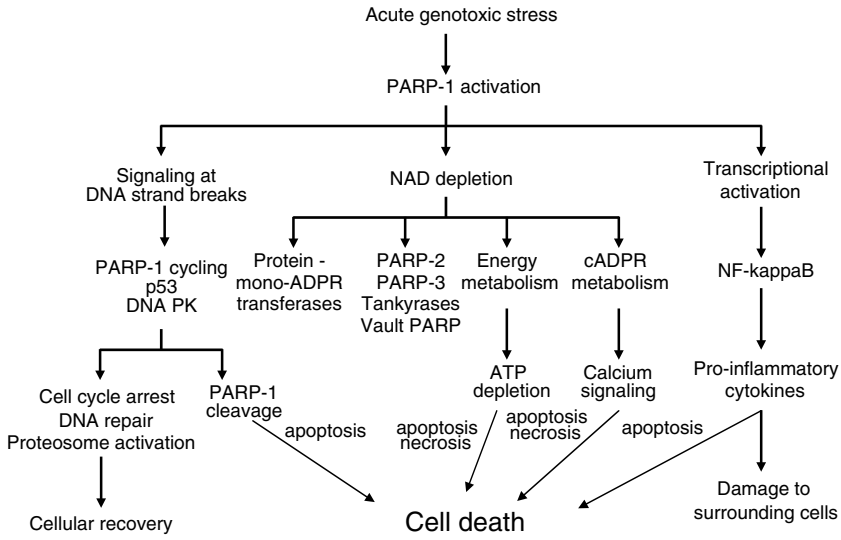

FIGURE 15.1 Involvement of PARP-1 in pathways of cell death following genotoxic stress.

proteosome.[2,3] All of these pathways promote cell survival following relatively low levels of genotoxic stress, but their significance during extensive DNA damage as occurs following reperfusion after ischemia is unclear. For some apoptotic pathways, a requirement for PARP-1 has been demonstrated[4] and PARP-1 cleavage has become a hallmark of apoptosis.[5] This cleavage may play a role in both sparing energy needed to complete apoptosis by limiting NAD and subsequently ATP depletion or by allowing the DNA binding domain of PARP-1 to block repair of DNA strand breaks introduced during the apoptotic program.

PARP-1-catalyzed NAD depletion following high levels of DNA damage allows multiple alternative routes to cell death. Compelling evidence indicates that cellular energy status is an important determinant of cellular life and death decisions, and the ability of hyperactivated PARP-1 to rapidly reduce NAD content has been postulated to result in necrosis as described in several of the preceding chapters. However, cellular energy status, particularly as reflected in the mitochondrial permeability transition, also is closely linked to apoptosis,[6] and thus NAD depletion contributing to cell death via apoptosis may be occurring in some systems. The effects of NAD depletion on other cellular ADP-ribosyltransferases also need to be considered. For example, NAD is the substrate for the synthesis of cyclic ADP-ribose (cADPR), which is involved in calcium signaling.[7] Alterations of calcium homeostasis have been linked to both apoptosis and necrosis.[8] Since PARP-2 also is activated by DNA strand breaks, effects of NAD depletion on its function may affect cellular responses. Although possible effects of NAD depletion on the other

cellular PARPs and on mono-ADP-ribosyltransferases are difficult to predict, possible connections to cell death need to be evaluated as the roles of these PARPs become more apparent.

The discovery of the function of PARP-1 in transcription mediated by nuclear factor kappa B (NF-κB) has major implications with regard to the targeting of PARP-1 for therapeutic development. This mechanism has ramifications not only for cells directly undergoing DNA damage but also for other cells in a tissue exposed to proinflammatory cytokines that result from NF-κB-stimulated transcription. Although considerable evidence supports a role for NAD depletion leading to necrosis as a primary mechanism resulting in cell death following many genotoxic stresses, the involvement of PARP-1 in NF-κB function dictates that this mechanism should be carefully examined as a contributing or alternative mechanism of cell death.

The involvement of PARP-1 in ADP-ribose polymer cycles creates additional potential targets whose modulation could lead to different therapeutic outcomes

PARP-1 initiates cycles of ADP-ribose polymer metabolism involving multiple enzymes.[9] Figure 15.2 shows an overview of these reversible cycles of protein modification. The enzyme that catalyzes the hydrolysis of ADP-ribose polymers is poly(ADP-ribose) glycohydrolase (PARG). PARG activity generates free ADP-ribose, and recent studies have suggested that this molecule may have a signaling function within cells.[10] Following DNA damage, the activities of PARP-1 and PARG are closely coordinated as evidenced by the observation that an ADP-ribose polymer residue has a half-life of less than 1 min.[11,12] Also of interest is that PARP-1 itself is a major acceptor of polymer residues in an automodification reaction.[1] The disassociation of automodified PARP-1 from DNA results in its inactivation, and PARG-catalyzed removal of polymers is required for reactivation of the enzyme. This has led to the concept that PARP-1 undergoes cycles of binding and disassociation as it performs its signaling function in DNA-damaged cells. The concerted activities of

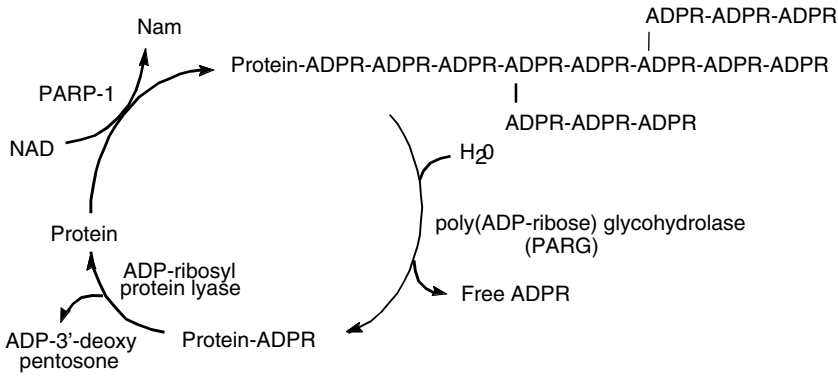

FIGURE 15.2 ADP-ribose polymer cycles.

TABLE 15.1
Comparison of Possible Effects of Inhibition of PARP-1 and PARG

Condition	NAD Depletion	PARP-1 Automodification	PARP-1 on DNA breaks
PARP-1$^{+/+}$	Yes	Yes	Cycling between bound and unbound
PARP-1$^{-/-}$	Depends upon presence of PARP-2	No (Absent)	Unbound (Absent)
PARP-1 Inhibitor	No	No	Bound
PARG Inhibitor	No (?)	Yes (?)	Unbound (?)

PARP-1 and PARG in DNA damage response-signaling raises the possibility that the targeting of PARG represents a future opportunity for therapeutic development. The cDNAs encoding mammalian PARGs have been isolated and the structural biology of the enzyme is emerging.[9,13] Table 15.1 compares possible outcomes that might result from targeting ADP-ribose polymer cycles at different reactions and speculates that inhibition of PARG may result in biological responses different from those that result from inhibition of PARP-1. In the case of normal PARP-1 activity, acute high levels of DNA damage result in NAD depletion, PARP-1 automodification/reactivation cycles, and cycling of binding to DNA strand breaks. In PARP-1$^{-/-}$ cells, PARP-1 protein is absent and although the degree of NAD depletion is reduced, the amount of depletion can vary upon the expression level of PARP-2, which also is activated by DNA strand breaks and can cause NAD depletion.[14] In the presence of a PARP-1 inhibitor, the residence time of PARP-1 on DNA breaks increases as the formation of the automodified enzyme is inhibited. It may be that some of the differences observed between responses of PARP-1$^{-/-}$ animals and PARP-1$^{+/+}$ animals in the presence of PARP-1 inhibitors relates to the residence time of PARP-1 at DNA strand breaks. Because PARG activity is required for maintaining PARP-1 in an active (unmodified) state, inhibition of PARG is likely to have the same NAD-sparing effect as inhibition of PARP-1. However, in contrast to direct inhibition of PARP-1, inhibition of PARG is predicted to result in the accumulation of automodified PARP-1, thereby decreasing residence time of PARP-1 on DNA breaks. This could result in a quite different cellular outcome than that observed with PARP-1 inhibitors. Future studies aimed at target validation of PARG are needed to determine if PARG represents a new opportunity for therapeutic development.

A third enzyme, ADP-ribosyl protein lyase, has been postulated to complete ADP-ribose polymer cycles by catalyzing removal of the protein-proximal ADP-ribose residue from acceptor proteins.[9] There is very limited information about the properties of this enzyme and no structural information about the protein has been reported. Lack of information concerning this enzyme represents a significant gap in our understanding of ADP-ribose polymer metabolism that should be investigated.

The presence of multiple PARPs may require PARP-1 specific inhibitors

The realization that PARP-1 is but one of a family of PARPs[15,16] may dictate a major paradigm shift in the approach to the therapeutic targeting of PARP-1. While much remains to be learned about the function of the newly discovered PARPs, a pattern of involvement in mechanisms that affect genomic stability seems to be emerging as a function of all PARPs.[15] This raises potential challenges for therapeutic development targeting PARP-1 because all PARP-1 inhibitors currently under development target the nicotinamide-binding region of the NAD-binding site of PARP-1. This site is similar in each of the known PARPs based on the observation that several known PARP-1 inhibitors also inhibit the other known PARPs.[14,17-19] The question that needs to be addressed is whether PARP-1 inhibitors that also affect other members of the PARP family will be suitable for human therapy. The answer to this question may relate to whether the goal of PARP-1 inhibition is acute, short-term therapy such as treatment of heart attack or stroke or longer-term therapy such as the treatment of chronic inflammatory disease. Acute, nonspecific inhibition of all PARPs including PARP-1 may not compromise therapy, whereas long-term inhibition of other PARPs involved in the maintenance of genomic integrity may be unacceptable. In the event that PARP-1 specific inhibitors are needed, the emerging domain structures of the PARPs clearly indicate large differences in domains apart from the catalytic domain. These differences, combined with the tools of modern medicinal chemistry, should allow the development of PARP-1-selective inhibitors if such are necessary for therapy.

Tissues with cells undergoing both acute and chronic genotoxic stress may benefit from strategies to enhance PARP-1 activity

The ability of PARP-1 to consume a major fraction of the NAD pool following genotoxic stress raises interesting questions concerning how cellular responses to such stresses may be affected by niacin status, which also can affect the cellular content of NAD.[20] For conditions in which PARP-1-mediated NAD depletion is the primary mechanism of cell death, maintaining an optimal NAD content would be predicted to confer resistance in the absence of PARP-1 inhibition. Maintaining optimal cardiac NAD content in patients at risk of heart attack and maintaining optimal NAD content in bone marrow of patients undergoing cancer chemotherapy are two possible examples where enhancing substrate availability may have therapeutic benefit. In tissues chronically exposed to DNA damage, enhancement of the multiple protective roles of PARP-1 (see Figure 15.1) by maintaining optimal NAD also may be a viable therapeutic goal. This possibility is supported by a study demonstrating in a mouse model of ultraviolet-induced skin cancer that high-level niacin supplementation resulted in both elevated skin NAD content and reduced skin cancer incidence.[21] Enhancing PARP-1 activity by enhancing the availability of its substrate may emerge as a new therapeutic strategy for disease prevention.

Lessons learned from targeting PARP-1 may lead to future opportunities for therapeutic development

The discovery of a family of PARPs was partially an outgrowth of research designed to validate PARP-1 as a therapeutic target. Experience gained in the study of PARP-1 may lead to the development of PARP inhibitors targeted at other members of the PARP family. An exciting possibility is that of tankyrase, a PARP located in chromosome telomeres.[18] Telomeres are the terminal regions of chromosomes that contain unique repetitive DNA sequences with G-rich single-stranded overhanging regions that are stabilized by the telomere-specific proteins TRF1 and TRF2.[22] Since DNA replication enzymes can synthesize DNA only in a 5′ to 3′ direction, the single-stranded regions of telomeres cannot be completely replicated by the normal replication machinery. Telomeres can be extended by a specialized cellular reverse transcriptase termed *telomerase*, but most normal human tissues do not contain this activity. Thus, in most cells telomeres shorten with each round of replication. This "telomere erosion" leads to cellular senescence and cell death when the cell can no longer maintain telomere structures that normally prevent telomeres from being detected by DNA-damage checkpoint proteins such as p53.[23] The manner in which telomeres normally escape detection by DNA damage checkpoint proteins has long been an enigma, but recently a telomere structure[22] was proposed that postulates that TRF1 and TRF2 align telomeric DNA to form a loop structure that allows the single-stranded overhangs to invade and form a displacement loop that no longer has single-

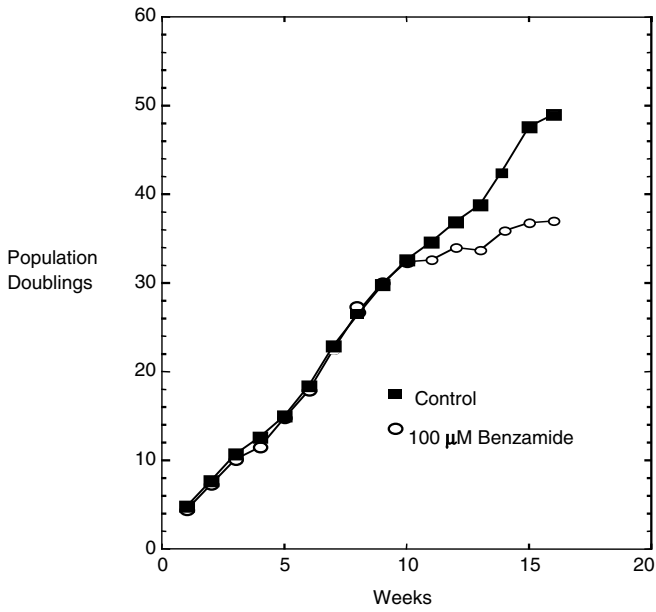

FIGURE 15.3 Long-term growth of human breast cancer cells in the presence of the tankyrase inhibitor benzamide.

stranded regions or free DNA ends, thus avoiding detection by DNA damage checkpoint proteins. In this model of telomere structure, cellular senescence or cell death results when telomere length is too short to form a telomere loop. In contrast to most normal cells, cancer cells contain telomerase and thus maintain unlimited proliferation potential by avoiding telomere erosion. Although the function of tankyrase is still poorly understood, modulation of tankyrase activity results in changes in telomere length,[24] consistent with a role of tankyrase in modulating changes in telomere structure necessary for access of telomerase to telomeric DNA. This possibility is supported by an experiment shown in Figure 15.3 in which MCF-7 cells derived from a human breast cancer were cultured long term in the presence of 100 μ*M* benzamide, which is an inhibitor of tankyrase. The results show that the proliferation potential of these cells is unaffected by the presence of benzamide for more than 30 population doublings, after which the proliferation potential greatly decreases. This result suggests that inhibition of tankyrase does not interfere with chromosome replication, but limits access to telomerase. If tankyrase is required for telomerase access to telomeric DNA, the possibility of targeting tankyrase as an approach to cancer prophylaxis becomes an intriguing possibility.

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