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Research Article

Oxygen free radical activity is elevated in diabetes mellitus and has been implicated in the etiology of vascular complications. Recent studies have shown that impaired perfusion of nerve endoneurium is a major cause of nerve fiber dysfunction in experimental diabetes. Free radical scavenger treatment prevents the development of nerve conduction abnormalities in diabetic rats. In vitro experiments suggest that autoxidation reactions of glucose, catalyzed by free transition metal ions, are a potential source of free radicals in diabetes. We investigated whether chronic treatment with deferoxamine and trientine, transition metal chelating agents which can prevent autoxidation, could correct nerve conduction and blood flow changes in streptozotocin-diabetic rats. A 20% reduction in sciatic nerve motor conduction velocity after 2 mo diabetes was 90% ameliorated by 2 wk of treatment with deferoxamine or trientine. Sciatic endoneurial nutritive blood flow was 45% reduced by diabetes, but was completely corrected by treatment. In contrast, transition metal chelation had no effect on blood flow or conduction velocity in nondiabetic rats. Thus, the data support the hypothesis that increased free radical activity by glucose autoxidation as a result of impaired transition metal handling is a major cause of early neurovascular deficits in diabetes.

Neurovascular Dysfunction in Diabetic Rats

Potential Contribution of Autoxidation and Free Radicals Examined Using Transition Metal Chelating Agents

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Abstract

Oxygen free radical activity is elevated in diabetes mellitus and has been implicated in the etiology of vascular complications. Recent studies have shown that impaired perfusion of nerve endoneurium is a major cause of nerve fiber dysfunction in experimental diabetes. Free radical scavenger treatment prevents the development of nerve conduction abnormalities in diabetic rats. In vitro experiments suggest that autoxidation reactions of glucose, catalyzed by free transition metal ions, are a potential source of free radicals in diabetes. We investigated whether chronic treatment with deferoxamine and trientine, transition metal chelating agents which can prevent autoxidation, could correct nerve conduction and blood flow changes in streptozotocin-diabetic rats. A 20% reduction in sciatic nerve motor conduction velocity after 2 mo diabetes was 90% ameliorated by 2 wk of treatment with deferoxamine or trientine. Sciatic endoneurial nutritive blood flow was 45% reduced by diabetes, but was completely corrected by treatment. In contrast, transition metal chelation had no effect on blood flow or conduction velocity in nondiabetic rats. Thus, the data support the hypothesis that increased free radical activity by glucose autoxidation as a result of impaired transition metal handling is a major cause of early neurovascular deficits in diabetes. (*J. Clin. Invest.* 1995. 96:1159–1163.) Key words: blood flow • diabetes • free radicals • metal chelators • nerve conduction

Introduction

An early reduction in peripheral nerve conduction velocity (NCV)[1] in animal models of diabetes mellitus depends primarily on endoneurial hypoxia resulting from a deficit in nerve perfusion (1, 2). Decreased nerve blood flow and hypoxia have also been identified in diabetic patients with neuropathy (3). Recently, attention has focused on the role of oxygen free radicals (OFRs) in vascular dysfunction in diabetes, particularly regarding the destruction of nitric oxide (NO) and impairment of endothelium-dependent relaxation (2, 4, 5). Antioxidant treatment (6–8) prevents the development of NCV deficits in diabetic rats. In some experiments, the degree of protection was almost complete (7, 8), suggesting that OFRs have a central role in the etiopathogenesis of early diabetic neurovascular change.

Sources of increased free radical activity in diabetes include advanced glycosylation processes which follow from the nonenzymatic glycation reaction between glucose and amino groups of proteins (9, 10), altered prostanoid production (11) and ischemia/reperfusion effects (12, 13). Autoxidation reactions of molecules like glucose and vitamin C, catalyzed by small amounts of free transition metals such as iron and copper (14), are a powerful source of OFRs for in vitro experiments (15). Advanced glycosylation and autoxidation are closely interconnected, the OFRs accelerating the formation of advanced glycosylation products (AGPs), which in turn supplies more OFRs, the process being termed autoxidative glycosylation or glycoxidation (9, 14). The potential importance of autoxidation in vivo has mainly been inferred from in vitro experiments rather than direct observation. It is not known whether this mechanism contributes to neurovascular dysfunction in diabetes. Given that transition metal handling is impaired by diabetes (16) we investigated whether short term treatment with the transition metal chelators, deferoxamine and trientine, could correct established NCV and endoneurial blood flow deficits in streptozotocin-diabetic rats.

Methods

Experimental groups and diabetes induction. Male Sprague-Dawley rats (Aberdeen University breeding colony), 19 wk old at the start of the study were used. Nondiabetic animals acted as onset controls. Diabetes was induced by intraperitoneal administration of streptozotocin (Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK) at 40–45 mg/kg, freshly dissolved in sterile saline. Diabetes was verified 24 h later by estimating hyperglycemia and glycosuria (Visidex II and Diastix; Ames, Slough, UK). Diabetic rats were tested weekly and weighed daily. Animals were rejected if the plasma glucose concentration was < 20 mM or if body weight consistently increased over 3 d. Samples were taken from the carotid artery after final experiments for plasma glucose determination (GOD-Perid method; Boehringer Mannheim, Mannheim, Germany).

After 6 wk of untreated diabetes, groups of rats were treated for a

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1. *Abbreviations used in this paper:* AGP, advanced glycosylation end product; ARI, aldose reductase inhibitor; NCV, nerve conduction velocity; NO, nitric oxide; OFR, oxygen free radical.

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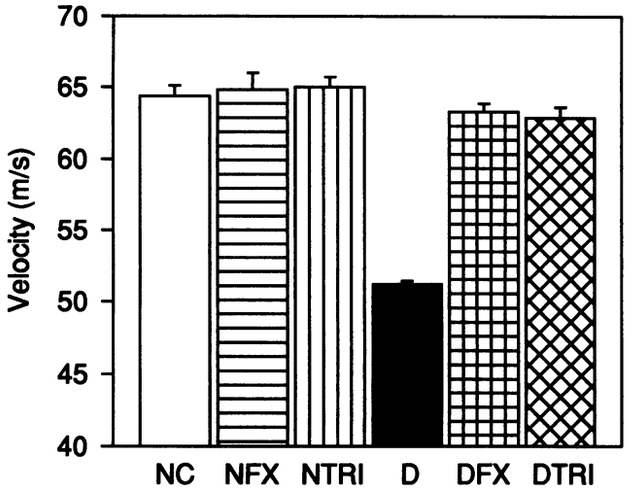

Figure 1. Sciatic motor nerve conduction velocity in nondiabetic and diabetic rats treated with deferoxamine and trientine. *NC*, nondiabetic controls, $n = 10$; *NFX*, nondiabetic group treated with deferoxamine (8 mg/kg) for 2 wk, $n = 8$; *NTRI*, nondiabetic group treated with trientine (20 mg/kg) for 2 wk, $n = 8$; *D*, 8 wk diabetic group, $n = 10$; *DFX*, diabetic group untreated for 6 wk and then treated with deferoxamine (8 mg/kg) for a further 2 wk, $n = 12$; *DTRI*, diabetic group untreated for 6 wk and then treated with trientine (20 mg/kg) for a further 2 wk, $n = 10$. Data are means + SE.

further 2 wk with deferoxamine mesylate (Sigma, Poole, Dorset, UK) by daily subscapular subcutaneous injection of 8 mg/kg freshly dissolved in sterile saline, or with trientine (triethylenetetramine dihydrochloride; Sigma) dissolved in the drinking water such that the daily dose was 20 mg/kg. To ascertain whether transition metal chelation had any effect on neurovascular function in the absence of diabetes, deferoxamine (8 mg/kg) or trientine (20 mg/kg) treatments were given for 2 wk to separate groups of nondiabetic rats.

Sciatic motor conduction velocity and endoneurial blood flow. At the end of the treatment period, rats were anesthetized with thiobutabarbitone (Zeneca) by intraperitoneal injection (50–100 mg/kg). The trachea was cannulated for artificial ventilation and a carotid cannula was used to monitor mean systemic blood pressure. Motor NCV was measured as previously described (7) between sciatic notch and knee in the nerve branch to tibialis anterior muscle, which is representative of the whole sciatic nerve in terms of susceptibility to diabetes and treatment effects. In this model, NCV effects are established within 2 wk of diabetes induction, and have stabilized by 1 mo without further significant alteration for at least 4 mo (17, 18). For a subset of rats in nondiabetic, diabetic and deferoxamine-treated diabetic groups, sensory saphenous NCV was also measured (7). Rectal and nerve temperatures were monitored, and regulated between 36.5 and 37.5°C.

Sciatic endoneurial blood flow was measured in the contralateral leg using microelectrode polarography and hydrogen clearance as previously described (8, 17). Briefly, core temperature of the rat was monitored and regulated between 37 and 38°C, using a rectal probe and radiant heat. The skin around the sciatic nerve incision was sutured to a metal ring and used to form a pool which was filled with mineral oil at 37°C to a depth of at least 1 cm to minimize gas diffusion. Rats were given neuromuscular blockade using d-tubocurarine (Sigma, 2 mg kg^{-1} via the carotid cannula) and artificially ventilated. The level of anesthesia was monitored by observing any reaction of blood pressure to manipulation, and supplementary anesthetic was given as necessary. A glass-insulated platinum microelectrode (tip diameter 2–8 μm, 45° bevel) was inserted into the middle portion of the sciatic nerve, above its trifurcation, and polarized at 0.25 V with respect to a subcutaneous reference electrode. 10% H_2 was added to the inspired gas, the proportions of O_2 and N_2 being adjusted to 20 and 70%, respectively. When the H_2 current recorded by the electrode had stabilized (15–40 min), indicating equilibrium with arterial blood, the H_2 supply was shut off and N_2 delivery was increased appropriately. The H_2 clearance curve was recorded until baseline values were reached (25–75 min), the latter being defined as no systematic decline in electrode current over 5 min. This procedure was then repeated at another nerve site. After the experiment, clearance curves were digitized and monoexponetial or biexponential curves were fitted to the data by computer using nonlinear regression software that employed the Marquardt algorithm and the least squares method for optimizing goodness-of-fit (Inplot, Graphpad, San Diego, CA). The slow exponent, representing nutritive (capillary) flow (17, 19, 20), was accepted. Vascular conductance was calculated by dividing flow by the mean arterial blood pressure during the recording period.

Table I. Body Weights and Plasma Glucose Concentrations

Group	*n*	Body weight (g) Start	Body weight (g) End	Plasma glucose (mmol/l)
Nondiabetic control	10	452±8		7.6±0.5
Nondiabetic + 2 wk deferoxamine	8	465±5	474±6	7.6±0.5
Nondiabetic + 2 wk trientine	8	469±12	480±11	8.3±0.5
8 wk diabetic	10	461±9	314±11	40.1±2.5
6 wk diabetic + 2 wk deferoxamine	12	441±8	303±6	39.6±1.7
6 wk diabetic + 2 wk trientine	10	451±8	307±12	40.3±1.8

Data are mean±SE.

Statistical analysis. Data are expressed as mean±SE. Using standard statistical software (Inplot, Graphpad, San Diego, CA), data were first subjected to Bartlett's test for homogeneity of variances and were given a log transformation if necessary. One-way ANOVA was then performed, followed by the Student-Newman-Keuls test to estimate the significance of differences for individual between-group comparisons. $P < 0.05$ was considered statistically significant.

Results

Diabetic rats were hyperglycemic and lost weight over the experimental period (Table I). This was not affected by deferoxamine or trientine treatments. Data for sciatic motor NCV are shown in Fig. 1. There was a 20.4% ($P < 0.001$) reduction with untreated diabetes, which was ameliorated by both deferoxamine and trientine treatments to the extent of 91.9% ($P < 0.001$) and 88.8% ($P < 0.001$), respectively. The resultant NCV values were not significantly different from those of the nondiabetic control group. Deferoxamine or trientine treatment of nondiabetic rats did not affect NCV. Saphenous sensory NCV was also measured in a subset ($n = 8$) of nondiabetic, diabetic and deferoxamine treated diabetic groups. Sensory NCV was reduced from 60.2±0.4 m/s to 51.5±0.5 m/s ($P < 0.001$) by untreated diabetes. With deferoxamine treatment, sensory NCV was 59.7±0.8 m/s, not significantly different from the nondiabetic control group and improved ($P < 0.001$) compared with untreated diabetes.

Nutritive endoneurial blood flow (Fig. 2, *upper panel*) was 44.8% ($P < 0.001$) reduced by diabetes. This was completely restored to the nondiabetic range by both deferoxamine ($P < 0.001$) and trientine ($P < 0.001$) treatments. Neither deferox-

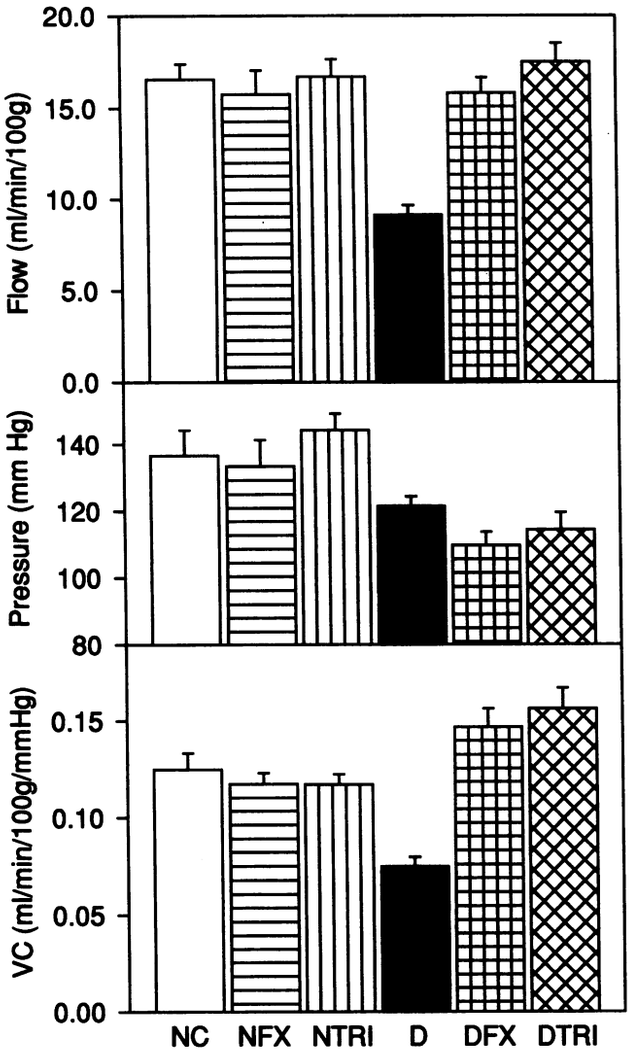

Figure 2. Sciatic nutritive endoneurial blood flow, mean systemic arterial blood pressure, and endoneurial vascular conductance (VC) in nondiabetic and diabetic rats treated with deferoxamine and trientine. *NC*, nondiabetic controls, $n = 10$; *NFX*, nondiabetic group treated with deferoxamine (8 mg/kg) for 2 wk, $n = 8$; *NTRI*, nondiabetic group treated with trientine (20 mg/kg) for 2 wk, $n = 8$; *D*, 8 wk diabetic group, $n = 10$; *DFX*, diabetic group untreated for 6 wk and then treated with deferoxamine (8 mg/kg) for a further 2 wk, $n = 12$; *DTRI*, diabetic group untreated for 6 wk and then treated with trientine (20 mg/kg) for a further 2 wk, $n = 10$. Data are means + SE.

amine nor trientine altered blood flow in nondiabetic rats. There was some indication of reduced systemic arterial pressure (Fig. 2, *middle panel*) in the diabetic groups, in agreement with previous findings (8, 17), although this only reached statistical significance ($P < 0.05$) for deferoxamine treatment compared with both treated and untreated nondiabetic groups and between trientine treated nondiabetic and diabetic groups ($P < 0.01$). Variations in blood pressure are taken into account when data are expressed as sciatic nutritive vascular conductance (Fig 2, *lower panel*). With untreated diabetes, there was a 39.9% ($P < 0.001$) conductance deficit compared with the nondiabetic control group. Both deferoxamine and trientine treatment tended to cause supranormal conductance, which was statistically significant for the latter group ($P < 0.05$). Deferoxamine and trientine did not significantly alter vascular conductance in nondiabetic rats.

Discussion

The data show for the first time that compounds with a common action as chelating agents correct the early NCV and perfusion deficits in diabetic rats. Reduced nerve blood flow has been characterized using a variety of invasive and non-invasive techniques in diabetic rats, including hydrogen clearance (8, 17, 20–24), laser-Doppler flowmetry (24–28), and [^{14}C]butanol (29) or [^{14}C]iodoantipyrine (28) accumulation. Hydrogen clearance measures give an estimate of nutritive (capillary) flow, which is crucial for neuronal function. Impaired nutritive perfusion is an early event, preceding or paralleling the development of NCV abnormalities (17). Further evidence for a neurovascular etiology includes prevention of early NCV changes by peripheral vasodilators (2) or chronic hyperbaric oxygenation (1). Moreover, diabetes-like NCV deficits are produced in nondiabetic rats by drugs causing vasa nervorum constriction (8, 30) or by chronic exposure to reduced atmospheric oxygen (1). Parallels may be drawn with the situation in diabetic neuropathic patients, where sural nerve perfusion is impaired, the endoneurium is hypoxic, and NCV is improved by vasodilator treatment (3).

The high degree of correction of NCV and perfusion abnormalities by deferoxamine and trientine and the relative lack of effect on flow in nondiabetic rats suggests that impaired transition metal handling in diabetes (16) is likely to be an important cause of early oxidative stress related vascular abnormalities. Iron and copper are particularly potent catalysts of OFR formation by glucose autoxidation in vitro (9, 14). However, the limited selectivity of the chelating agents used in this in vivo study precludes determination of which, if any, of these or other metals may be most critical for neurovascular dysfunction. In nondiabetic rats, natural defence mechanisms (superoxide dismutase, catalase, glutathione peroxidase and free radical scavengers such as vitamins C and E) are sufficient to limit OFR activity under normoglycemic conditions. In contrast, the combined effect of hyperglycemia and increased free transition metals (16, 31) in diabetes is exacerbated by impaired defences. For example, superoxide dismutase levels are reduced in sciatic nerves of diabetic rats (32). Vascular endothelium appears to be a vulnerable target for hyperglycemia-induced metabolic changes (2, 4, 5, 11). Endothelial cells cultured under high glucose conditions have a diminished GSH content which increases susceptibility to peroxide-induced damage (33). Defective NO-mediated endothelium-dependent relaxation in aortas from diabetic rats, or from nondiabetic rabbits acutely exposed to high glucose in vitro is corrected by free radical scavengers (4, 11). This is likely to be relevant for vasa nervorum as perfusion deficits in diabetic rats were prevented by the lipophilic free radical scavenger, probucol (8). In nondiabetic rats, pro-oxidant treatment caused a reduction in nerve blood flow, diminished endoneurial oxygen tension and NCV abnormalities similar to those found in diabetes (8), further emphasizing the link between elevated free radical activity and neurovascular dysfunction. The low chelator dose (< 20 mg/kg) used, compared with that needed for comparable NCV effects with free radical scavengers (500–1,000 mg/kg for butylated hydroxytoluene, probucol and vitamin E (2, 7, 8), suggests that preven-

tion of OFR generation via improved transition metal handling in diabetes could be a better therapeutic proposition than scavenging the OFRs once formed.

The nutritive flow deficit was corrected by trientine and deferoxamine, despite a tendency for reduced blood pressure in diabetic rats. As there is little pressure autoregulation by vasa nervorum (1) maintenance of flow requires a greater than normal vasodilation, reflected in the high vascular conductance values. This effect was also seen for aldose reductase inhibitor (ARI) treatment, particularly in a reversal experimental design (2, 24). One plausible explanation is that treatment revealed an adaptation to the initial period of untreated diabetes, consisting of an upregulation of basal NO release, which is normally masked by increased NO destruction due to elevated OFR activity. Such an effect was noted for aortas from diabetic rats *in vitro* when superoxide dismutase was added to the bathing fluid (4).

Endothelium-dependent relaxation defects in diabetes are prevented by treatment with OFR scavengers, ARIs, and aminoguanidine (4, 5, 11, 34). Although experiments were performed on large vessels in vitro or the general circulation *in vivo*, the mechanisms are probably relevant for nerve blood flow changes. Thus, these agents prevent endoneurial blood flow deficits in diabetic rats (8, 22, 24). A deficiency in the NO system, probably resulting from elevated OFR activity, causes a marked increase in vasa nervorum epi/perineurial vessel reactivity to norepinephrine in vivo (2). This may be mimicked acutely by NO synthase inhibition in nondiabetic rats (2), chronic treatment causing reduced NCV (30). Polyol pathway blockade may also improve OFR scavenging. Conversion of glucose to sorbitol by aldose reductase consumes NADPH, which is required for GSH formation by glutathione reductase. The resultant reduction in GSH concentration, which also leads to superoxide dismutase downregulation (35), would compromise OFR protection. This chain of events is corrected by ARIs, which may, therefore, have an indirect antioxidant action (2, 24). NCV effects of ARIs are prevented by NO synthase inhibitor cotreatment (36), providing further evidence of polyol pathway involvement in oxidative stress-related diabetic NO deficits.

Aminoguanidine inhibits AGP formation. However, the intimate interaction with oxidative stress means that it is difficult to determine whether AGPs have a direct neurovascular action as opposed to an indirect effect as a source of OFRs. AGP reactions are enhanced by OFRs and their progression produces OFRs. Some of these reactions are catalyzed by transition metals (9), therefore, the effects of deferoxamine and trientine might, at least in part, depend on a reduction of the AGP process, hence reducing OFR production. Conversely it could be argued that the main effect of antioxidant treatment in vivo is to slow AGPs formation rather than a more direct action, for example to attenuate OFR degradation of NO. AGPs have been shown to quench NO in vitro (34). The impaired depressor response to acetylcholine in diabetic rats is prevented by aminoguanidine treatment (34). To explain the latter finding it was postulated that a buildup of AGPs in subendothelial collagen neutralized NO released by the endothelium before it reached vascular smooth muscle (34). However, AGP accumulation sufficient to affect the depressor response to acetylcholine took at least 1 month to develop, therefore, this mechanism is unlikely to play a major role in the very early neurovascular defects. Reduced endoneurial blood flow develops to at least 90% of its chronic extent within 7 d of diabetes induction (17). In addition, the rapid reversal of NCV and perfusion deficits by deferoxamine and trientine is incompatible with expectations based on the longevity of AGPs and a low collagen turnover rate (34). Thus, any AGP involvement in early neurovascular changes, evidenced by the preventive effect of aminoguanidine (22, 37), would probably result indirectly from OFR generation. However, a putative influence of rapid intracellular AGP formation by reactive glucose metabolites (10) for proteins with a rapid turnover cannot be ruled out. Fructose, produced by the polyol pathway, is a potent glycosylating agent (10) which could potentially provide a link between ARI effects, aminoguanidine's anti-AGP action, and oxidative stress in neurovascular function. A further plausible explanation for the vascular effects of antioxidants and aminoguanidine relates to prevention of LDL oxidation (38), which is toxic to endothelial cells and would trigger dysfunction. Aminoguanidine also inhibits inducible and brain constitutive NO synthase isoforms in vitro, an action that was put forward to explain the beneficial microvascular effects of treatment as an alternative to inhibition of AGP formation (39). However, aminoguanidine does not acutely affect endothelium-dependent vessel relaxation in vitro (2). Chronic treatment does not alter blood pressure (39), in contrast to conventional NO synthase inhibitors such as N^G-nitro-λ-arginine or its methyl ester, which cause a marked and sustained elevation (30, 36). Thus, it is unlikely that aminoguanidine inhibits the endothelial constitutive isoform of NO synthase in vivo. In addition, aminoguanidine has opposite effects to NO synthase inhibitors on nerve blood flow and NCV in diabetic rats and does not alter these parameters in the absence of diabetes (2, 22, 30, 37). Therefore, the neurovascular actions of aminoguanidine are likely to depend on prevention of AGP formation and consequent sequelae that would otherwise impair NO-mediated endothelium-dependent vasorelaxation.

In addition to the NO system, other endothelium-related changes deleteriously affect nerve perfusion in diabetes. OFR-related mechanisms may also be implicated which could, therefore, be amenable to deferoxamine or trientine treatments. Thus, impaired vasa nervorum prostacyclin synthesis (12) could partially depend on high levels of lipid peroxidation (40). Vasoconstrictor angiotensin II (25) and endothelin-1 (23) activities are elevated. Antioxidant treatment prevents an increase in plasma angiotensin converting enzyme (8). OFR-mediated endothelial damage stimulates endothelin-1 production (41).

In conclusion, the short-term neurovascular changes which cause nerve dysfunction in experimental diabetes can be reversed by transition metal chelator treatment. This suggests that impaired transition metal homeostasis plays an important role in elevated free radical generation. It is plausible that chelators could have a therapeutic role in the chronic neuropathic and micro/macrovascular changes in diabetic patients, which requires study in clinical trials. In addition to early neurovascular deficits highlighted in this report, elevated OFR activity may, via lipid peroxidation, cause chronic cumulative damage to nerve fibers (32), as well as being involved in vessel atherosclerosis (42).

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