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Review

The Role of Oxidative Stress in Diabetic Vascular and Neural Disease

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This review will focus on the impact of hyperglycemia-induced oxidative stress in the development of diabetes-induced vascular and neural dysfunction. Oxidative stress occurs when the balance between the production of oxidation products and the ability of antioxidant mechanisms to neutralize these products is tilted in the favor of the former. The production of reactive oxygen species has been shown to be increased in patients with diabetes. The possible sources for the overproduction of reactive oxygen species is widespread and include enzymatic pathways, autoxidation of glucose and the mitochondria. Increase in oxidative stress has clearly been shown to contribute to the pathology of vascular disease not only in diabetes but also in hypertension, stroke and ischemia. Since the etiology of diabetic neuropathy is considered to have a large vascular component, prevention of oxidative stress in diabetes is considered by many investigators to be a primary defense against the development of diabetic vascular disease. Potential therapies for preventing increased oxidative stress in diabetes and the neural vasculature will be discussed.

Keywords: Oxidative stress; Diabetic vascular and neural disease; Reactive oxygen species; Superoxide dismutase

INTRODUCTION

Increased oxidative stress has been implicated in the pathology of a variety of diseases including diabetic vascular and neural complications.[1–3] Oxidative stress is a condition resulting from an imbalance between the generation of reactive oxygen species (ROS) and the ability of antioxidant mechanisms to neutralize these compounds. Therefore, increased oxidative stress is the consequence of enhanced ROS production and/or attenuated ROS scavenging capacity, resulting in tissue damage.[4] The most common forms of ROS are superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), and peroxynitrite ($ONOO^-$).[5]

Hypochlorous acid (HOCl), reactive aldehydes, and lipid peroxides are among other oxidants that have relevance to vascular biology.[6] These compounds are produced endogenously, and the levels are increased under conditions of oxidative stress.[5,6] Enzymes and pathways located throughout the cell, including the plasma membrane, cytosol, mitochondria, and peroxisomes have been demonstrated to generate these compounds under both normal and pathological conditions.[5] Superoxide can be produced by the electron transport chain of the mitochondria, and by NADH oxidase, NAD(P)H oxidase, xanthine oxidase, cyclooxygenase, lipoxygenase, cytochrome P-450, and, during periods of tetrahydrobiopterin deficiency, by nitric oxide synthase.[5] Superoxide can spontaneously acquire an electron to form H_2O_2. The formation of H_2O_2 from O_2^- can also occur via a reaction catalyzed by superoxide dismutase (SOD) of which there is three isoforms: Mn-SOD, which is located in the mitochondria and two isoforms of Cu, Zn-SOD, which are located either in the cytosol or extracellularly.[5] Hydrogen peroxide can be converted to water by

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the action of catalase or by glutathione peroxidase in the presence of reduced glutathione.[5] However, in the presence of trace metals such as Fe, H_2O_2 can form OH^- via a process known as the Fenton reaction.[5] The formation of $ONOO^-$, which is the result of a reaction between O_2^- and nitric oxide (NO), is also important in vascular disease and has been demonstrated to be enhanced in diabetes.[5,6]

DIABETES AND THE GENERATION OF REACTIVE OXYGEN SPECIES

Superoxide is considered to be the most biologically important ROS.[7] It can directly react with smooth muscle cells causing contraction and rapidly scavenges NO, thereby diminishing its biological half-life.[8,9] In diabetes, the overproduction of O_2^- has been attributed to increase activity of several enzymes including nitric oxide synthase and NADH/NAD(P)H oxidase[7] (Fig. 1). Some sources have suggested that increased NAD(P)H activity constitutes the main enzymatic source of O_2^- generated by vascular tissue in diseases including hypercholesterolemia and hypertension.[10,11] In conditions related to diabetes, high glucose and free fatty acid levels have been shown to stimulate ROS production in cultured vascular cells through a protein kinase C (PKC)-dependent activation of NAD(P)H oxidase.[12] Activation of NAD(P)H oxidase has also been linked to the increased production of advanced glycation end-products (AGEs).[13] Exposing cultured human endothelial cells to increased concentrations of AGEs caused an increase in intracellular formation of H_2O_2 and expression of vascular cell adhesion molecule-1, which was suppressed by diphenyliodonium (DPI).[13] In aortas from diabetic rats Hink *et al.* found an activation of NAD(P)H oxidase and a 7-fold increase in gp91^{phox} mRNA levels, a subunit of the NAD(P)H complex.[14] Angiotensin II, which is increased in diabetes, through activation of angiotensin-1 receptors has also been demonstrated to up-regulate several subunits of NAD(P)H oxidase and increase intracellular levels of O_2^-.[15] Direct evidence that increased NAD(P)H oxidase activity impairs vascular function comes from recent studies by Hamilton *et al.*[16] They demonstrated that inhibiting of NAD(P)H activity with apocynin decreased O_2^- production by human mammary arteries and saphenous veins, increased NO production and induced vasodilation.[16] Increased formation of O_2^- in diabetes has also been linked to increased activity of xanthine oxidase.[17] The activity of xanthine oxidase is increased in liver and plasma of diabetic animals, and in diabetic rabbits, increased O_2^- formation has been demonstrated to be blocked by allopurinol, an inhibitor of xanthine oxidase.[17] Another enzyme system that produces ROS in the vascular wall is nitric oxide synthase (NOS). Nitric oxide synthase exists in three isoforms; endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS). In endothelial cells, arginine is converted to NO and citrulline via

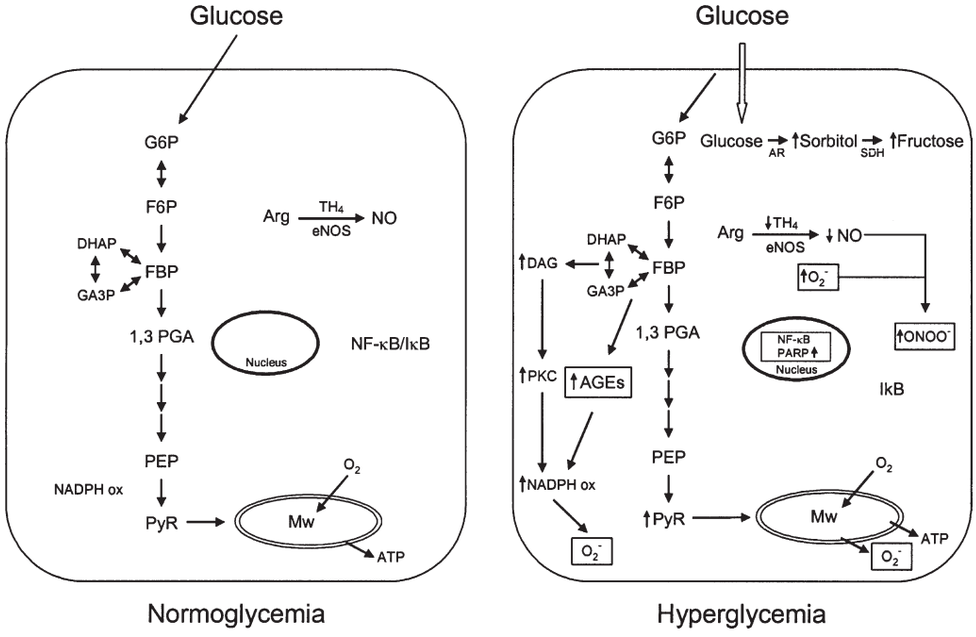

FIGURE 1 Representation of sources of superoxide (O_2^-) formation and enzymatic pathways altered by hyperglycemia in the endothelium. This figure illustrates that following exposure to hyperglycemic conditions a variety of pathways and enzymes are activated that can contribute to the formation of O_2^- and an increase in oxidative stress as discussed in the text of this review.

eNOS. Endothelial NOS and nNOS are constitutive to endothelial tissue and neurons, respectively, and produce small amounts of NO in a short time.[18] Acting as a signal, this NO activates guanylate cyclase in target tissues, and in the case of eNOS, the end result is smooth muscle relaxation and increased blood flow.[18] Much of the NO produced by eNOS is scavenged by hemoglobin and eventually reduced to nitrate.[18] As part of the inflammatory response, iNOS produces large amounts of NO, which is lethal to pathogens and injurious to cells.[18] Even when produced in small amounts by eNOS, some NO can be reduced or oxidized to more reactive nitrogen species and initiate lipid peroxidation in cell membranes, where it is concentrated.[18] A more important reaction of excess NO is combination with O_2^- producing $ONOO^-$.[18] It is $ONOO^-$ that is responsible for much of the cytotoxicity of NO. Peroxynitrite has a short half-life but is able to diffuse across cell membranes and depending on the cell environment can cause a nitrosylation of proteins, which generally reduces enzyme activity, oxidation of glutathione, an important antioxidant, and increased peroxidation of lipids.[18] In diabetes the generation and/or bioactivity of NO by eNOS is reduced.[19] This is due in part to the quenching of NO by O_2^- to form $ONOO^-$. However, a decrease in substrate and co-factor availability, arginine and tetrahydrobiopterin (TH_4), respectively, may also contribute to this deficit. The administration of arginine has been demonstrated to improve vascular function in diabetic patients and animal models.[19–24] Likewise, the administration of tetrahydrobiopterin derivative has been shown to improve diabetes-impaired vascular function.[25,26] In diabetes and other pathological conditions a suboptimal concentration of tetrahydrobiopterin reduces the formation of NO and favors "uncoupling" of NOS leading to NOS-mediated reduction of oxygen and formation of O_2^- anions and H_2O_2.[26] In diabetes and other pathologic conditions associated with increased oxidative stress the accelerated catabolism of tetrahydrobiopterin may contribute to endothelial dysfunction.[26]

Excessive amounts of ROS may also arise from dysregulation of the mitochondrial electron transport chain. Recently, four main molecular mechanisms have been linked to hyperglycemia-induced vascular dysfunction (Fig. 1). These four mechanisms are increased flux of glucose through the polyol pathway, increased formation of AGEs, increased activity of PKC, and increased flux through the hexosamine pathway.[27] These mechanisms have in common one feature which is the overproduction of O_2^- by the mitochondrial electron-transport chain.[27,28] Since these four mechanisms have been linked to the overproduction of O_2^- by the mitochondria it opens up new possibilities and avenues of research for preventing diabetes-induced vascular disease. Our studies support the paradigm that mitochondria are a major source for O_2^- production in the vasculature of diabetic rats.[29] Using epineurial arterioles of the sciatic nerve from diabetic rats we determined that complex 1 of the electron-transport chain is a site of O_2^- formation in the mitochondria of vascular tissue.[29] Using cultured bovine aortic endothelial cells, Brownlee and colleagues demonstrated that hyperglycemia increased O_2^- production.[28] Hyperglycemia, due to an overproduction of electron donors derived from glycolysis and the TCA cycle, has been demonstrated to increase the proton gradient across the mitochondrial inner membrane above a threshold level causing a prolonged period of O_2^- generation.[2,30,31] Brownlee and colleagues have gone on to show that overexpression of Mn-SOD abolishes the signal generated by ROS, and overexpression of uncoupling protein-1 collapses the proton electrochemical gradient, thereby preventing the overproduction of ROS by endothelial cells.[28]

In vascular tissue and especially the endothelium one of the major consequences of hyperglycemia and increase in the generation of ROS is the activation of the transcription factor nuclear factor κB (NF-κB).[32–36] Upon activation, NF-κB translocates from the cytosol to the nucleus where it influences the regulation of many genes associated with oxidative stress. In endothelial cells, one of the most documented effect of NF-κB activation is the increased expression of adhesion molecules leading to increased attachment of monocytes to the endothelial cell monolayer.[32,33,37,38] We have demonstrated that the effect of hyperglycemia on the activation of NF-κB and increased adhesion of monocytes to endothelial cells is blocked by the antioxidant α-lipoic acid.[32] This suggests that hyperglycemia-induced oxidative stress and generation of ROS is responsible for the activation of NF-κB in cultured endothelial cells.

Overall, these studies demonstrate that multiple sources exist for overproduction of ROS in pathological conditions such as diabetes and illustrate the challenges facing investigators in designing strategies to prevent the development of oxidative stress.

REACTIVE OXYGEN SPECIES AND DIABETIC VASCULAR AND NEURAL DISEASE

Endothelial dysfunction contributes significantly to diabetic vascular disease and is an important factor in the development of diabetic neuropathy. Cameron and Cotter have demonstrated that reduced nerve perfusion is a contributing factor in the etiology of diabetic neuropathy.[39] Free radicals such as O_2^- and OH^- cause vascular endothelial damage and reduced

NO-mediated vasodilation. Inhibition of advanced glycosylation and autoxidation, major sources of free radicals, by aminoguanidine and transition metal chelators, or antioxidants and free radical scavengers have been demonstrated to improve the diabetes-induced decrease in endoneurial blood flow and improve neural dysfunction.[39–48] The endothelium, via the release of vasodilators and vasoconstrictors, controls the vascular tone. The three major factors produced by the endothelium that contribute to the regulation of vascular relaxation are NO, prostacyclin and the as yet an unidentified factor referred to as endothelium-derived hyperpolarizing factor (EDHF). Impaired endothelium-dependent vasodilation has been demonstrated in various vascular beds of animal models of diabetes and humans with type 1 and type 2 diabetes.[49,50] Some of the mechanisms attributed to diabetes-induced endothelium dysfunction include impaired signal transduction pathways or substrate availability, impaired release or increased metabolism of vasodilatory mediators, increased release of vascular constricting factors, and decreased reactivity of the smooth muscle to vasodilatory mediators.[49,50] The mechanisms induced by hyperglycemia/diabetes considered to contribute to endothelial dysfunction are the activation of PKC, increased activity of the polyol pathway, increased formation of AGEs and increased oxidative stress. Interestingly, studies by Brownlee and colleagues have suggested hyperglycemia-induced production of O_2^- by mitochondria of endothelial cells as the common link for mechanisms of diabetes-induced vascular dysfunction.[27,28] Our studies conducted with intact vascular tissue consisting of epineurial arterioles of the sciatic nerve lend support to the studies by Brownlee and colleagues conducted with cultured endothelial cells.[29] Recently, studies by my laboratory have provided evidence that the generation of oxidative stress through the production of O_2^- and peroxynitrite impairs vascular function and endothelium-dependent vascular relaxation of epineurial arterioles of the sciatic nerve from diabetic rats, which precedes the slowing of motor nerve conduction velocity.[40–43,51] Studies designed to investigate the source of superoxide formation provided results suggesting that complex I of the mitochondrial electron transport chain and possibly NAD(P)H oxidase are responsible for the increase in O_2^- formation observed with epineurial arterioles from the sciatic nerve.[29] It was shown that pretreating epineurial arterioles from diabetic rats with the PKC inhibitor bisindolylmaleimide (GF 109203X) improved acetylcholine-mediated vascular relaxation but did not prevent the increase in O_2^- formation suggesting that activation of PKC by oxidative stress is downstream of O_2^- formation.[29] We have also demonstrated that treating diabetic rats with three different types of antioxidants prevented the diabetes-induced increase in O_2^- and peroxynitrite formation in aorta and epineurial arterioles of the sciatic nerve and diabetes-induced vascular and neural dysfunction, thereby providing additional evidence that increased oxidative stress contributes to diabetes-induced vascular and neural disease.[40,42] Studies from other laboratories have provided further evidence that antioxidants may prevent vascular complications in diabetes. Treating diabetic rats with tempol, a stable superoxide dismutase mimic compound, abolished the diabetes-induced increase in vascular O_2^-, malondialdehyde and 8-epi-prostaglandin F(2α), and also the impairment in relaxation of aortic rings to acetylcholine.[52] Cameron and colleagues have demonstrated that treating diabetic rats with α-lipoic acid or the metal chelators hydroxyethyl starch deferoxamine or trientine prevented the diabetes-induced impairment in vascular relaxation associated with hyperalgesia and neurovascular deficits.[44,45,53–55] In addition, Keegan *et al.* demonstrated that treating diabetic rats with α-lipoic acid improved endothelium-dependent vascular relaxation of corpus covernosum smooth muscle.[47] These studies imply that increased O_2^- formation via the mitochondrial electron transport chain and perhaps NAD(P)H oxidase are partially responsible for reduced vascular reactivity observed in epineurial arterioles of the sciatic nerve from diabetic rats.[29] Because metals chelators and OH^- scavengers have also been demonstrated to be effective in preventing diabetes-induced vascular and neural dysfunction it is likely that the formation of OH^- may also contribute to impairment of vascular reactivity and nerve function in diabetes.[39,44,45,48,53–56] Besides antioxidants, therapies designed to improve NO formation have also been shown to be beneficial in improving diabetes-induced vascular dysfunction. Treating diabetic rats with arginine or a tetrahydrobiopterin derivative improved vascular function in diabetic rats.[20–26] This implies that reduced availability of NO due to increased degradation, such as scavenging of NO by O_2^- to form $ONOO^-$ or reduced production of NO in the diabetic state, may lead to vascular impairment.

PREVENTION OF OXIDATIVE STRESS AS TREATMENT FOR DIABETIC VASCULAR AND NEURAL DYSFUNCTION

Many therapeutic approaches for the prevention of diabetic neuropathy have been successfully applied in animal models of diabetes indicating that the etiology of diabetic neuropathy is complex. This has lead investigators to propose a wide range of mechanisms for the cause of diabetic neuropathy. Some of the different treatments that have been demonstrated to improve or prevent neuropathy in

diabetic animal models include aldose reductase inhibitors, aminoguanidine, antioxidants, inhibitors of PKC, vasodilators, nerve growth factor (NGF), γ-linolenic acid, acetyl L-carnitine, myo-inositol, gangliosides, inhibitors of poly(ADP-ribose) polymerase (PARP), and others.[57–69] At this time not all of these treatment protocols have been applied to human diabetes, although the results from those that have been used have been disappointing. This raises the question of why these treatment protocols generally failed in human trials after being successful in studies with diabetic animal models. There are a number of reasons that could explain this outcome. First, due to side effects or other concerns the dosage of the drugs used in human studies may have been inadequate and the efficacy to low to be effective against the targeted mechanism compared to animal studies. This is a valid concern and outcomes from studies using aldose reductase inhibitors, NGF and antioxidants may have been influenced by this problem. Second, the targeted mechanisms for treatment may be incorrect and have no direct contribution to diabetic neuropathy. This seems unlikely since each of the compounds mentioned above and their targeted mechanism are based on valid scientific data obtained from cells and/or animal studies. However, whether each of the targeted mechanisms plays a significant role in the development of diabetic neuropathy in humans is a point of conjecture. In addition, it is unlikely that one single mechanism is solely responsible for the development of diabetic neuropathy and that combination therapy targeted at two or more deficits will probably be necessary to prevent diabetic neuropathy. Third, the patients used in the human studies were inappropriate for the targeted mechanism because their complications have progressed to the point that the symptoms or end-points being examined are not readily reversible. This is certainly a valid concern in studies of human diabetic neuropathy. Drug intervention in human diabetic neuropathy when patients have advanced clinical symptoms will likely have minimal benefit since most of these patients will likely have nerve damage that is either irreversible or only slowly reversed and positive clinical outcomes would probably take longer to achieve than the period applied in the clinical trial. For instance, many of the clinical trials conducted with aldose reductase inhibitors were designed with a year or less of treatment before measuring clinical outcomes. Considering the severity of the progression of diabetic neuropathy in the patients used in these studies this treatment period is likely too short. Lastly, outcomes were based on invalid end-points. For instance, measurement of sorbitol for interventions using aldose reductase inhibitors may not be a good outcome predictor for the patient. Measurement of sorbitol may provide good evidence of the current metabolism but may not reflect the severity or progression of the disease. In the case of human diabetic neuropathy, treatment with aldose reductase inhibitors may provide control of glucose flux through the aldose reductase pathway and measurement of the level of sorbitol in tissue samples or cells may provide information on the efficacy of aldose reductase inhibitor treatment, but likely will not provide any indication of the present extent of nerve damage or progression of the disease. In order to better analyze the progression of diabetic neuropathy several relevant markers of nerve function and biological status including oxidative stress will probably be required.

One of the most promising approaches for intervention and halting of diabetic neuropathy is the prevention of oxidative stress. A variety of antioxidants including vitamin E have been demonstrated to have beneficial effects in treating diabetic neuropathy in diabetes patients and diabetic animal models.[70–72] More recently α-lipoic acid has shown promise as a potential antioxidant treatment for diabetic neuropathy and is undergoing clinical trials in Europe.[73,74] Our studies have demonstrated that α-lipoic acid provides good protection against oxidative stress in diabetic rats of 4–6 week duration.[40] The treatment of diabetic rats with α-lipoic acid significantly improved diabetes-induced decrease in endoneurial blood flow, endothelium-dependent vascular relaxation in arterioles that provide circulation to the region of the sciatic nerve, and motor nerve conduction velocity. α-Lipoic acid treatment also reduced the production of superoxide by the aorta and superoxide and peroxynitrite by arterioles that provide circulation to the region of the sciatic nerve. Treating diabetic rats with α-lipoic acid prevented the diabetes-induced increase in thiobarbituric acid reactive substances in serum and significantly improved lens glutathione levels. α-Lipoic acid is a good metal chelator and is capable of scavenging hydroxyl radicals, hypochlorous acid and singlet oxygen, but not superoxide or peroxyl radicals.[74–77] However, in its reduced form, as dihydrolipoic acid, it is a good scavenger of superoxide and prevents initiation of lipid peroxidation.[74–77] *In vivo*, α-lipoic acid can be converted into dihydrolipoic acid.[74,75] This reaction requires NADPH, which is reduced in diabetes due to the increased flux of glucose through the aldose reductase pathway.[78,79] Therefore, one potential form of combination therapy for the treatment of diabetic neuropathy may be combining an aldose reductase inhibitor with α-lipoic acid. This combination should promote the formation of dihydrolipoic acid, thereby enhancing the antioxidant potential of α-lipoic acid and possibly providing a synergistic effect. In a study by Nakamura *et al.* of diabetic neuropathy in

streptozotocin-induced diabetic rats, they found that treating diabetic rats with the aldose reductase inhibitor NZ-314 improved nerve function and reduced oxidative stress.[80] They concluded that the efficacious effect of aldose reductase inhibition on diabetic neuropathy may be mediated by decreasing oxygen free radicals.[80] This would agree with our studies demonstrating that treating diabetic rats with sorbinil improves glutathione levels presumably by correcting the redox imbalance.[41] We purpose that a similar mechanism may apply for the conversion of α-lipoic acid to dihydrolipoic acid. Correcting the redox imbalance in diabetic rats with an aldose reductase inhibitor may promote the production of dihydrolipoic acid *in vivo*.

The development of superoxide dismutase mimetics are another class of antioxidants with potential for treatment of diabetic complications including diabetic neuropathy.[42,81,82] Because of limitations associated with enzyme therapies these non-peptidyl compounds may offer advantages resulting in better clinical therapies and outcomes for diseases mediated by O_2^- radicals such as diabetes.[83] In our studies we demonstrated that treating diabetic rats with M40403 inhibited the generation of superoxide by aorta and epineurial vessels of the sciatic nerve, the formation of peroxynitrite by epineurial vessels of the sciatic nerve, the reduction in endoneurial blood flow, the slowing of motor nerve condition velocity and impairment of endothelium-dependent vasodilation of arterioles that provide circulation to the sciatic nerve. It also improved the diabetes induced increase in serum TBARS and sciatic nerve conjugated diene level, two additional markers of oxidative stress.[42] M40403 is a prototypic example of a stable, low molecular weight, manganese-containing, non-peptidic molecule possessing the function and catalytic rate of native SOD enzymes, but with the advantage of being a much smaller molecule (molecular weight 483 *vs* 30,000 for M40403 and the native enzyme, respectively).[42,83,84]

Another form of treatment yet to be thoroughly examined for diabetic neuropathy, but has showed promise for treatment of diabetic nephropathy, is intervention with angiotensin converting enzyme (ACE) inhibitors and/or angiotensin receptor antagonists.[85–88] It has been demonstrated that these drugs have antioxidant properties, neuroprotective potential and may reduce the accumulation of AGEs.[89–94] Angiotensin II causes endothelium dysfunction by increasing NAD(P)H oxidase-mediated vascular O_2^- production.[95,96] It has been shown that two isoforms of the NAD(P)H oxidase family (Nox), Nox1 and Nox4, are involved in the vascular oxidative stress pathways in response to angiotensin II.[96] Evidence suggests that intrarenal renin-angiotensin system activation is responsible for non-ACE-dependent angiotensin II production within the kidney and the resulting low-renin state in patients with diabetes. The renin-angiotensin system is highly activated in patients with type 2 diabetes.[97] Hyperglycemia (both acute and sustained) and obesity activate the renin-angiotensin system.[97] A wide range of evidence now exists that blocking the renin–angiotensin system in diabetic patients reduces the progression of diabetic nephropathy.[85–88,97] However, little information is available whether treatment of diabetic patients with ACE inhibitors or angiotensin receptor antagonists will improve or interrupt the progression of diabetic vascular and neural disease. To provide a rational answer as to whether blocking the renin–angiotensin system can prevent diabetic neuropathy two issues must be addressed: (1) Does vascular dysfunction cause diabetic neuropathy, and (2) can ACE inhibitors and/or angiotensin receptor antagonists ameliorate diabetic vascular dysfunction and hence neuropathy.[98] Existing data suggests that vascular dysfunction contributes significantly to the development and progression of diabetic neuropathy.[40–43] It has also been demonstrated that treatment with ACE inhibitors improves endothelial dysfunction and reduces oxidative stress in diabetes.[89,93,99] Therefore, it seems likely that ACE inhibitors and/or angiotensin receptor antagonists may improve diabetic neuropathy. In studies by Cameron and colleagues with diabetic animal models, treatment of diabetic rats with an ACE inhibitor or with an angiotensin receptor antagonist improved motor and sensory nerve conduction velocities, nerve blood flow and stimulated endoneurial angiogenesis.[100,101] Aggarwal *et al*. have also demonstrated that treating streptozotocin-induced diabetic rats with lisinopril, an ACE inhibitor, improved diabetic neuropathy.[102] These studies provide a rationale for more studies to determine the potential benefits of ACE inhibitor and angiotensin receptor antagonist treatment for diabetic vascular and neural disease.

Other potential treatment for diabetic vascular and neural disease that may involve protection of neural tissue from oxidative stress is inhibition of PKC and PARP.[103–110] The PKC family includes at least 12 isoforms and a number of other proteins with homology to either the phorbol ester/diacyglycerol domain or the PKC-terminal region.[103] One specific isoform PKCβ2 has been found to be activated in retina, heart and aorta of diabetic rats.[103] Using a specific inhibitor of the PKCβ isoform, researches found that retinal and renal vascular reactivity was normalized and renal function was improved in diabetic rats.[103] Recently, studies have demonstrated that PKCβ inhibition also improved nerve conduction velocity and endoneurial blood flow in

diabetic rats.[105] At present the mechanism responsible for PKCβ-mediated vascular dysfunction in diabetes is unknown. However, it has been shown that high concentrations of vitamin E can reverse some of the changes in retinal and renal vessels caused by diabetes as well as inhibit the activation of PKCβ by diabetes or hyperglycemia.[101,104] Furthermore, Brownlee and colleagues have demonstrated that the activation of PKC by hyperglycemia in cultured bovine aorta endothelial cells is downstream of O_2^- generation, indicating that increased oxidative stress may influence the activation of PKC in vascular tissue.[28] Another possible downstream mediator of hyperglycemia-induced oxidative stress is PARP. PARP is an abundant nuclear enzyme of eukaryotic cells that participates in DNA repair in response to genotoxic stress.[107,108] When activated by DNA single-stranded breaks, PARP initiates an energy-dependent cycle by transferring ADP ribose units from NAD^+ to nuclear proteins. This process results in rapid depletion of the intracellular NAD^+ and ATP pools by slowing the rate of glycolysis and mitochondrial respiration and eventually leading to cellular dysfunction and death.[108] Over activation of PARP represents an important mechanism of tissue damage in various pathological conditions associated with oxidant stress, including diabetes.[107] Recently, Pacher *et al.* have reported that the activation of PARP contributes to the development of endothelium dysfunction in streptozotocin-induced diabetic mice.[108] The development and use of specific inhibitors of PARP have indicated that PARP may be a novel target for the intervention of diabetes-induced endothelial dysfunction.[107–110]

In summary, diabetes causes an increase in oxidative stress in the vasculature of neural tissue. Preventing the diabetes-induced increase in oxidative stress with antioxidants or with drugs having antioxidant properties and/or inhibit downstream mediators of oxidative stress have been shown to improve vascular activity and neural function in diabetic animal models, indicating that an increase in oxidative stress in diabetes is associated with neural dysfunction. Future studies should focus on determining the most effective therapeutic approach for preventing the diabetes-induced increase in oxidative stress and thus, the development and/or progression of diabetic neuropathy. In patients, this will likely require early intervention, prior to the development of severe clinical symptoms, with a combination of drugs that may include antioxidants, aldose reductase and/or PKC inhibitors, and possibly ACE inhibitors and/or angiotensin receptor antagonists. Additional studies will be required in animal models to determine the most effective drugs, such as an antioxidant, and combinations to use. This will have to be followed by extensive clinical trials to address issues related to safety, dosage and synergy as well as efficacy in diabetes patients. The development of reliable clinical end-points or markers will also be required in order to effectively evaluate the efficacy of these drugs and outcomes during the clinical studies.

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