OXIDATIVE STRESS AND ANTIOXIDANT TREATMENT IN DIABETIC NEUROPATHY

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SUMMARY

Oxidative stress is one of the factors contributing to the development of diabetic neuropathy. Several mechanisms, among which glucose autooxidation, glycation of antioxidant enzymes like superoxide dismutase, catalase and glutathione peroxidase, and increased polyol pathway activity, lead to increased production of reactive oxygen species. Both direct toxicity of oxygen free radicals to the peripheral nerve as well as changes in endothelial function and vascular reactivity, possibly by the quenching of nitric oxide, may lead to nerve dysfunction. Antioxidant drugs like iron chelators, N-acetylcysteine, probucol, α-lipoic acid and vitamin E can prevent nerve dysfunction in experimental diabetes. The present review focuses on the potential mechanisms explaining the association between oxidative stress and diabetic neuropathy, and summarizes the studies in which the effects of antioxidant treatment on the diabetic peripheral nerve have been evaluated.

Key words: Diabetes mellitus, neuropathy, oxidative stress, nitric oxide, antioxidants, review

INTRODUCTION

Diabetes is associated with an increased production of reactive oxygen species (ROS), and enhanced oxidative stress has been postulated as one of the contributing factors in the pathogenesis of neuropathy and other late complications of diabetes (1-3). In the present review we will summarize the mechanisms that may be responsible for the increased production of ROS in diabetes and we will give an overview of the available data concerning pro- and antioxidant changes in the diabetic nerve. Furthermore, as microvascular oxidative damage may also lead to nerve dysfunction in diabetes (4), we will focus on the role of the endothelium and the blood vessel wall, in particular in relation to nitric oxide (NO\(^*\)). Finally, experimental studies with antioxidant compounds for the prevention of diabetic neuropathy will be reviewed.

ORIGINS OF INCREASED OXIDATIVE STRESS IN DIABETES

ROS, although essential in low concentrations for normal cellular physiology, can cause peroxidation of lipid membranes, DNA and proteins, and thus negatively affect cellular homeostasis and function (5,6). The formation of ROS like the superoxide anion (O\(^2-\)), hydroxyl radicals (\(^*\)OH), hydrogen peroxide (H\(_2\)O\(_2\)), and peroxynitrite (ONOO\(^-\)) is limited by the presence of ROS scavenging compounds like catalase, glutathione peroxidase (GPx), superoxide dismutase (SOD), vitamin E and reduced glutathione (GSH) (7,8). Hyperglycaemia can induce changes in the balance between pro- and antioxidant compounds, leading to increased production and reduced scavenging of ROS (2,9,10). The mechanisms responsible for increased ROS generation in...
diabetes have been reviewed elsewhere (2). We will summarize these mechanisms, which include glucose autooxidation, protein glycation, and increased polyol pathway flux.

**Glucose autooxidation and autooxidative protein glycosylation**

Monosaccharides have the capacity to form enediols, which can generate \( \text{H}_2\text{O}_2 \) and \( \text{O}_2^- \) while simultaneously oxidising reduced free metal ions. In the presence of oxidised free iron as a consequence of the Haber-Weiss reaction \( \cdot \text{OH} \) radicals are formed (11,12). Subsequently, the interaction between \( \cdot \text{OH} \) and the initially formed product of the reaction between glucose and protein amino groups (the Amadori product) can lead to accelerated protein glycation and oxidation, a mechanism which has been referred to as autooxidative glycosylation [review (13)]. Increased activity of this pathway as a consequence of chronic hyperglycaemia is considered to contribute to the pathogenesis of diabetic complications (1).

**Non-enzymatic glycation of antioxidant enzymes**

The major ROS scavenging enzymes are SOD, catalase and GPs, while glutathione reductase (GRed) is essential to assure the availability of GSH (14). As mentioned above, hyperglycaemia is associated with non-enzymatic glycation of proteins, and glycated forms of SOD with reduced enzymatic activity have been observed (15,16). Glycation of GRed leads to enzyme inactivation (17). Non-enzymatic glycation of ROS scavenging proteins may account for their reduced activity observed in diabetic patients and animals (2,18-20), and secondarily lead to the increased presence of ROS.

**The polyol pathway and glutathione**

Increased polyol pathway activity, leading both to a rise in nerve sorbitol levels as well as to endoneurial

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**Figure 1.**

Schematic overview of potential mechanisms contributing to the increased production of reactive oxygen species in diabetes, leading to enhanced oxidative stress, and alterations in nerve microcirculation and nerve function.
myo-inositol depletion, has been postulated to play a major role in the pathogenesis of diabetic neuropathy. Blockade of this pathway by aldose reductase inhibitors (ARI) has been extensively studied. Administration of ARI can improve experimental and, to a more limited extent, clinical diabetic neuropathy (21,22). As the reduction of glucose to sorbitol by aldose reductase is dependent on the oxidation of NADPH to NADP⁺, and as the reduction of glutathione by GRed is NADPH-dependent, altered polyol pathway activity has also been associated with attenuated GSH availability in diabetes (23). Therefore, the polyol pathway may be an additional mechanism leading to increased oxidative stress in diabetes. The link between increased polyol pathway flux and oxidative damage in diabetes was confirmed by the observation, that lipid peroxidation in the diabetic rat lens could be attenuated by the ARI sorbinil (24). Moreover, increased GSH levels have been observed in red blood cells from ARI-treated diabetic patients (25).

THE OXIDATIVE BALANCE IN DIABETIC PERIPHERAL NERVE

The peripheral nerve is relatively poor in enzymatic antioxidant capacity. Romero et al. demonstrated that, in comparison to both liver and brain tissue, the enzymatic activity of glutathione transferase, GPs and GRed was 10- to 100-fold lower in rat sciatic nerve (26,27). The nerve GSH content was only 10 % of GSH levels in the brain (27). SOD activity in the nerve was 50 % reduced in comparison to liver tissue, but similar in brain tissue (26,27). The available data on peripheral nerve antioxidant concentrations in different pathological conditions have recently been reviewed (28).

To evaluate the contribution of oxidative stress to the development of experimental diabetic neuropathy, antioxidant concentrations have been measured in sciatic nerve homogenates of streptozotocin (STZ)-diabetic rats. Low and Nickander observed decreased Cu,Zn-SOD activity in the sciatic nerve four weeks after STZ injection (29). This effect could be prevented by insulin treatment. Nickander et al. extensively studied antioxidant levels in STZ-diabetic rat nerve (30). GSH levels were attenuated after one, but not after three months, while glutathione reductase and peroxidase remained unchanged. The diabetic animals had a five- to tenfold increase in sciatic nerve α-tocopherol concentrations. The same group demonstrated, that the decrease in GSH after four weeks of diabetes was associated with an increase in nerve oxidised glutathione (GSSG), and that these changes in GSH/GSSG balance did not occur after preventive treatment with α-lipoic acid (LA) (31). Hemeneno observed reduced sciatic nerve GPx activity after 7 and 21 days in alloxan-diabetic mice (32). GSH levels were not affected. Our own data showed a small increase in nerve catalase levels, a tendency towards increased total glutathione and GSSG levels, and no change in Cu,Zn-SOD in long-term (12-16 weeks) diabetic rats (19,33). The observation that the initial decrease in nerve GSH in diabetes disappears later or suggests that compensation mechanisms lead to increased GSH synthesis in long-term hyperglycemic rats.

In most of the studies that have focused on antioxidant changes in the diabetic nerve, the result of the toxic effects of ROS have been measured as lipid peroxidation. Conjugated dienes were initially found to be increased in diabetic nerve homogenates (29,34), but this observation was not confirmed in consecutive studies (30). Malondialdehyde (MDA), a degradation product of lipid peroxidation, was not increased in diabetic rat nerve (19,29,33). In contrast, we have demonstrated that in vitamin E-deficient rats, increased nerve MDA levels can be found without changes in nerve function, suggesting that oxidative damage to the nerve is not
invariably associated with functional deficits (33). In summary, these data suggest that either the currently used parameters for lipid peroxidation are insufficient to demonstrate increased oxidative damage in the diabetic nerve, or that endoneurial ROS-induced damage is relatively insignificant. However, the finding that in vitro lipid peroxidation in brain homogenates is increased in high glucose media (35) indicates that direct ROS-mediated damage in the diabetic nerve cannot be not excluded.

**DIABETIC MICROVASCULAR CHANGES AND OXIDATIVE STRESS**

Endoneurial capillary changes, attenuated endoneurial blood supply and nerve hypoxia have been demonstrated in both diabetic animals and patients (4,36,37). Microvascular changes are a major finding in diabetes. In several studies, the contribution of oxidative stress to alterations in blood vessel wall structure or reactivity has been shown. For example, decreased antioxidant levels, which were corrected by insulin treatment, have been observed in aortic endothelial cells from diabetic rabbits (38). Alterations in antioxidant activity in the blood vessel wall of diabetic rats could also be corrected by pancreatic islet transplantation (39). Furthermore, it has been demonstrated that the disturbed endothelium-dependent vascular relaxation of isolated aorta from diabetic rats can be corrected in the presence of SOD (40) or the antioxidant dimethyldithiothioure (41), and accentuated after exposure to a free radical generating system (42). Changes in cell proliferation, which are generally observed in endothelial cells cultured in high glucose media, could be prevented by antioxidants (43). In these cell cultures, alterations in antioxidant metabolism and increased H_2O_2-mediated cytotoxicity have been demonstrated (44-47). Although no studies have specifically focused on endoneurial blood vessels, there is no reason to suppose that nerve microcirculation is exempted from hyperglycaemia-induced oxidative alterations. As antioxidant treatment can prevent both changes in nerve function and nerve blood flow in experimental diabetes (31,48-50), there is at least indirect evidence that ROS-mediated endoneurial microvascular changes contribute to the development of diabetic neuropathy.

**NITRIC OXIDE**

Most studies evaluating the role of altered NO* status in diabetes and its contribution to the development of diabetic neuropathy have focused on vascular mechanisms. The formation of the endothelium-derived relaxing factor NO* by nitric oxide synthase (NOS) is NADPH-dependent, and increased GSH concentrations can stimulate NOS activity (51). Therefore, alterations in NADPH and GSH levels due to increased polyol pathway flux and changes in glutathione redox cycle may lead to attenuated NO* synthesis in diabetes. Furthermore, O_2^* inactivates NO*, and may cause both reduced availability of NO as a vasodilating factor as well as an increase production of the highly toxic peroxynitrite radical ONOO* (52). It has been shown that, despite increased vascular NO*-synthesis in diabetic endothelium, NO* -mediated relaxation in diabetic rat aorta in vitro is attenuated; relaxation could be corrected by the addition of SOD, which suggests a role for O_2^* in the inactivation of NO* in diabetes (40,53). Others have demonstrated that advanced glycosylation end products could quench NO*, and they suggested that this is a consequence of the formation of ROS during the glycosylation process (54). In contrast, but probably as a consequence of a different mechanism, it has been claimed that aminoguanidine, which inhibits the formation of advanced glycosylation end products, could inhibit NOS
activity (55).

Decreased synthesis or increased quenching of NO⁺ in diabetes, leading to reduced endoneurial microcirculation and functional nerve deficits, is a mechanism possibly linking oxidative stress to the NO⁺ pathway. It has been demonstrated that the beneficial effects of ARI treatment in diabetic rats on nerve blood flow and nerve function could be abolished by the simultaneous administration of an NO⁺-synthase inhibitor, and that long-term NO⁺-synthase inhibition in non-diabetic rats causes nerve conduction deficits (56-58). Interestingly, NO⁺-synthase inhibition in non-diabetic rats also caused reduced sciatic nerve Na⁺-K⁺-ATPase activity (58). Beneficial effects of the NO⁺-donor isosorbide nitrate on nerve conduction and nerve blood flow in diabetic rats have also been shown (59).

Most studies have focused on the vasodilating effects of NO⁺, and have shown that attenuated NO⁺ availability in diabetes may contribute to the development of neuropathy. Although NO⁺ probably plays a major role in the pathogenesis of neurodegenerative diseases (60, 61), no specific data on endoneurial NO⁺ in the diabetic nerve are available. In vitro studies have demonstrated that human neuroblastoma cells in high glucose media express reduced NO⁺-dependent cGMP production, suggesting that high glucose leads to neuronal cell NO⁺ deficit (62). These glucose-mediated effects could be corrected by the addition of myo-inositol, protein kinase-C agonists or sodium nitroprusside. Although additional studies should further elucidate the role of endoneurial NO⁺ in diabetic neuropathy, these data suggest that diabetes leads to reduced NO⁺ availability in nerve cells. Therefore, both endothelial and neuronal NO⁺ depletion, possibly as a consequence of increased O₂⁻ production, may contribute to the pathogenesis of diabetic neuropathy. This would implicate that NO⁺ as a free radical does not contribute to increased oxidative stress in diabetes, but that changes in NO⁺ status are rather a consequence of hyperglycaemia-induced ROS production.

**EXPERIMENTAL ANTIOXIDANT TREATMENT IN DIABETIC NEUROPATHY**

Clinical experience with antioxidant treatment in patients suffering from diabetic neuropathy is still limited. However, one promising study concerning the beneficial effects of the previously mentioned ROS scavenger LA on neuropathic symptoms in diabetic patients has been reported (63). The major problems in all clinical studies concerning diabetic neuropathy are the difficulty to obtain a homogeneous patient group, the large variety of parameters that may be evaluated during follow-up, and the long duration of experimental, placebo-controlled treatment.

In experimental models, usually in the STZ-diabetic rat, effects of several antioxidant drugs on nerve conduction velocity (NCV) and nerve blood flow (NBF) have been evaluated. These studies have demonstrated that several antioxidant compounds can both prevent and correct NCV and NBF deficits in hyperglycaemic rats (30, 31, 48-50, 64-69). We previously showed that GSH administration to STZ-diabetic rats could prevent, but not reverse sciatic NCV defects (65), but we did not measure NBF or oxidative stress-mediated damage. Four weeks of treatment with N-acetylcysteine (NAC), a hydrophilic antioxidant and sulphydryl donor, could reverse NCV deficits and sciatic NBF after one month of untreated diabetes (66). In the same study, improvement in sciatic nerve myelinated fibre regeneration was reported in the diabetic animals. Prevention and reversal of NCV deficits by NAC, as well as almost complete normalisation of sciatic nerve myelinated fibre, has also been
observed in combination with inhibition of enhanced serum tumor necrosis factor activity in diabetic rats (68). These data indicate, that NAC has systemic and neurovascular effects in experimental diabetes. However, the question whether these systemic effects lead to changes in nerve morphology, or whether additional direct endoneurial antioxidant effects occur, remains unanswered.

Cameron et al. have demonstrated a preventive effect of the antioxidant butylated hydroxytoluene on decreased NCV and NBF as well as on increased sciatic nerve hypoxic resistance in STZ-diabetes (64,67). They also demonstrated a beneficial effect of this drug on NCV and NBF in galactosaemic rats, which have increased flux in the first step of the polyol pathway only (67). Furthermore, using the ROS scavenger probucol, they observed a reversal of attenuated NCV and NBF in both STZ-diabetic rats as well as in rats treated with the pro-oxidant drug primaquin (48). In this study, beneficial effects of probucol were also observed on sciatic nerve oxygen tension. Similar reversal of decreased NCV and NBF in STZ diabetes was observed using the probucol analogue BM15.0639 (70). These effects could be abolished by the co-administration of a NO*-synthase inhibitor, suggesting that the beneficial effects of antioxidant treatment are mainly neurovascular.

As free metal ions contribute to the synthesis of ROS, metal chelators have been evaluated in experimental diabetic neuropathy. Both deferoxamine and trientine could rapidly reverse NCV and NBF deficits in experimental diabetes, after only two weeks of treatment (49). The beneficial effects of trientine on NCV and NBF have also been demonstrated in galactosaemic rats (67).

Vitamin E, the major lipophilic antioxidant that scavenges*OH radicals, could also prevent NCV and NBF decreases in STZ-diabetic rats (50). However, very high doses of 500 to 1000 mg/kg were necessary to obtain significant effects. Combined treatment of vitamin E with vitamin C was more effective than vitamin E alone, but vitamin C alone was ineffective. Others have shown that STZ-diabetic rats fed with a vitamin E-deficient diet had further decreased NCV in comparison to normally fed diabetic animals, suggesting that further enhancement of ROS activity can lead to additional nerve dysfunction (30).

Finally, LA treatment has also been evaluated in experimental diabetes (31). Although sciatic NBF was increased in diabetic animals in a dose-dependent manner, only interdigital nerve conduction deficits could be corrected, while sciatic-tibial and caudal NCV remained at diabetic levels. As LA has also been shown to improve neuropathic symptoms in diabetic patients (63), the outcome of this study is somewhat confusing. The authors demonstrated that sciatic nerve GSH levels were increased in the diabetic animals, which may also occur in more distally located nerves. However, the discrepancy between NBF and NCV correction in this study remains to be explained.

**CONCLUDING REMARKS**

The origin of increased production of ROS in diabetes probably involves several mechanisms. Enhanced oxidative stress contributes to the multifactorial pathogenesis of diabetic neuropathy, and can lead to alterations in neurovascular supply. Furthermore, although less evident, ROS may also directly affect the peripheral nerve in diabetes. Antioxidant treatment is beneficial in animal studies, leading to improvement of both NCV and NBF. The outcome of extensive trials should be awaited to inform us about the clinical usefulness of antioxidant treatment for the prevention and treatment of neuropathy in diabetic patients.
REFERENCES