Oxidative Stress in Applied Basic Research and Clinical Practice

Samar Basu
Lars Wiklund *Editors*

Studies on Experimental Models

Humana Press

Oxidative Stress in Applied Basic Research and Clinical Practice

Editor-in-Chief
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All books in this series illustrate point-of-care testing and critically evaluate the potential of antioxidant supplementation in various medical disorders associated with oxidative stress. Future volumes will be updated as warranted by emerging new technology, or from studies reporting clinical trials.

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Preface

The notion that reactive oxygen species are involved in diseases originated in the 1930s when scientists realized that radiation injury may lead to formation of free radicals and in turn initiate cancer and other pathologies. Even though research in free radicals has been stretched exponentially in the last three decades, there are still major questions as to what extent free radicals are involved in health and various diseases. In recent years, there is a growing recognition that free radicals, and thereby oxidative stress, is involved in atherosclerosis, cardiovascular diseases, cancer, ischemia–reperfusion injury, radiation injury and neurological diseases, etc. In addition, free radicals are perhaps involved in both aging and redox process. Despite the great amount of research being carried out in the field, there are still uncertainties about the overall mechanisms behind free radical-related pathologies, as well as how free radicals may play a fundamental role in protecting cells through redox signaling.

With these concepts in mind, there is a wide-spread consensus today that the use of antioxidants as a therapeutic approach may counteract free radical-mediated pathologies. However, the role of antioxidants in normal physiology and redox signaling is still in its infancy. Since oxidative stress is related to various diseases and pathologies, scientists are eager to study the disease in humans. However, it is not always ethical to study all the aspects of the disease in humans; consequently, it is mandatory to study the disease process and the mechanisms behind it through experimental models. The models generally involve animals, in vitro/cell culture studies, and even in primates and humans to a certain extent.

This book contains mainly data on the experimental models or review of such models of oxidative stress in various diseases, and is divided into six parts that present a sketch of models in humans, animals and in vitro methods. Part I deals with diabetes, which includes the role of oxidative stress and antioxidant therapies in experimental models of diabetes, diabetes complications, micronutrient intake and its relevance to atherosclerosis and insulin resistance. Part II deals with stroke, including arachidonic acid metabolism and the role of alpha-tocotrienol as a therapeutic agent, assessment of oxidative stress, heat-shock proteins and doxorubicin-induced oxidative stress in the heart, as well as biomarkers of oxidative stress in cardiovascular diseases. Part III deals with neurology, which includes MPTP and

oxidative stress, oxidative stress in Alzheimer's disease, retinal disturbances in patients and animals models with Huntington's, Parkinson's and Alzheimer's disease, stress gene regulation in Alzheimer's blood cells, Gpx4 knockout mice and transgenic mice in aging, experimental models of myocardial, and cerebral ischemia. Part IV deals with ocular diseases, including neovascular models of the rabbit eye and purinergic signaling in volume regulation of glial cells in rat retina. Part V is concerned with toxicological/environmental aspects that contain *Helicobacter Pylori*-induced oxidative stress and inflammation, experimental models for ionizing radiation research, cigarette smoke-induced oxidative stress in preclinical models, smokers and patients with airways disease, exhaled breath condensate biomarkers in airway inflammation in COPD, induction of oxidative stress by iron/ascorbate in isolated mitochondria and by UV irradiation in human skin, carbon tetrachloride-induced oxidative stress, lipid metabolites after exposure of toxicants, oxidative stress in porcine endotoxemia and exercise as a model to study oxidative stress. The final part concerns in vitro/cell culture models that contain mitochondrial oxidative stress, protection against oxidant-induced neuronal cell injury, oxidative DNA biomarkers, oxidative stress in cell culture, animals and humans, role of cAMP and G protein signaling in cardiovascular dysfunction, cellular and chemical assays of antioxidants, and arsenic-induced oxidative stress.

We hope that this book will contribute to the advancement of oxidative stress research using appropriate animal models and will serve as a valuable reference for basic and clinical scientists.

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Part VI In Vitro/Tissue Culture

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Part I
Diabetes

Role of Oxidative Stress and Targeted Antioxidant Therapies in Experimental Models of Diabetic Complications

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Abstract Diabetic patients, whether of type 1 or type 2 origin, are at greater risk of developing complications of the vasculature than non-diabetic patients. Macrovascular complications such as diabetes-associated atherosclerosis lead to accelerated and often more advanced lesions than seen in the general population. Microvascular complications such as nephropathy, retinopathy and neuropathy as well as diabetic cardiomyopathy are further complications associated with the diabetic milieu. Understanding the mechanisms leading to and accelerating these complications is a major research initiative of many laboratories. To facilitate these studies, the design and use of appropriate animal models has been central to the study of these diabetic complications. A new and emerging concept underpinning many of these end-organ complications is oxidative stress, particularly of mitochondrial origin, which is understood to play a critical role in the initiation and progression of these diabetic complications. Thus the development of experimental models that specifically delineate the cause and role of ROS in diabetic complications is now becoming a major research area. This chapter focuses on some of the latest oxidative stress-driven experimental models of diabetic complications. Use of the ApoE/GPx1 double-knockout mouse has revealed the importance of antioxidant defense in limiting accelerated diabetes-associated atherosclerosis and diabetic nephropathy, while RAGE knockout mice have shown that oxidative stress is inextricably linked with pathophysiological cell signaling, particularly through RAGE. The use of NOX knockout mice is shedding light on the contribution of the NADPH oxidases to the ROS milieu as well as the contribution of the various isoforms (NOX 1, 2 and 4) to the individual diabetic complications. Furthermore, these models are helping to understand the types of ROS involved and their cellular location, which may help in the specific targeting of these ROS to reduce ROS-mediated pathogenesis. For example, antioxidants that target mitochondrial ROS (location) or ROS such as hydrogen peroxide (specificity) may offer an alternate

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approach to reduce diabetes-driven oxidative stress. It is only via manipulation of experimental models of diabetes-driven oxidative stress that the contribution of the various ROS will be revealed, and only then that effective treatment regimens can be designed to lessen the effect of oxidative stress on diabetic complications.

Keywords Antioxidant defense • Diabetic complications • Ebselen • Experimental models • Glutathione peroxidase • Oxidative stress

1 Introduction

Diabetes mellitus is a metabolic disorder that is characterized by chronic hyperglycemia with disturbances in carbohydrate, fat and protein metabolism and occurs as a result of defects in insulin secretion and/or action [1]. Whether diabetes occurs as a result of type 1, the early-onset and predominantly insulin-dependent form, or type 2, the late-onset form that is associated with metabolic syndrome, obesity and insulin-resistance, individuals with diabetes are at greater risk of developing diabetes-associated complications [2, 3].

The predominant complications include cardiovascular disease, nephropathy, retinopathy, neuropathy and a specific impairment in the heart muscle leading to cardiomyopathy [4]. It is now well accepted that most diabetic complications arise from chronic hyperglycemic damage to the vascular system [5]. Vascular disease can be separated into that affecting the macrovasculature resulting in atherosclerosis of major vessels and/or stroke, and microvascular complications resulting in retinopathy, nephropathy and neuropathy. Diabetic cardiomyopathy is understood to occur as a result of abnormal myocardial metabolism in diabetes, rather than as a result of micro- or macro-vascular disease [6–8].

While many studies address the causative mechanisms of the individual diabetes-driven complication, it is now becoming increasingly apparent that oxidative stress is an important underpinning phenomenon that assists with the progression towards more severe and often fatal complications. Evidence from numerous studies suggests an important causal role for increased reactive oxygen species (ROS), particularly mitochondrial ROS, in the pathogenesis of the major complications associated with diabetes [9]. Several pathways, including the polyol pathway [10], increases in advanced glycation end products (AGEs) [11], activation of protein kinase C [12] and increases in hexosamine flux, have been identified where hyperglycemia triggers increased ROS production that in turn may initiate, progress or amplify end-organ damage in diabetes. One postulate suggests that all of these pathways are activated as a result of glucose-induced overproduction of superoxide by the mitochondrial electron transport chain [2]. Indeed it has been estimated that 1–2% of all electrons passing through the respiratory chain contribute to the formation of superoxide [13, 14], with the rate of production varying greatly depending on the environment and/or disease state [14]. Other potential sources of ROS production include nitric oxide synthases (NOS), nicotinamide adenine

dinucleotide phosphate (NADPH) oxidases (NOX), xanthine oxidase, lipoxygenase and cytochrome P450 mono-oxygenases [13, 15]. Particular attention has focused on the members of the NOX/DUOX family of NADPH oxidases since these enzymes mediate physiological functions such as host defense, cell signaling, and thyroid hormone biosynthesis through the generation of ROS, including superoxide anion and hydrogen peroxide [16]. However, it is becoming increasingly apparent that alterations in diabetes-driven cellular ROS arise not only as a consequence of overproduction of ROS, but also as a result of ineffective removal by antioxidant defenses [17, 18].

The design and use of appropriate animal models to study diabetic complications has been examined in several reviews covering mouse models of type 1 and type 2 diabetes as well as related phenotypes such as obesity and insulin resistance [5, 19–22]. However, the development of experimental models that specifically delineate the cause and role of ROS in diabetic complications is now becoming a major research area, enabling both an understanding of the mechanisms of ROS-mediated damage as well as facilitating the design of targeted therapeutic approaches to limit diabetes-mediated ROS action. This chapter will focus on some of the latest oxidative stress-induced experimental models of diabetic complications with particular emphasis on cardiovascular disease, nephropathy, and diabetic cardiomyopathy.

2 Diabetes-Associated Atherosclerosis

Diabetes mellitus is a major pro-atherosclerosis risk factor, with a two- to fourfold higher incidence of cardiovascular disease in diabetic patients than in the general population [23]. Other risk factors include hyperglycemia, dyslipidemia, hypertension and obesity. However, these risk factors only partly explain the more advanced lesions [24] and increased incidence of cardiovascular disease observed in these patients. Understanding the underlying mechanisms that accelerate diabetes-associated atherosclerosis remains an important research area and various pathways have been implicated that include the biochemical process of advanced glycation [25] and the receptor for AGEs, RAGE [26]. In addition, various proteins that have been implicated in the atherosclerotic process per se have been shown to be upregulated in the diabetic condition. These include vascular cell adhesion molecule-1 (VCAM-1), monocyte chemoattractant protein-1 (MCP-1) and connective tissue growth factor (CTGF) [27].

Strong evidence now suggests that ROS derived from the hyperglycemia-driven increase in mitochondrial electron transport chain activity [9], glucose autoxidation [28] and enzymes such as NAD(P)H oxidase play a causal role in mediating many of the pro-atherogenic changes observed [29]. Indeed, ROS are known to upregulate a number of pro-atherogenic processes such as monocyte infiltration, platelet activation [30], smooth muscle cell migration [31], cell adhesion [32], release of CTGF [33] and increased production of AGEs [28]. Specifically, the accumulation

of oxidized low-density lipoproteins (oxLDL) in vessel walls is an early initiator of atherosclerotic events [34]. In turn, oxLDL is atherogenic for several reasons, namely (1) it is chemotactic for circulating monocytes, the cellular precursors of arterial macrophages, (2) it is responsible for inhibiting the migration of macrophages from the aortic wall, thus trapping them inside the lesion and (3) it is directly cytotoxic to endothelial cells, thus aiding in the erosion of the endothelial surface and promoting thrombosis [35–37]. Furthermore, macrophages preferentially take up oxLDL, thereby promoting atherosclerosis [38], while the oxidative bursts of macrophages and neutrophils recruited to inflammatory sites further compound the oxidative insult [37]. Importantly, a heightened state of oxidative stress has been observed in diabetic patients [39].

Several laboratories, including ours, have focused on the role of antioxidant defense in regulating the flow of ROS during the atherogenic process. Initial studies focused on the levels of the various antioxidant enzymes known to play a role in ROS removal. One study showed that migrating smooth muscle cells and macrophages in atheromatous plaques express these enzymes intensively [40], while a different study assessed antioxidant levels in ApoE−/− mice prior to and during visible aortic lesion formation [41]. In that study, there was a coordinated increase in a wide range of antioxidant enzymes prior to visible atherogenic changes, while the expression of many antioxidant enzymes decreased during the period of lesion formation. This led these authors to suggest that the induction of antioxidant activities partially prevents the progression of atherogenesis, while the subsequent decline in antioxidant capacity may contribute to lesion formation. It is now increasingly recognized that knowledge of antioxidant enzyme involvement in limiting oxidative processes may delineate where potential therapeutic targets exist to reduce non-diabetes and diabetes-associated atherosclerosis. Indeed, overexpression of the antioxidant enzyme catalase, which removes hydrogen peroxide, reduced the severity of lesions in ApoE-deficient mice [42].

Recent attention has focused on the most abundant isoform of the glutathione peroxidase (GPx) family, glutathione peroxidase-1 (GPx1), based on a number of important clinical observations that strongly support a major role for GPx1 in limiting atherosclerosis. Blakenberg et al. [43] first described a patient cohort where blood GPx1 activity was the strongest predictor of cardiovascular disease risk, with an inverse association between GPx1 activity and cardiovascular events. Based on this data, these authors suggested assessment of GPx1 for prognostic value in addition to that of traditional risk factors. Furthermore, they suggested that increasing GPx1 activity might lower the risk of cardiovascular events. Schnabel et al. [44] demonstrated that plasma homocysteine (HCys) was related to future cardiovascular events in a patient cohort with coronary artery disease and that this occurred mainly in patients with low erythrocyte GPx1 activity. These authors proposed that HCys elicits its cardiovascular effect by directly affecting GPx1 activity. Winter et al. [45] found a significant association between a polymorphism in the human GPx1 gene and the risk of coronary artery disease, while Hamanishi et al. [46] found additional GPx1 polymorphisms within a diabetic population that correlated with reduced GPx1 activity and an increased risk of atherosclerosis. Recently, Nemoto et al. [47]

reported that the presence of a Pro197Leu substitution within the GPx-1 gene may play a crucial role in determining genetic susceptibility to coronary arteriosclerosis in type 2 diabetic patients since this polymorphism was associated with increased coronary artery calcification as assessed by multi-slice computed tomography.

Although in vitro and clinical data are highly supportive of a role for ROS in underpinning diabetes-associated atherosclerosis, it is only through interrogation of appropriate animal models that a true picture of the role of ROS and antioxidant defense is revealed.

2.1 Experimental Models of Diabetes-Associated Atherosclerosis with an Emphasis on Oxidative Stress

2.1.1 The GPx1 Knockout Mouse

Glutathione Peroxidase-1 and Its Role in the Antioxidant Pathway

Glutathione peroxidase-1 is a ubiquitously expressed antioxidant enzyme present in the cytosol and mitochondria of all living cells. One of its major functions is the second-step detoxification of hydrogen peroxide within the antioxidant pathway. A build-up of hydrogen peroxide and its subsequent non-enzymatic conversion to noxious hydroxyl radicals is prevented by the rapid interaction of GPx1 with its substrate, H_2O_2, and its co-factor, reduced glutathione (GSH). GPx1 is also involved in the removal of lipid peroxides [48] and it acts as a peroxynitrite reductase in the reduction of potentially damaging peroxynitrite radicals [49] (Fig. 1). In the absence of this antioxidant enzyme, a build-up of ROS ensues that are known to damage DNA, proteins and lipids [49].

GPx1 knockout (–/–) mice, generated in our laboratory [50] and by others [51, 52], have become an excellent research tool with which to establish a role for ROS in the progression and promotion of oxidant stress-mediated pathogenesis. Furthermore they have allowed us to draw meaningful conclusions about the protective role of this isoform of the GPx family of antioxidant enzymes, since standard assays do not discriminate between the different isoforms. In addition, most studies investigating the role of the GPxs do so by limiting selenium intake, which results in non-specific reductions in selenium-dependent enzymes [53], including all the selenium-dependent isoforms of GPx. The GPx1 knockout model also facilitates the distinction between the contributions of Gpx1, catalase (a peroxisomal H_2O_2 metabolizing enzyme) and thioredoxin peroxidase in the peroxidation of H_2O_2 to water.

Our initial studies using this experimental model of oxidative stress showed an important role for GPx1 in the protection against ischemic-reperfusion mediated stroke [54]. In this instance, Gpx1–/– mice that were subjected to focal cerebral ischemia for 2 h via occlusion of the mid-cerebral artery showed significantly elevated lipid hydroperoxide levels compared with stroked control brains as well as

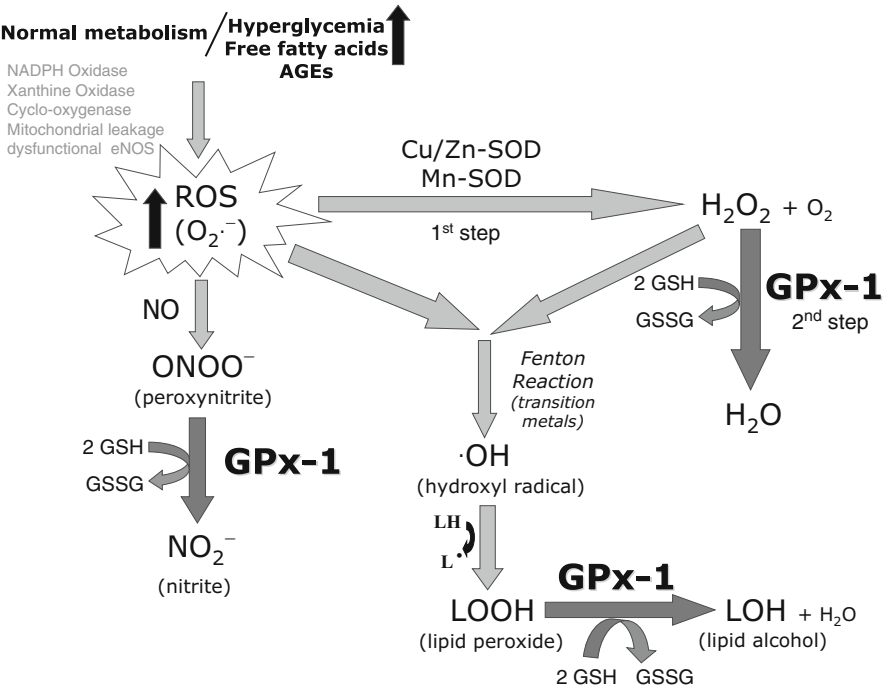

Fig. 1 Removal of ROS by GPx1. The generation of superoxide radicals ($^{-}O_2^{\cdot}$) is greatly enhanced in the diabetic milieu via enzymes such as NADPH oxidase. $^{-}O_2^{\cdot}$ is neutralized to water via a two-step process involving superoxide dismutase (SOD) in a first step, and glutathione peroxidase-1 (GPx1) or catalase in a second step. An imbalance in this pathway favors the build-up of hydrogen peroxide (H_2O_2). Fenton-type reactions occur when H_2O_2 or $^{-}O_2^{\cdot}$ interact with transition metals such as iron (Fe^{2+}), resulting in the production of noxious hydroxyl radicals (OH). These radicals initiate peroxidative damage to lipids, forming lipid hydroperoxides (LOOH). The functional importance of GPx1 rests in its ability to remove both hydrogen peroxide and lipid peroxides and neutralize these to water and lipid alcohol (LOH) respectively. In addition, GPx1 removes peroxynitrite radicals that form as a result of the interaction of $^{-}O_2^{\cdot}$ with nitric oxide (NO). Two reduced glutathione (GSH) are consumed each time GPx1 reduces ROS, generating oxidized glutathione (GSSG)

accelerated caspase-3 activation and apoptosis of neuronal cells. Similar results were obtained in GPx1−/− mice subjected to cold-induced cerebral damage as a model of head trauma [55].

Given the strong evidence for a role for ROS in the initiation and progression of atherosclerosis, we felt that the GPx1−/− mouse model was ideally suited to studying the consequences of a lack of GPx1 on pro-atherogenic processes associated with diabetes. However, in order to understand the role of GPx1 in diabetes-associated atherosclerosis, it was important to first consider the contribution of GPx1 to pro-atherogenic processes not limited to the diabetic milieu. An underlying assumption would then be that the diabetic milieu, with its highly pro-oxidant environment, would facilitate even greater responses than those seen in a non-diabetic environment.

GPx1–/– Mice Fed High Fat Diets as a Model to Study Pro-atherogenic Mechanisms

Our initial studies were performed in control mice and Gpx1–/– mice fed high fat diets (15% fat and 1% cholesterol) for 20 weeks on a C57Bl/J6 background [56]. In these animals, our biochemical analysis confirmed increased uptake of cholesterol into the vasculature of control and Gpx1–/– mice. Despite increases in non-enzymatic antioxidants after HFD-feeding (α-TOH in both plasma and vasculature, and total CoQ levels in vasculature), a rise in α-tocopheryl quinone (α-TQ), a well established marker of oxidative stress, suggested enhanced oxidative events within the vasculature of Gpx1–/– mice. However, Gpx1–/– mice failed to show an increase in aortic root lesions, nor were peroxidative events increased in plasma or aortic wall lipids compared with that seen in control animals. These results suggested that increased oxidative events within vasculature did not translate into increased lipid peroxidative damage and did not influence lipid deposition within the aortic sinus region in this strain of mice.

However, the importance of GPx1 in the protection against atherosclerosis became apparent to us [57] and others [58] when the lack of GPx1 was coupled with a lack of apolipoprotein E (ApoE) in ApoE/GPx1 double-knockout (dKO) mice. ApoE–/– mice are now recommended as the murine model in which to study atherosclerosis, since these mice develop more extensive and more pathophysiologically relevant lesions throughout the aortic tree [59] that are not restricted to the aortic root as observed in high-fat-fed, non-genetically altered mice [60–62]. This allows for a more robust analysis of pathophysiologically relevant factors affecting atherosclerosis throughout the aortic tree. Furthermore, ApoE–/– mice develop atherosclerosis over a relatively short period of time as a consequence of their impaired clearance of plasma lipoproteins [63]. It should however be highlighted that LDL-R knockout mice are also a useful model in which to study pro-atherogenic processes [64], particularly since diabetes does not have as great a dyslipidemic effect in this strain of mice [19].

ApoE/GPx1 Double-Knockout Mouse Model

After the establishment of our ApoE/GPx1 dKO colony (on a C57Bl/J6 background), we initially investigated the effect of a lack of GPx1 on atherosclerosis during aging. Our data showed increased lesion formation in the aortic arch and sinus region of 6- and 12-month-old female ApoE/GPx1 dKO mice fed a regular diet (4% fat) compared with age- and sex-matched ApoE–/– controls (Fig. 2a, b). In addition, Torzewski et al. [58] provided evidence that a lack of GPx1 accelerated atherosclerosis in their ApoE/GPx1 dKO mice after high fat feeding. In their study, atherosclerotic lesions were significantly increased in female ApoE/GPx1 dKO mice placed on a Western-type diet for 24 weeks. Moreover, their lesions showed increased cellularity, with an increase in macrophage content in early lesions and an increase in smooth muscle cells in advanced lesions. Furthermore, a deficiency

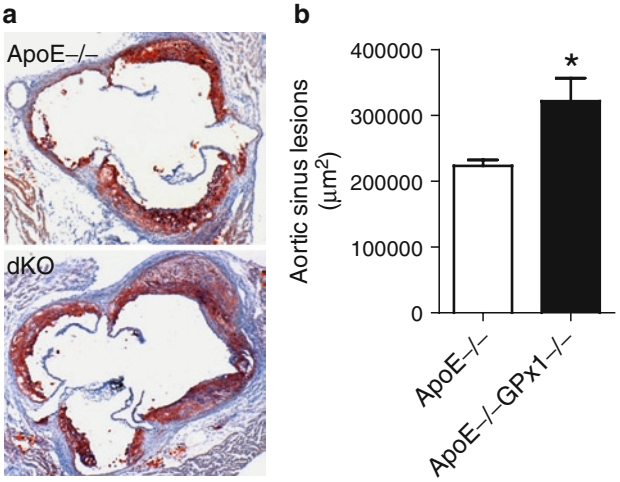

Fig. 2 Aortic sinus lesions, detected after staining with Oil Red O, of 6-month-old female ApoE-deficient and ApoE/GPx1 double-knockout (dKO) mice are shown in (**a**). Quantitation of lesions (**b**) show that sinus lesions are significantly increased in ApoE/GPx1 double-knockout mice compared with age and sex-matched ApoE-deficient controls. $n = 10$ mice/group. $^*P < 0.05$ vs. ApoE–/– aortas

of GPx1 led to an increase in ROS within the aortic wall, lower levels of bioactive nitric oxide and increased nitrotyrosine, a marker of peroxynitrite production, clearly demonstrating that oxidative stress is increased in this pro-atherogenic model. ApoE/GPx1 dKO peritoneal macrophages also showed increased in vitro proliferation in response to macrophage-colony-stimulating factor [58]. Collectively, these results, together with our results in aging mice, suggest that a deficiency of GPx1 accelerates and modifies atherosclerotic lesion progression in apolipoprotein E-deficient mice.

Diabetic ApoE/GPx1 dKO Mice as a Model of Accelerated Diabetes-Associated Atherosclerosis

Based on recent clinical studies that suggest a major protective role for GPx1 in diabetes-associated atherosclerosis [43, 44, 46], we induced diabetes in our ApoE/GPx1 dKO mice to determine whether this is merely an association or whether GPx1 has a direct effect on diabetes-associated atherosclerosis. ApoE-deficient and ApoE/GPx1 dKO mice were rendered diabetic using the diabetogenic agent streptozotocin (STZ). STZ destroys the pancreatic β-islet cells, thus providing a robust model of type 1 diabetes. Furthermore, the National Institutes of Health (NIH), in collaboration with the Juvenile Diabetes Research Foundation, established the Animal Models of Diabetic Complications Consortium (AMDCC),