[Frontiers in Bioscience S3, 709-729, January 1, 2011]

Targeted antioxidant therapies in hyperglycemia-mediated endothelial dysfunction

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1. ABSTRACT

Although intensive glycaemic and blood pressure control have reduced the risks of micro- and macrovascular complications, diabetes remains a major cause of cardiovascular events, end-stage renal failure, blindness and neuropathy. It is therefore imperative to understand the underlying mechanisms and to establish effective treatments to prevent, retard or reverse diabetic complications. One area of increased focus is the diabetic vascular endothelium. Hyperglycaemia triggers a cascade of events, not least an increase in reactive oxygen species (ROS) leading to enhanced oxidative stress, with its negative impact on endothelial function. In this review, we explore a unifying hypothesis that increased glucose-mediated ROS leads to endothelial dysfunction as the underpinning causative event triggering accelerated micro- and macrovascular complications. In particular, the consequences of deficiencies in the antioxidant enzyme, glutathione peroxidase, on endothelial dysfunction as a trigger of diabetic micro- and macrovascular complications, will be reviewed. Furthermore, novel antioxidant therapies will be highlighted. Specifically, use of Gpx1-mimetics holds promise as a targeted antioxidant approach and an alternative adjunct therapy to reduce diabetic complications.

2. INTRODUCTION

Diabetes mellitus is a metabolic disorder that occurs as a result of defects in insulin secretion and/or action and is characterised by chronic hyperglycaemia with disturbances in carbohydrate, fat and protein metabolism (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 which is associated with the metabolic syndrome, obesity and insulin-resistance, individuals with diabetes are at greater risk of developing microvascular and/or macrovascular complications (2, 3). Macrovascular disease in most instances, leads to atherosclerosis of the major vessels and/or stroke, while microvascular injury results in retinopathy, nephropathy and neuropathy. Hyperglycaemia is now well accepted as an independent risk factor for the development of cardiovascular disease (1, 4).

Vascular cells are particularly susceptible to fluctuations in glucose levels since glucose uptake into these cells is largely insulin-independent. Thus, large increases in glucose concentrations render vascular cells, and in particular endothelial cells, vulnerable to glucose-mediated injury. Indeed, endothelial dysfunction, as a result

of glucose-mediated injury, is postulated to accelerate atherosclerosis, and is an area of intense interest as a first line of defence against vascular complications (5, 6).

It is now becoming increasingly apparent that oxidative stress is an important underlying phenomenon that assists with the progression towards more severe and often fatal diabetic complications (7). 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 (2, 8). Several pathways, including the polyol pathway (9), increases in advanced glycation end products (AGEs) (10), activation of protein kinase C (11) and increases in hexosamine flux, have been identified where hyperglycaemia triggers increased ROS production that in turn may initiate, progress or amplify end-organ damage in diabetes (2). 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 (12, 13), with the rate of production varying greatly depending on the environment and/or disease state (13). Other potential sources of ROS production include nitric oxide synthase (NOS), nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (Nox), xanthine oxidase, lipoxygenase and cytochrome P450 mono-oxygenase (12, 14). Animal models generally support the concept that proatherogenic states such as hypertension, diabetes and hypercholesterolemia are associated with increased expression and activity of Nox2 (and possibly Nox1)-containing NADPH oxidases within the vessel wall (15). Specifically, ROS production is known to increase after exposure to high glucose in endothelial cells (16).

Oxidative stress is mainly caused by an imbalance between the activity of endogenous pro-oxidative enzymes (such as NADPH oxidase, xanthine oxidase or the mitochondrial respiratory chain) and antioxidant enzymes (such as superoxide dismutase, glutathione peroxidase, heme oxygenase, thioredoxin peroxidase/peroxiredoxin, catalase and paraoxonase) (17). Since the role of oxidative stress on endothelial function and dysfunction has recently been extensively reviewed by Thomas *et al.* (15), the current review, although drawing on the knowledge gained in a non-diabetic milieu, will focus on hyperglycaemia-driven endothelial dysfunction and its consequences on diabetic micro- and macrovascular complications. The term endothelial dysfunction refers to the loss of a range of normal homeostatic functions of the endothelium such as vasodilation, inhibition of platelet aggregation and leukocyte adhesion (15). This review will not only critically evaluate the role of various endogenous antioxidant defences in limiting diabetes-associated oxidative stress, but will also assess various novel antioxidant strategies being tested pre-clinically to reduce diabetes-associated oxidative stress with the aim to lessen, ameliorate or prevent diabetic complications. In particular, this review will highlight the usefulness of the diabetic atherosclerosis-prone Glutathione peroxidase-1 (Gpx1)/ApoE double knockout mouse, a model that combines oxidative stress, hyperglycaemia and hyperlipidemia to assess the role of oxidative stress in micro- and macrovascular disease. This model is proving useful in the delineation of the types of ROS and their *modus operandi* in these diabetic complications, and is facilitating both an understanding of the mechanisms leading to ROS-mediated damage, as well as allowing the design of targeted therapeutic approaches to limit oxidative damage.

3. OXIDATIVE STRESS AND DIABETES-MEDIATED ENDOTHELIAL DYSFUNCTION

3.1. The vascular endothelium and vascular tone

Vascular endothelial cells line the vessels of the circulatory system, providing a layer that separates the blood from the other layers of the blood vessel. The endothelium plays an important role in the vascular milieu by regulating vascular tone modulators (the vasodilators and vasoconstrictors), inflammation, the growth and differentiation of vascular smooth muscle cells and the maintenance of hemostasis after injury (18). Endothelial cells produce endothelial derived relaxing factor (EDRF) that include endothelium-derived hyperpolarising factor (EDHF), prostaglandins, nitric oxide (NO) and hydrogen sulphide (19), the latter being added as a recent potential candidate. The best characterized is NO, an important contributor to vascular tone and the preservation of endothelial integrity. In addition, NO also inhibits smooth muscle cell migration and proliferation and acts as an antioxidant. These attributes contribute to NO being an integral component of healthy vessels and making NO bioavailability the subject of intense investigation for the maintenance of healthy vascular tone (20). Endothelial nitric oxide synthase (eNOS), the critical enzyme responsible for the conversion of L-arginine to L-citrulline and NO, is tightly regulated under physiological conditions requiring the co-factors (6R)-5,6,7,8-tetrahydrobiopterin (BH4) and NADPH (15). NO bioavailability is reduced either by decreased formation or by enhanced removal of NO. Evidence points most strongly to the loss of NO with prevention of NO reaching its molecular target, rather than its attenuated production as being critical for NO bioavailability. An increase in ROS within the endothelial cell is one of the most significant factors known to decrease NO.

3.2. The role of reactive oxygen species and NADPH Oxidase in diabetic vascular tissue

An understanding of the role of ROS in atherogenic processes *per se* is assisting with investigations into the effects of increased ROS in the diabetic vasculature. It is now well accepted that increased ROS such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and peroxynitrite (ONOO-), occur in cardiovascular tissue, and are particularly associated with a pro-inflammatory state (1), the occurrence of atherosclerotic lesions (21-23) and pro-atherogenic thrombus formation (24). The level of ROS is determined by numerous factors such as the extent of mitochondrial oxidation, the activity and cellular location of ROS generating enzymes, as well as the presence of

ROS-removing antioxidants. Mitochondrial oxidation is driven by fatty acid oxidation, which is particularly enhanced in obesity-driven diabetic complications (25).

Much attention has focused on the NADPH oxidases as a major source of vascular superoxide (26). NADPH oxidase, first identified in leukocytes, is composed of several subunits ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, $p22^{phox}$, $gp91^{phox}$). It is a membrane-associated enzyme that catalyses the 1-electron reduction of oxygen using NADPH or NADH as the electron donor to generate the superoxide anion. Besides $gp91^{phox}$, also known as Nox2, vascular cells contain isoforms of Nox such as Nox1, Nox4 and Nox5 (27). Furthermore, the regulatory subunits p22phox, p47phox, Noxa1 and p67phox are also present in the vasculature. Stressors such as hormones, vasoactive agents, and cytokines control the expression and activity of these enzymes (28). Several reports have shown that expression of some of the NADPH oxidase subunit proteins ($p47^{phox}$, $p67^{phox}$, $p22^{phox}$) is upregulated in the aortas of diabetic rodent models (29, 30). Indeed, evidence suggests that high glucose stimulates ROS production via PKC-dependent activation of the NADPH oxidases in vascular and renal mesangial cells (31).

Vascular endothelial cells (VEC) are known to protect their intracellular environment against an increased influx of glucose in a hyperglycaemic environment by reducing the expression and plasma membrane abundance of their glucose transporter-1 (GLUT-1). Cohen *et al.* (32) investigated whether glucose-derived free radicals induce this down-regulatory mechanism in VEC, but discovered that pro-oxidants significantly increased the expression and abundance of GLUT-1 and the rate of glucose transport in VEC. Indeed, the resulting uncontrolled influx of glucose followed by the overproduction of glucose-derived ROS further up-regulated the rate of glucose transport. These authors hypothesized that the perpetuating glycoxidative stress finally leads to the collapse of the auto-regulatory protective mechanism and accelerates the development of a dysfunctional endothelium in blood vessels exposed to high glucose (32).

3.2.1. Consequences of increased superoxide: reductions in NO bioavailability

The major risk factors for diabetic complications such as hypertension, hypercholesterolemia and atherosclerosis are all associated with increased steady-state flux of $O_2^{-\cdot}$ (33). A major consequence of increased superoxide within the vasculature is its propensity to react with NO. This leads to diminished NO bioavailability with major implications for vascular tone such as impaired endothelium-dependent relaxation and vascular injury (33). Furthermore increased $O_2^{-\cdot}$, via enzymatic antioxidant conversion (see Fig.1) gives rise to increased levels of H_2O_2, an important ROS implicated in pro-inflammatory processes that are further amplified as diabetes develops (34). In particular, endothelial derived H_2O_2 has been shown to upregulate VCAM-1, an important adhesion molecule that aids in the migration of leukocytes from the blood into the tissue (35).

3.2.2. Consequences of increased peroxynitrite: implications for vascular function and inflammation

The interaction of NO with $O_2^{-\cdot}$ produces toxic peroxynitrite radicals that react with a variety of biological macromolecules (36). Peroxynitrite is a potent trigger of oxidative protein, lipid and DNA damage, including DNA strand breakage and base modification. Vascular endothelial dysfunction is a complex phenomenon that might be caused by a deficiency of NO and an overproduction of ONOO-. Indeed, a perturbed [NO]/[ONOO-] balance is seen by some as central to endothelial dysfunction, and targeting by statins is effective in restoring this balance (37). In addition, peroxynitrite also plays a significant role in vascular inflammation (38). Indeed, treatment with peroxynitrite decomposition catalysts, which selectively inhibit peroxynitrite, prevents *in vivo* tissue injury and inflammation (39). Importantly, the increase in cellular glucose together with the increase in peroxynitrite, triggers the uncoupling of eNOS due to reduced substrate availability, to form a dysfunctional superoxide-generating enzyme that contributes further to the oxidative stress (40). Elevated glucose also mediates an upregulation of cyclooxygenase-2, which together with dysfunctional eNOS, additionally reduce NO bioavailability (40). Furthermore, H_2O_2 and ONOO- have been implicated in the post-translational modifications of proteins, a process integrally linked with the control of many biological processes through the activation, inactivation, or gain-of-function of the modified proteins (41, 42).

3.3. A role for antioxidant defence

Eukaryotic cells have evolved an extensive array of antioxidant defences to regulate the flux of ROS and to limit oxidative damage (43). Antioxidant enzymes found to play a protective role in the vasculature include the superoxide dismutases, glutathione peroxidases, catalase, heme oxygenase, thioredoxin peroxidase, glucose-6-phosphate dehydrogenase and paraoxonase (17). Evidence suggests that glucose alters antioxidant defences in endothelial cells (44) and in patients with diabetic complications such as diabetic nephropathy (45, 46). Indeed, fibroblasts derived from type 1 diabetic patients susceptible to microvascular complications, were unable to upregulate their protective antioxidative defences after exposure to high glucose compared with skin fibroblasts from normal subjects, demonstrating a failure of the antioxidant defensive mechanism in diabetic patients with nephropathy (44). Blood vessels *per se* are known to express antioxidant enzymes to counteract oxidant stress (47). However, reductions in antioxidant defence or failure to respond to increased oxidative stress, leading to increased ROS accumulation, can elicit pathophysiological consequences (48). For example, recent evidence from ApoE-deficient mice suggest that the levels of several antioxidant enzymes decline during atherogenesis (49), implying a link between reduced antioxidant capacity and increased macrovascular lesion formation. This review will now focus on the role of the major antioxidants of the antioxidative pathway (see Figure 1) and their potential role in the protection against diabetes-mediated oxidative stress.

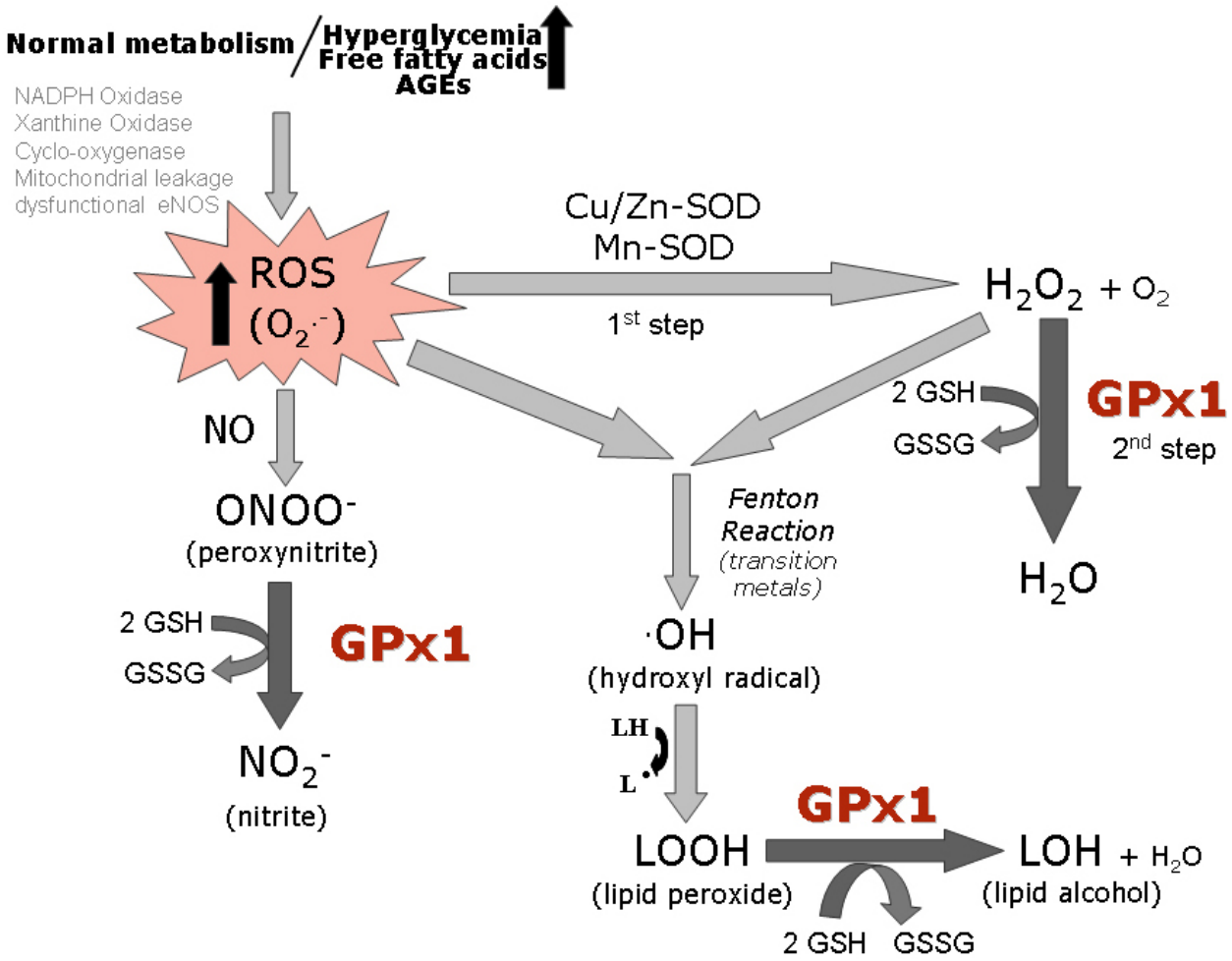

Figure 1. Removal of ROS by GPx1. The generation of superoxide radicals ($O_2^{\cdot-}$) is greatly enhanced in the diabetic milieu via enzymes such as NADPH oxidase. $O_2^{\cdot-}$ is neutralised to water via a two-step process involving superoxide dismutase (SOD) in a first step, and glutathione peroxidase-1 (GPx1) or catalase in a second step. An imbalance in this pathway favours 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 ($OH^{\cdot}$). These radicals initiate peroxidative damage to lipids, forming lipid hydroperoxides (LOOH). The functional importance of GPx1 resides in its ability to remove both hydrogen peroxide and lipid peroxides and neutralise 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 oxidised glutathione (GSSG).

3.3.1. Superoxide dismutase

The superoxide dismutase family of enzymes catalyse the dismutation of superoxide into oxygen and hydrogen peroxide in the first step of the antioxidant pathway (see Figure 1), thereby performing an important antioxidant function in the removal of superoxide. Three isoforms exist in humans, SOD1, SOD2 and SOD3 with distinct cellular localizations, namely cytosolic, mitochondrial and extracellular respectively. Mice with a Sod1 gene deletion are more susceptible to reperfusion injury in a model of myocardial ischemia, suggesting that the Sod1 gene constitutes an important antioxidant defense for the heart (50). SOD3 appears to play an important protective role in the cardiovascular and renal systems. Jung *et al.* (51) recently showed that inactivation of extracellular SOD3 contributes to the development of hypertension via a decrease in vascular NO levels, while ablation of Sod3 in male knockout mice significantly reduced renal blood flow and increased renal injury suggestive of an important protective role of Sod3 in renal ischemia/reperfusion injury, and in keeping with its high level of expression in renal tissue (52).

Diabetes is associated with reduced Sod activity in most animal studies (53-55), with reductions reported in serum and urine of STZ-treated Sprague-Dawley rats (56), and specific reductions in SOD1 and SOD3 suggested to play a key role in the pathogenesis of diabetic nephropathy (54). Indeed, overexpression of mitochondrial-specific Sod targeted to the endothelium prevented diabetic retinopathy in eMnSOD-Tg mice via a mechanism that involved downregulation of VEGF (57). Further strong evidence that

reductions in Sod accelerate diabetic complications came from a study of diabetic renal injury in Sod1 knockout mice (58). After 5 months of diabetes, these authors noted significant mesangial matrix expansion, renal cortical malondialdehyde content, and severe tubulointerstitial injury compared with controls, effects that were reduced in the presence of the Sod-mimetic, tempol (58).

Human studies looking at SOD levels are more variable. In type 2 diabetic patient studies, the level of serum extracellular SOD3 was significantly increased compared with non-diabetic subjects and correlated with the severity of micro- and macrovascular disease (59). It should however be remembered that elevations in SOD levels could, via the antioxidant pathway (see Figure 1), lead to increased hydrogen peroxide, with subsequent downstream effects due to these elevated ROS. These authors suggest that serum SOD3 levels may be a marker of vascular injury, possibly reflecting hyperglycaemia-induced oxidative injury to the vascular endothelium. In addition, polymorphisms within the SOD family of genes have been investigated to establish a link between SOD and vascular injury. Investigations into the genetic variability of the SOD3 gene have shown that the frequency and number of subjects with a Thr allele (Ala/Thr+Thr/Thr) in the Ala40Thr polymorphism was significantly higher in Japanese type 2 diabetic patients than in non-diabetic Japanese controls (60). Furthermore, patients with a Thr allele showed earlier age at diagnosis of diabetes, higher prevalence of hypertension and lower insulin sensitivity than those without the allele (60). These results suggest that a particular genetic variant of the SOD3 gene is associated with insulin resistance and the susceptibility to type 2 diabetes. Others report that serum SOD activity was significantly decreased in both type 1 and type 2 diabetic patients compared to control subjects and that SOD1 and SOD2 polymorphisms detected in these patients may have affected their SOD activity (61). However, this area remains controversial with other patient studies reporting no association between antioxidant polymorphisms and macro- or microangiopathy (62), as well as no significant difference in SOD activity compared to controls (63).

3.3.2. Catalase

Catalase is present mainly in the peroxisomes of mammalian cells as a tetrameric enzyme of four identically arranged subunits, each containing a heme group and NADPH at its active centre. The enzyme decomposes hydrogen peroxide to water and oxygen. Depending on the concentration of H_2O_2, catalase either acts catalytically, i.e. removes H_2O_2 by forming H_2O and O_2, or at a low concentration of H_2O_2, catalase acts peroxidically, removing H_2O_2 but oxidizing its substrate (64). Over-expression of catalase reduced the severity of lesions in ApoE-deficient mice (65), implying a role for hydrogen peroxide-scavenging enzymes in atherosclerotic processes. Interestingly, most studies show that the onset and progression of diabetes is accompanied by reductions in catalase activity (66-69), although some studies show increased activity (70, 71). Importantly, catalase gene mutations have been detected in association with diabetes mellitus and hypertension (72). Acatalasemia, an inherited yet mild deficiency of catalase (<10% of normal activity) is widespread and is associated with an increased risk of diabetes mellitus (72). Hypocatalasemia (~50% of normal activity) is associated with a greater risk of developing diabetes (73). In a Hungarian study of patients with both acatalasemia and hypocatalasemia, the incidence of diabetes was 12.7% compared with no occurance in normocatalasemic family members (74). Studies investigating whether mutations within the catalase gene predispose to diabetic complications are varied. Most studies show no association with gene polymorphisms and vascular complications of DM (61, 75). In particular, there was no association with the -262C/T polymorphism within the catalase gene and the development of diabetic retinopathy, diabetic nephropathy or ischemic heart disease in patients with type 2 diabetes (76).

3.3.3. Glutathione peroxidase

The glutathione peroxidase (GPx) family of enzymes play an important role in the cellular protection against oxidant stress by utilizing reduced glutathione (GSH) to reduce hydrogen and lipid peroxides to water and their corresponding alcohol respectively (43, 77). The majority of the isoforms are selenocysteine-containing proteins that differ in their cellular localisation, substrate specificity and tissue-specific functions (77). Glutathione peroxidase-1 (GPx1) is a major and ubiquitously expressed isoform present in the cytosol and mitochondria. It is involved in the second-step detoxification of hydrogen peroxide (see Figure 1) and lipid peroxides (43) and acts as a peroxynitrite reductase in the reduction of potentially damaging peroxynitrite radicals (78). In the absence of this antioxidant enzyme, a build-up of ROS ensues that are known to damage DNA, proteins and lipids (78). Clinical evidence now suggests a potential role for GPx1 in diabetes-associated atherogenesis (79, 80). Polymorphisms identified within the GPx1 gene resulting in reduced GPx1 activity, have been linked with increased intima-media thickness of carotid arteries and an increased risk of cardiovascular and peripheral vascular disease in type 2 diabetic patients (79). Furthermore, additional studies suggest a protective role for GPx1 in the atherogenic process *per se*. For example, reductions in red blood cell GPx1 activity were associated with an increased risk of cardiovascular events in a prospective cohort study (81,82), while atherosclerotic plaques of patients with carotid artery disease have reduced GPx1 activity (83). In animal studies, reduced GPx1 expression increased cell-mediated oxidation of low-density lipoproteins (84) and decreased the bioavailability of nitric oxide leading to endothelial dysfunction (85). These findings imply that GPx1 is a key enzyme for the protection of vessels against oxidative stress and atherogenesis, and that GPx1 may be of particular importance in the highly pro-oxidant diabetic environment.

3.3.3.1. The GPx1 knockout mouse: a model of increased oxidative stress

GPx1 knockout (-/-) mice, generated in our laboratory (86) and by others (87, 88), have become an excellent research tool with which to establish a role for ROS in the progression and promotion of oxidant stress-mediated injury. Furthermore studies in these mice 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 (89). 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 (90), 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.

3.3.3.2. GPx1-/- mice are more susceptible to endothelial dysfunction

Assessment of mesenteric vascular reactivity in control versus GPx1-/- mice after treatment with two endothelium-dependent vasodilatory compounds, namely β-methacholine and bradykinin, resulted in paradoxical arteriolar vasoconstriction in GPx1-/- arterioles compared with vasodilated control vessels (85). Superfusion of these vessels with sodium nitroprusside, an endothelium-independent vasodilator, resulted in dose-dependent arteriolar vasodilatation that was similar in both wild-type and Gpx1-/- arterioles. These results led Forgione *et al.* (85) to conclude that GPx1-/- mice have impaired endothelium-dependent vasodilation, yet intact endothelium-independent vasodilation, implying that a lack of GPx1 leads to a depletion of bioavailable NO. The observed endothelial dysfunction was accompanied by increased levels of oxidative stress, as assessed by increased levels of nitrotyrosine in the endothelial layer of the vessel wall and elevated levels of plasma isoprostanes. The arteriolar vasoconstriction in GPx1-/- arterioles could be reversed through the addition of OCT, a compound known to increase GSH, cysteine and glutathione levels in vascular tissue and plasma.

This marked impairment of endothelium-dependent relaxation was also detected in GPx1-/- carotid arteries (91). In addition, Chrissobolis *et al.* (91) showed that carotid arteries from GPx1-heterozygous mice are remarkably sensitive to angiotensin (Ang) II, suggesting that GPx1 haploinsufficiency greatly increases sensitivity to Ang II-induced endothelial dysfunction and emphasized the need for both copies of the GPx1 gene to protect blood vessels against Ang II-mediated endothelial dysfunction. Furthermore, over-expression of GPx1 protects against endothelial dysfunction, while treatment with PEG-catalase to remove H_2O_2 prevented the Ang II-mediated dysfunction (91). Collectively, these findings strongly suggest that Ang II mediates endothelial dysfunction via signalling pathways that involve ROS such as H_2O_2, and that GPx1 plays a major protective role against Ang II-mediated endothelial dysfunction, most likely through its antioxidant properties in the removal of H_2O_2.

3.3.3.3. Diabetic ApoE/GPx1 double knockout mice as a model of accelerated diabetes-associated-atherosclerosis

Based on the premise that hyperglycaemia accelerates atherosclerosis by induction of endothelial dysfunction (5), and recent clinical studies that suggest a major protective role for GPx1 in diabetes-associated atherosclerosis (79, 81, 92), we hypothesized that a lack of GPx1 in a pre-clinical model, would accelerate diabetes-associated atherosclerosis. Furthermore, we predicted that this would involve inflammatory pathways where ROS are known to play an important pro-atherogenic role. Such experiments would allow us to determine whether the clinical observations were merely associative or whether GPx1 has a direct effect on diabetes-associated atherosclerosis. In order to achieve these aims, we crossed our GPx1-/- mice with ApoE-deficient mice that were also on a C57/BL6 background (89). ApoE-deficient and ApoE/GPx1 double knockout (dKO) mice were then rendered diabetic using the diabetogenic agent streptozotocin (STZ). STZ destroys the pancreatic β-islet cells, thus providing a robust model of insulin deficient diabetes resembling type 1 diabetes (93).

Aortic lesion formation and atherogenic pathways were assessed after 10 and 20 weeks of diabetes. In our study, we showed that atherosclerotic lesions within the aortic sinus region, as well as lesions within the arch, thoracic, and abdominal region, were significantly increased in diabetic ApoE/GPx1 dKO aortas compared with diabetic ApoE-/- aortas (89). This increase in aortic lesion deposition was accompanied by increases in macrophages, alpha-smooth muscle actin, RAGE and various proinflammatory (VCAM-1 and MCP-1) and profibrotic markers such as vascular endothelial growth factor (VEGF) and CTGF (89). Quantitative reverse-transcription polymerase chain reaction analysis showed increased gene expression of RAGE, VCAM-1, VEGF and CTGF in diabetic dKO aortas compared with diabetic controls. In addition, nitrotyrosine levels, consistent with local enhanced ROS production, were significantly increased in diabetic ApoE/GPx1 dKO mouse aortas. These findings were observed despite upregulation of other antioxidants. Our results clearly showed that a lack of functional GPx1 accelerates diabetes-associated atherosclerosis via upregulation of proinflammatory and profibrotic pathways in ApoE-/- mice and established that GPx1 is an important antiatherogenic therapeutic target worthy of further study for patients with or at risk of diabetic macrovascular disease (89).

3.3.3.4. Diabetic ApoE/GPx1 dKO mice as an oxidative stress model of diabetic nephropathy

Endothelial dysfunction is closely associated with diabetic nephropathy (DN) in type 1 and type 2 diabetes, with DN considered a clear clinical expression of diabetic microangiopathy (94). Indeed, this microvascular complication is now the most common cause of chronic kidney disease (CKD) and accounts for up to 50% of renal cases (95). Up to one-third of diabetic patients develop end-stage kidney disease necessitating renal replacement therapy within 25 years of disease onset (95). Long term prospective and interventional studies have shown that the risk of developing kidney disease is directly related to the exposure to elevated blood glucose levels (96-98). Therefore, therapies aimed at reducing the toxic effects of high glucose may be beneficial in reducing DN in diabetic patients. Although recent clinical studies have shown that

intensive glucose control and blockade of the renin-angiotensin system reduce the risk and progression of DN (99, 100), such therapies have not fully eliminated DN in diabetic patients. Therefore, the development of other novel therapeutics that specifically target DN may be helpful for a significant proportion of subjects with diabetes.

There is accumulating evidence to suggest an important role for oxidative stress in DN (101). Oxidative stress has been linked with hyperglycaemia. Indeed, the increased oxidative stress as a consequence of the high glucose levels elicits vascular inflammation and alters gene expression of growth factors and cytokines within the kidney (102). In particular, high glucose induces intracellular ROS directly via auto-oxidation and glucose metabolism, and indirectly through the formation of AGEs that then bind to RAGE to promote upregulation of pro-oxidant enzymes (103). ROS have been shown to mimic the stimulatory effects of high glucose and upregulate transforming growth factor-beta 1 (TGF-beta1), plasminogen activator inhibitor-1 (PAI-1), and extracellular matrix (ECM) proteins in glomerular mesangial cells, leading to mesangial expansion (104). ROS also activate other signalling molecules such as MAP kinases and protein kinase C, as well as transcription factors such as nuclear factor-kappa B, activator protein-1 and specificity protein-1, leading to the upregulation of cytokines, growth factors and ECM proteins (105). Furthermore, oxidative stress may arise in this hyperglycaemic milieu as a result of reduced removal by antioxidant enzymes.

Diabetic patients have a reduced capacity to attenuate oxidative stress as a result of lowered antioxidant function (106-109). Under physiological conditions, the kidney expresses abundant amounts of the major antioxidants, superoxide dismutase-1 (SOD1) (110), GPx1 (86, 111) and catalase (112). However, under pathophysiological conditions, these enzymes decrease leaving the kidney vulnerable to the increased ROS known to accompany diabetes (113). Strategies that limit oxidative stress may prevent or lessen the development and progression of DN. The importance of the GPx family of enzymes in limiting DN has been shown in STZ-treated diabetic rats where a dietary deficiency of the essential trace element selenium (selenium is an integral part of the active site of the GPx enzymes) led to decreased GPx activity and enhanced DN (114). There are several GPx isoforms present in the kidney; however, GPx1 accounts for >96% of the renal GPx activity (86). Protection against oxidative stress is therefore most likely as a result of the function of the GPx1 isoform. However, to date, no study has directly linked GPx1 to the protection against DN. Our initial studies using diabetic C57Bl/J6 GPx1-/- mice, surprisingly failed to show accelerated DN (115). In this setting, the significance of a lack of GPx1 may not have been properly revealed since lipid levels are unaffected in this diabetic model. Indeed, elevated lipids may be critical in accelerating DN since clinical observations suggest that hyperlipidemia is an important contributory factor to the progression of diabetic renal disease (116, 117). Furthermore, Lassila *et al.* (118) have shown accelerated nephropathy in diabetic ApoE-/- mice. Therefore, mice with both ApoE and Gpx1 deficiencies may represent a more advanced model in which to study the consequences of a lack of GPx1.

In our studies using diabetic ApoE/GPx1 dKO mice, we demonstrate increased albuminuria which is associated with pathological changes that include mesangial expansion and up-regulation of pro-fibrotic (collagen I and III, fibronectin and TGF-beta) and pro-inflammatory mediators (VCAM-1 and MCP-1) in the diabetic ApoE/GPx1 dKO kidneys (119). Importantly, we show enhanced staining for the oxidative stress marker nitrotyrosine, an indicator of the amount of peroxynitrite damage, in diabetic ApoE/GPx1 dKO glomeruli and tubules of the kidney compared with diabetic ApoE-/- controls. This occurred against a background of elevated lipids, highlighting the involvement of both lipids and oxidative stress in the progression of diabetic nephropathy (119). Thus, we have established a role for GPx1 in limiting and/or preventing diabetic nephropathy in the pathophysiologically relevant milieu of increased lipids known to accompany diabetes (117-119).

In summary, our studies in the diabetic ApoE/GPX1 dKO mice have revealed accelerated diabetes-associated-atherosclerosis and increased diabetic nephropathy. Clearly the lack of GPx1 with its associated increased oxidative stress appears to play a pivotal role in mediating these hyperglycaemic-associated pathophysiological events. It is tempting to speculate that the development of endothelial cell dysfunction, which has been demonstrated in these mice (85, 91), represents a common pathophysiological pathway leading to both of these diabetic complications, namely renal disease and macrovascular complications (see Figure 2).

4. NOVEL ANTIOXIDANTS TO LIMIT DIABETIC MICRO- AND MACROVASCULAR DISEASE

Early studies have suggested that endothelial dysfunction may be therapeutically reversible (120). Indeed, in patients with diabetes, pharmacological intervention with hypolipidemic agents, insulin sensitizers, angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARB) as well as dietary and lifestyle modifications were found to improve flow-mediated endothelium-dependent vasodilation (121, 122). Currently, the design of drugs that specifically improve endothelial dysfunction is an achievable and desirable, yet mostly unmet need. Novel and improved antioxidant therapies may be an attractive alternative or adjunct therapy to reduce the undesirable effects of diabetes-induced increases in ROS.

Although results from basic research suggest a protective role for vitamins in the prevention of cardiovascular disease, evidence from clinical trials indicate no overall benefit for major cardiovascular events (123). In some trials the use of vitamins increased cardiovascular mortality. For example, the HOPE trial showed an increase in the incidence of congestive heart

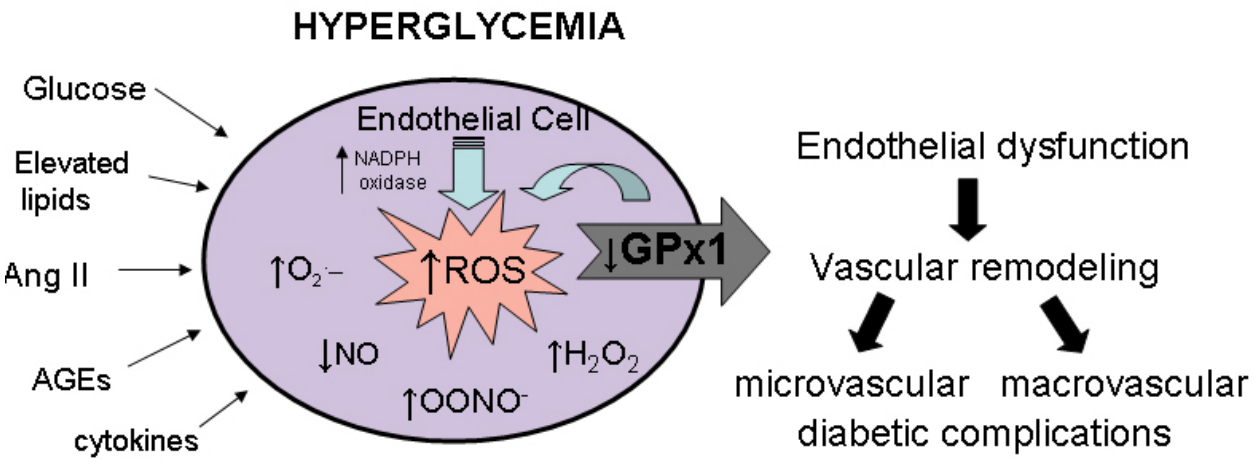

Figure 2. The hyperglycaemic milieu, which includes elevations in the levels of glucose, AGEs, oxidised lipids, angiotensin II, and cytokines, impacts on the ROS-producing machinery of the endothelial cell. Pro-oxidant enzymes such as NAPDH oxidase upregulate their production of superoxide ($O_2^{\cdot-}$) with detrimental downstream effects. Superoxide interacts with nitric oxide (NO) to produce peroxinitrite radicals ($ONOO^-$). This interaction depletes NO levels, reducing its bioavailability. $ONOO^-$ in turn is highly reactive resulting in oxidative damage to proteins. Furthermore, eNOS is uncoupled due to reduced substrate availability (L-arginine and BH_4) resulting in increased $O_2^{\cdot-}$ and H_2O_2 production. The antioxidant GPx1 normally removes excess ROS such as H_2O_2 and $ONOO^-$. Under diabetic conditions, where GPx1 activity is known to decrease, ROS such as H_2O_2 and $ONOO^-$ are not efficiently removed, leading to enhanced oxidative stress and exacerbating endothelial dysfunction. A compromised endothelium results in vascular remodelling triggering micro- and macrovascular complications, such as diabetic nephropathy and diabetes-associated-atherosclerosis respectively.

failure (CHF) in patients treated with vitamin E (124), while the use of vitamin E has also been reported to be contra-indicated in patients with left ventricular dysfunction (125). Failure of vitamins in the clinical setting may arise as a result of a lack of specificity in targeting the correct ROS and/or their cellular location. Indeed, Vitamins E and C that target radical or 1-electron oxidants may not have adequately removed critical ROS and ROS-generating pathways in major clinical trials, thus explaining their equivocal results (126). It is becoming increasingly clear that enzymatically generated non-radical or 2-electron oxidants such as H_2O_2 and peroxynitrite are more important (127), since these oxidants modulate redox-sensitive thiol and methionine residues in proteins (128). Indeed, protection of these often critical residues in enzymes and/or structural proteins prevents inactivation and/or destruction of vital protein function.

Several novel antioxidant approaches are being investigated to reduce ROS, and potentially offer promise as more effective therapeutic strategies than the administration of vitamins such as Vit E and C. One strategy involves preventing or limiting ROS production in the first instance. As already mentioned, the NADPH oxidases are one of the primary sources of ROS in the vasculature, and much interest has focused on ways to minimise ROS production without compromising the important role played by physiological ROS. Another is the use of SOD mimetics to remove superoxide radicals. A third strategy is to bolster the function of antioxidants such as GPx1 that target the removal of H_2O_2 and peroxynitrite. Indeed, our studies with ebselen, a GPx1 mimetic, highlight the potential effectiveness of this approach by demonstrating reduced diabetes-associated-atherosclerosis in our experimental models (23). Other strategies involve the targeting of key organelles such as the mitochondria, the attachment of antioxidant-conjugates to endothelial cells, the upregulation of the stress response transcription factor Nrf2 and antioxidant gene transfer therapy. This review will now evaluate the merits and potential pitfalls of these varied approaches to reduce cellular ROS.

4.1. Nox-inhibitors

Evidence now suggests that increased NADPH oxidase activity in tissues vulnerable to hyperglycaemia occurs downstream of the advanced glycation end products and protein kinase C pathways, two important mechanisms involved in the pathogenesis of diabetic complications (129). Suppression of NADPH oxidase activity may offer therapeutic benefits to ameliorate diabetic complications, in particular diabetic nephropathy where NOX involvement is now recognised to play an important role (102). Furthermore, upregulation of NOX is associated with upregulation of the endothelin pathway in the pathology of DN and it is now recognised that the dual endothelin receptor antagonist, CPU0213, attenuates DN by additionally suppressing NOX in male Sprague-Dawley rats made diabetic with STZ (130). A further postulate is that inhibition of Nox1 may efficiently suppress neointimal formation in the prevention of vascular complications associated with diabetes (131).

These observations have led to the development of several small-molecule and peptide inhibitors of the NOX enzymes. Indeed, some of these have shown promise

in experimental studies, but issues of specificity, potency, and toxicity argue against the use of any of the existing published compounds as candidates for drug development (132). One compound, apocynin, a proven NADPH oxidase inhibitor, has been used widely in animal models of oxidative tissue injury and has shown improvements in vascular and renal complications. Nam *et al.* (133) have recently evaluated the effects of apocynin on diabetic nephropathy in a type 2 diabetic rat model. Using aged apocynin-treated Otsuka Long Evans Tokushima Fatty (OLETF) rats, these authors demonstrate improvements in glomerular and mesangial expansion, significantly decreased glomerular VEGF expression and reductions in the oxidative stress markers, urinary 8-OHdG and MDA. However, it should be remembered that apocynin is non-selective in its mode of action since it also targets other enzymes such as Rho-kinase (134).

Other inhibitors of NADPH oxidase include the PI3K inhibitor wortmannin, diphenylene iodonium (DPI), and 4-(2-amino-ethyl)-benzenesulfonyl fluoride (AEBSF) (135). These compounds have limitations as therapeutic strategies due to their effects on multiple enzymes and other potential targets. Indeed, AEBSF, has been shown to be an irreversible serine protease inhibitor (136). Use of novel tat peptide inhibitors (132) and the Vas2870 compound (137) are other approaches being investigated. gp91 ds-tat is one of the most specific inhibitors developed to date. It is an 18-amino acid peptide that interferes with NADPH oxidase assembly and activation (138). In particular, this peptide mimics the binding region of NOX2, and possibly NOX1, a region known to interact with p47phox. In this manner, NADPH oxidase subunit assembly is prevented, resulting in the specific inhibition of superoxide production from NADPH oxidase and not from other oxidases such as xanthine oxidase. Promising results have been obtained using gp91ds-tat in reducing vascular ROS associated with AngII-mediated hypertension in mice (138) as well as reducing endothelial dysfunction and vascular ROS in the Dahl salt-sensitive rat model (139). However, the mode of administration of this peptide, which at present can only be via intravenous injection due to its limited bioavailability, is one major limitation of this strategy (132). Recently, through the use of a high-throughput screening assay specific for NADPH oxidase activity, VAS2870 was discovered (138) which attenuates *in vitro* PDGF-dependent smooth muscle cell chemotaxis via a mechanism that includes the complete abolition of NADPH oxidase activation and ROS production (137). Further testing in experimental models is necessary to determine if these approaches represent feasible therapeutic strategies for diabetic complications.

Since it may not be beneficial to block all NOX isoforms in specific disease states such as diabetes considering the important signalling role provided by some of the NOX isoforms and the role of NOX2 in the innate immune response, a more preferable strategy in the design of NOX-inhibitors would be to develop agents with isoform specificity, although broadly active NOX inhibitors may prove to be useful in some settings.

4.2. SOD mimetics

Since SOD is the first line of defence against physiological ROS, bolstering this aspect of the cellular armoury, may protect against increased pathological ROS. Indeed, Sod mimetics such as 4-hydroxytetramethyl-piperidine-1-oxyl (tempol), protects animals and mammalian cells from cytotoxicity induced by oxygen radicals such as H_2O_2 and superoxide (140). One attractive attribute of tempol is its ability to penetrate cell membranes and hence react with ROS both intracellularly and extracellularly as well as within important organelles such as the mitochondria (20). Numerous studies performed in different animal models lend support to the concept that removal of ROS improves agonist-induced endothelium-dependent vasodilation. For example, tempol improved acetylcholine and arachidonic acid relaxation in skeletal muscle arteries and in coronary arteries from diabetic animals (141, 142). Furthermore, albeit in a non-diabetic setting, in small arteries exposed to high blood pressure in an *in vivo* one-kidney one-clip hypertensive rat model, improved endothelial function was noted after treatment with tempol (143). Similarly, alpha-lipoic acid (ALA) and L-propionyl cartinine, two compounds with intracellular superoxide scavenging properties, have also shown protection against mitochondrial DNA damage and improved endothelial function via mechanisms that include reduced levels of ceramide, neutral sphingomyelinase activity and increased glutathione levels in endothelial cells (144). Of note, in a recent patient study, intravenous ALA treatment improved endothelium-dependent vasodilatation in patients with type 2 diabetes (145). Furthermore, use of a synthetic manganese containing Sod mimetic, M40403 reversed endothelial dysfunction in ApoE-/- mice aortas *ex vivo* by decreasing NADPH oxidase-dependent superoxide levels (146).

However, recent data suggest that *in situ*ations where tempol greatly increases the level of hydrogen peroxide, this ROS in turn limits the efficacy of Sod mimetic treatment. For example, in an experimental model of glomerulonephritis, Lu *et al.*, (147) unexpectedly observed that tempol exacerbated the progression of disease as evidenced by intensification of proteinuria, presence of severe crescentic glomerulonephritis with leukocyte influx and accelerated mortality in the treated group. Furthermore, tempol treatment raised SOD activity and H_2O_2 levels in urine, up-regulated p65-NFkB and osteopontin in the kidney. These authors attributed the worsening of the disease by tempol to the increase in H_2O_2 which is a potent NFkB activator and as such can intensify inflammation and renal injury.

Similarly, in a rat hypertensive model induced by the inhibition of renal medullary SOD with diethyldithiocarbamic acid, tempol could not prevent the development of hypertension (148). In this instance, the increased formation of H_2O_2 constricted the medullary vessels counteracting the vasodilator actions of tempol.

4.3. The GPx1-mimetic ebselen

Ebselen (2-phenyl-1, 2-benzisoselenazol-3[2H]-one) is a synthetic, lipid-soluble, non-toxic seleno-organic

compound (149) with anti-inflammatory and antioxidant activities (150). Ebselen has been widely reported to act against membrane hydroperoxides, including hydrogen peroxide and lipid peroxides due to its glutathione peroxidase mimetic activity (151-153). Indeed, ebselen inhibits hydrogen peroxide-induced cell death in various cell types, including HepG2 (154), human HL-60 (155), PC12 cells (156) and human umbilical vein endothelial cells (157). Ebselen also scavenges other ROS including peroxyl radicals and peroxynitrite (149). Ebselen also directly inhibits several enzymes involved in inflammatory processes. These include 5-lipoxygenases, NOS, NADPH oxidase, protein kinase C and ATPase (149). This ability of ebselen to alter enzyme function within pro-inflammatory pathways is of particular relevance to pathologies such as atherosclerosis where inflammation plays a key role.

4.3.1. Ebselen in an experimental model of diabetes-associated atherosclerosis

Results from our laboratory support a role for targeted antioxidant defence against diabetes-associated atherosclerosis (23). We postulated that ebselen would reduce oxidative stress, expression of pro-inflammatory mediators and activation of pro-atherogenic pathways in diabetic ApoE-/- mice based on the knowledge that ebselen has strong anti-inflammatory effects. Previous studies supported a role for ebselen in reducing various oxidative-stress mediated pathologies in experimental models, including cerebral infarction (158), the protection of the endothelium in stroke-prone hypertensive rats (159), and cardiac dysfunction in a murine model of chronic iron overload (160). Of relevance to the diabetic milieu, ebselen reduced oxidative damage of proteins and partially restored endothelial dysfunction in Zucker diabetic rats (161). Furthermore, arterial lesions were reduced by ebselen in a superoxide-driven non-inflammatory transgenic murine model, consistent with a potential role for ebselen in reducing atherosclerosis (162). In addition, in limited patient studies, ebselen improved acute ischemic stroke outcome (158) and delayed neurological deficits after aneurysmal subarachnoid hemorrhage, supporting a role for ebselen in reducing oxidant-mediated injury (163) and suggesting that ebselen may be a promising neuroprotective agent (158). However, prior to our study in ApoE-/- mice (23), no study had directly evaluated the effect of ebselen on diabetes-associated atherosclerosis.

In our study, 8-week old male C57Bl/J6 $ApoE^{-/-}$ mice were rendered diabetic with STZ and divided into ebselen-gavaged and non-gavaged groups. Ebselen was gavaged twice daily at 10mg/kg body weight starting at 10 weeks of age. Animals were maintained for 20 weeks of diabetes for gene expression, immunohistochemical analyses, *en face* aortic lesion analysis as well as an assessment of lesions within the aortic sinus region.

We were able to demonstrate that gavage of ebselen to diabetic ApoE-deficient mice attenuated lesion formation in most regions of the aorta including the arch, thoracic and abdominal aortic regions. This reduction in lesion formation was accompanied by a decrease in oxidative stress, as reflected by reduced nitrotyrosine levels as well as a reduction in expression of the Nox2 subunit of NADPH oxidase (23). Our results support the observations of Brodsky *et al.* (161) where ebselen decreased nitrotyrosine levels in Zucker diabetic rats, albeit that the Zucker rat is a model which is not associated with atherosclerosis. Our data therefore highlight the importance of ebselen in its role as a peroxynitrite reductase and strengthen the notion that ebselen functions in a similar fashion to GPx1 (78). Furthermore, the cellularity associated with a pro-atherosclerotic phenotype (increased alpha-smooth muscle actin positive cells and increased macrophage infiltration) was decreased by ebselen in association with a reduction in aortic expression of the pro-atherosclerotic mediators, RAGE and VEGF (23).

In addition, our study showed that ebselen elicits its anti-atherogenic effects in a site-specific manner since ebselen did not affect lesions within the aortic sinus (23). Our results support a growing list of studies where site-specific effects of modulators of atherosclerosis have been observed (164). For example, Witting *et al.* (165) showed that the lipid-lowering antioxidant probucol decreased lesion formation in most aortic regions but resulted in increased lesion formation within the aortic sinus. Indeed, local hemodynamic factors such as low shear stress, turbulence, oscillating flow and inherent properties of the vessel wall may have contributed to these differential effects (164, 165). However, importantly, our data showing a reduction in atherosclerotic plaque in most regions of the aorta after 20 weeks of diabetes, support the notion of Blankenberg *et al.* (81) that bolstering GPx-like activity reduces atherosclerosis, and is in agreement with our findings in diabetic ApoE/GPx1 dKO mice where lack of GPx1 greatly accelerated plaque deposition in the aorta of these mice (89). Our results therefore strongly suggest that ebselen is an effective anti-atherogenic agent against diabetic macrovascular disease. Given that ebselen has already been shown to improve endothelial dysfunction in the Zucker diabetic fat rat (161) and under hypertensive conditions (159), the evidence would suggest that the anti-atherogenic effects of ebselen observed in our STZ-model of diabetes-mediated atherosclerosis most likely were initiated at least partly through improved endothelial function.

4.3.2. Mechanistic understanding of the anti-atherogenic action of ebselen

To further understand the anti-atherogenic actions of ebselen observed in our diabetic experimental models, we have investigated the effect of ebselen on pro-atherogenic signalling pathways in human aortic endothelial cells (HAEC) (23). In these experiments, HAEC were pre-treated with 0.03 μM ebselen prior to exposure to 100 μM H_2O_2 for 30 min. Pre-treatment with ebselen reduced the H_2O_2-mediated increase in IkB-kinase (IKK) complex phosphorylation on critical activatory residues. Since IKK is a key regulator of NF-kB activation (166), it is predicted that by reducing IKK phosphorylation, ebselen anchors NF-kB in the cytoplasm thereby preventing the activation of pro-inflammatory genes. These studies therefore suggest that one of the key mechanisms whereby ebselen confers its anti-atherogenic effects is via

modulation of the transcription factor NF-kB. It is therefore likely that ebselen affects downstream cellular targets regulated by NF-kB. To explore this notion further, we investigated the effects of ebselen on Nox2 expression in HAEC, since Nox2 is known to be regulated by NF-kB (167) and in our diabetic ApoE-/- animal model, ebselen was associated with decreased Nox2 gene and protein expression. Our *in vitro* data showed reduced Nox2 protein levels after ebselen treatment and supports the view that ebselen affects downstream targets of NF-kB.

The cytokine TNF-alpha is an important diabetes-associated pro-inflammatory mediator and is involved in the activation of NF-kB (168). We therefore explored the consequences of ebselen pre-treatment on the expression TNF-alpha in HAEC. Our *in vitro* data showed that the H_2O_2-induced upregulation of TNF-alpha was reduced by ebselen. Our results in HAEC support growing evidence that ebselen inhibits TNF-alpha induced pro-inflammatory responses in endothelial cells (156) and other cell types (169, 170). We also investigated the effects of ebselen on H_2O_2-mediated phosphorylation of c-Jun-N-terminal kinase (JNK), a kinase involved in the activation of the transcription factor AP-1. We felt this to be particularly relevant since several laboratories have suggested a role for JNK in TNF-alpha mediated endothelial cell activation (171), in particular through the interactions of AP-1 with NF-kB (172). Furthermore, others have suggested that the effect of ebselen on JNK may be cell type specific (157, 173). Our *in vitro* analysis has indeed shown that ebselen effectively reduced the H_2O_2-mediated phosphorylation of c-Jun-N-terminal. Collectively, our results with ebselen have implications not only for inflammatory genes known to be regulated by these pathways (1), but also on the pro-atherosclerotic pathway itself, since inflammatory events are integrally linked with the development and progression of atherosclerosis.

Although our data are strongly supportive of the use of ebselen in the treatment and/or prevention of diabetic complications, it is important that further tests establish its translational potential to the human context. One of the major benefits of ebselen and this class of compound is its relative stability even when thiol cofactors are depleted (174, 175). This lessens its toxicity when compared with other selenium containing compounds that release their selenium under conditions where thiol cofactors are reduced (175). Novel Gpx-like compounds, synthesized for their greater solubility and efficacy than ebselen are now available, and are currently a major focus of our laboratory, in particular in assessing pre-clinically their anti-atherogenic potential. This approach and specifically the screening of more efficient ebselen analogues, has already shown their greater protection against mitochondrial oxidative damage and cell death in fibroblasts derived from Friedreich ataxia patients (176). Indeed, in these studies, ebselen analogues were several-hundred fold more effective at preventing cell death than ebselen (176).

4.4. Mitochondrially-targeted antioxidants

During oxidative respiration, free radicals produced by the mitochondria are known to play an important role in the physiology of a cell. However, under certain circumstances these ROS contribute to the pathophysiology of a cell and have been implicated in various pathologies that include ischemia-reperfusion injury, diabetes, atherosclerosis, endothelial dysfunction and cardiovascular diseases. Strategies are being developed to effectively remove or reduce these pathogenic ROS and these include the targeted delivery of antioxidants to the mitochondria (177-179). Indeed, one explanation for the failure of antioxidants to produce beneficial effects in clinical trials is their lack of accumulation within the mitochondria. Recent advances in mitochondrially-targeted antioxidants have concentrated on those that specifically target the matrix-facing surface of the inner mitochondrial membrane and therefore protect against mitochondrial oxidative damage (180). In particular, antioxidants conjugated with a lipophilic triphenylphosphonium cation such as mitoquinone, mitovitamin E and mitophenyltertbutyline accumulate in the mitochondrial matrix at concentrations several-fold greater than cytosolic non-mitochondrially targeted antioxidants because of the high negative membrane potential of the inner mitochondrial membrane (181, 182). Indeed, several studies in mice fed mitochondrially-targeted antioxidant compounds for several weeks show accumulation of these compounds in the mitochondria of various tissues including brain, heart, liver and kidney (183). *In vitro* experiments using both mitoquinone and mitovitamin E have shown promising reductions in peroxide-mediated oxidant stress and apoptosis whilst maintaining proteasomal function in bovine aortic endothelial cells. Such studies provide evidence of a potential role for mitochondrially targeted antioxidants in the protection against oxidative stress-mediated endothelial cell dysfunction (178).

The specific targeting of the antioxidant enzymes and their mimetics to the inner mitochondrial space may provide an alternative approach to increase antioxidant defences in this cellular compartment. This technique recently reduced cardiomyopathy in transgenic mice expressing mitochondrially-targeted catalase. In their study, Kohler *et al.* (184) attenuated cardiac mitochondrial oxidative stress and left ventricular dysfunction after antiretroviral-induced cardiomyopathy in transgenic mice expressing mitochondrially-targeted catalase. Furthermore, a mitochondrially-targeted form of the GPx1-mimetic ebselen, MitoPeroxidase, has been synthesized which contains ebselen covalently linked to a triphenylphosphonium cation. MitoPeroxidase decreases glucose or H_2O_2-mediated apoptosis in cell lines. However, this effect was only slightly more effective than the parent compound, ebselen, in *in vitro* assays (185).

The effectiveness of these mitochondrially-targeted antioxidants still needs further rigorous testing in experimental models of diabetes before clinical studies are conducted to evaluate their effectiveness in humans.

Finally, another targeted antioxidant approach involves the use of antibodies directed against platelet-endothelial cell adhesion molecule-1 (PECAM-1) that are

conjugated to the antioxidant enzymes SOD and catalase. PECAM-1 binds to endothelial cells and in this manner the antioxidant enzymes are targeted to the endothelial cell. In a recent study by Shuvaev *et al.* (186), anti-PECAM/catalase protected mice from H_2O_2-mediated lung injury produced by glucose oxidase deposited in the pulmonary vasculature. Therefore, the use of endothelial cell-targeted antioxidants holds promise for mechanistically tailored antioxidant treatment of vascular pathologies (187).

4.5. Bolstering antioxidant defences: The transcription factor Nrf2

Cells have evolved endogenous defence mechanisms against sustained oxidative stress such as NF-E2-Related Factor 2 (Nrf2), a redox sensitive transcription factor, which regulates the expression of important cytoprotective enzymes (188). Nrf2 is broadly expressed in tissues but is only activated in response to a range of oxidative and electrophilic stimuli (189). Nrf2 upregulates important detoxifying phase II enzymes, such as NAD(P)H:quinone oxidoreductase (NQO1) and antioxidant proteins, such as heme oxygenase 1 (HO1), through an antioxidant response element (ARE)-dependent pathway. Recent evidence suggest that diminished Nrf2/ARE activity contributes to increased oxidative stress and mitochondrial dysfunction in the vasculature leading to endothelial dysfunction, insulin resistance and abnormal angiogenesis observed in diabetes. Counteracting the decreased Nrf2 levels in diabetes is one mechanism that provides a new avenue for targeted antioxidant therapy to bolster antioxidant defences. This may be highly pertinent to the diabetic milieu since Jiang *et al.* (190), showed that lack of Nrf2 worsens streptozotocin-induced renal damage in diabetic NRF-/- mice, implying a protective role for NRF-2 in DN. The Nrf2-mediated protection is mediated, at least, partially through inhibition of TGF-beta1, whilst in cultured human mesangial cells, overexpression of Nrf2 inhibited the promoter activity of TGF-beta1 in a dose-dependent manner. The data of Jiang *et al.* (190) would suggest that dietary (one example would be isoflavones (191)) or therapeutic activation of Nrf2 could be a useful strategy to prevent or slow down the progression of diabetic nephropathy.

It is also noteworthy that the Gpx1-mimetic, ebselen up-regulates the expression of phase 2 enzymes like the GSTs via a pathway that involves kelch-like ECH-associated protein (Keap1) (192). Keap1 anchors Nrf2 in the cytoplasm, thereby repressing the ability of Nrf2 to induce phase 2 detoxification enzyme genes. Indeed, ebselen has been shown to modify Keap1 (192), thereby releasing Nrf2. Increasing ebselen activity, as detailed above, may additionally act via Nrf2 in its protection against ROS.

4.6. Genetic manipulations to increase antioxidant defences

Adenovirus-mediated gene transfer of antioxidants offers a mode of gene delivery to bolster the reduced antioxidant defences in diabetes. Use of this technique to deliver Sod isoforms to diabetic rabbit aorta has shown improvements in endothelium-dependent relaxation (193). Indeed, Zanetti *et al.* (193), show that gene transfer of both SOD1 and SOD2 reduced the diabetes-mediated increase in superoxide as well as correcting the impaired response to acetylcholine in these vessels. In a subsequent study, these authors also showed that gene transfer of SOD2 reversed vascular dysfunction in the absence but not in the presence of atherosclerotic plaque, inferring the potential benefits of early intervention with antioxidant gene therapy (194). Interestingly, eNOS gene transfer improved endothelium-dependent vasorelaxation, even in the presence of plaque (194), reduced endothelial dysfunction in the aorta of angiotensin II-treated rabbits (195) and improved carotid artery relaxation in alloxan-induced diabetic rabbits (196). However, in the latter study, in contrast to that of Zanetti *et al.* (193), adenovirus-mediated gene transfer of SOD1 was unable to improve impaired NO-mediated relaxation in vessels from diabetic rabbits. To date, no studies have investigated the effect of adenoviral delivery of GPx1 or catalase in diabetic complications.

5. CONCLUSION

Increasing evidence suggests a role for hyperglycaemia-mediated endothelial dysfunction in the initiation and progression of micro- and macrovascular disease in diabetes. One key pathway that may trigger this cascade of events is increased oxidative stress. Several factors contribute to the glucose-mediated increase in ROS, including decreased NO bioavailability as a consequence of increased superoxide production, eNOS uncoupling and increased NADPH oxidase activity. New treatment options to reduce oxidative stress include targeting the source of ROS, the use of low molecular weight antioxidant mimetics, the specific targeting of vulnerable organelles such as the mitochondria and the manipulation of the genetic machinery to boost antioxidant defences. Future treatment options may need to look at a combination therapy approach of oxidant-minimizing drugs together with the more conventional approaches such as the statins, peroxisome proliferator-activated receptors and renin-angiotensin system (RAS) blockade for additive beneficial effects on endothelial dysfunction due to both distinct and interrelated mechanisms. Indeed, combination therapy may be an important paradigm for treating and slowing the progression of diabetes-mediated micro and macro-vascular disorders characterized by endothelial dysfunction. Promising data from our laboratory have highlighted the potential use of GPx-mimetics as effective drugs against both diabetic nephropathy and diabetes-associated atherosclerosis. It is expected that newer generation ebselen-mimetics, with their predicted greater *in vivo* efficacy, will achieve even further reductions in diabetes-associated complications, making this strategy of antioxidant repletion an attractive alternative adjunct therapy in the fight against diabetic vascular complications. However, it is always important to recognize that physiological levels of ROS play a significant role in regulating vascular function. Thus, future therapeutic approaches that alleviate the burden of oxidative stress

need to be delicately balanced to ensure that important cellular processes remain intact.

6. ACKNOWLEDGEMENTS

The authors would like to acknowledge the Australian National Health and Medical Research Council (NH&MRC grant # 526656) as well as the Juvenile Diabetes Research Foundation for their support of this work.

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Key Words: Oxidative Stress, Glutathione Peroxidase, Diabetic Complications, Microvascular Disease, Macrovascular Disease, Endothelial Dysfunction, Targeted Antioxidant Defences, Ebselen, Review

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