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Anti-oxidant treatment prevents the development of peripheral nerve dysfunction in streptozotocin-diabetic rats

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Summary. We tested the notion that oxidative stress makes an important contribution to the aetiology of diabetic neuropathy. The effect of treatment with a 1% dietary supplement of the anti-oxidant butylated hydroxytoluene was studied during 2 months of streptozotocin-induced diabetes mellitus. In final experiments, sciatic motor and saphenous sensory conduction velocities were measured in vivo, and resistance to hypoxic conduction failure for sciatic trunk was examined in vitro. There were 20% and 12% decreases in motor and sensory conduction velocity, respectively after 2 months of diabetes ($p < 0.001$). These were completely prevented by butylated hydroxytoluene treatment ($p < 0.001$). Resistance to hypoxic conduction failure, shown by the time taken for sciatic compound action potential amplitude to decline by 80%, was 55% increased by diabetes, and this was limited to 31% ($p < 0.01$) by treatment. There were no significant effects of treatment on the 9–10 fold elevation of sciatic nerve sorbitol and fructose levels with diabetes, or on the non-significant 22% reduction in myo-inositol content. Butylated hydroxytoluene treatment also did not affect sciatic nerve capillary density. We conclude that oxidative stress makes an important contribution to the aetiology of early experimental diabetic neuropathy. Amelioration of oxidative stress could potentially be a final common mechanism whereby a number of diverse treatments exert a beneficial effect on diabetic nerve function.

Key words: Neuropathy, nerve conduction, ischaemic resistance, oxidative stress, butylated hydroxytoluene, polyol pathway, streptozotocin, diabetic rat.

An early reduction in nerve conduction velocity (NCV) and an increase in resistance to ischaemic conduction failure (RICF) are observed in rats following induction of diabetes mellitus. An important aetiological contribution comes from the vascular effects of diabetes, leading to hypoperfusion of the vasa nervorum and the development of a hypoxic microenvironment for neurons and Schwann cells [1]. Nerve dysfunction may be prevented or corrected by vasodilator treatment which restores endoneurial blood flow [2–5]. However, other seemingly unrelated treatments are also effective and a number of alternative mechanisms have been suggested to underlie the aetiology of diabetic neuropathy. Thus, nerve function is improved by chronic electrical stimulation [6], evening primrose oil [7–9], aminoguanidine (10, 11), and aldose reductase inhibitors [12, 13]. Evening primrose oil is a complex natural product which could have several potential actions ranging from alterations in cell membrane fluidity to promotion of vasoactive prostanoid synthesis [7]. Aminoguanidine inhibits the formation of advanced glycation end-products [10, 14, 15]. Under some conditions aminoguanidine can also function as an aldose reductase inhibitor [16] and has also been suggested to be an inhibitor of nitric oxide synthase [17]. Aldose reductase inhibitors reduce the metabolic consequences of elevated polyol pathway flux [12, 13]. They may also correct abnormalities in phosphoinositide metabolism and nerve Na^+-K^+-ATPase activity [18], although considerable doubt has been cast over these putative effects [3, 19, 20]. Aldose reductase inhibitors may also have vascular actions, which improve nerve blood flow [21] and vascular smooth muscle relaxation [22].

Thus, multiple therapeutic strategies correct nerve dysfunction in the early stages of experimental diabetes. It is possible to argue that a vascular action underlies at least some of these diverse treatments, and a common endpoint may be the amelioration of oxidative stress. Several factors promote oxidative stress in diabetes, including nerve ischaemia-reperfusion [23, 24], increased free radical production caused by autoxidation reactions of sugars with proteins and unsaturated lipids [14], and impairment of tissue anti-oxidant protection systems [25–27].

Anti-oxidants such as glutathione, vitamin E, and butylated hydroxytoluene (BHT) prevent or slow the develop-

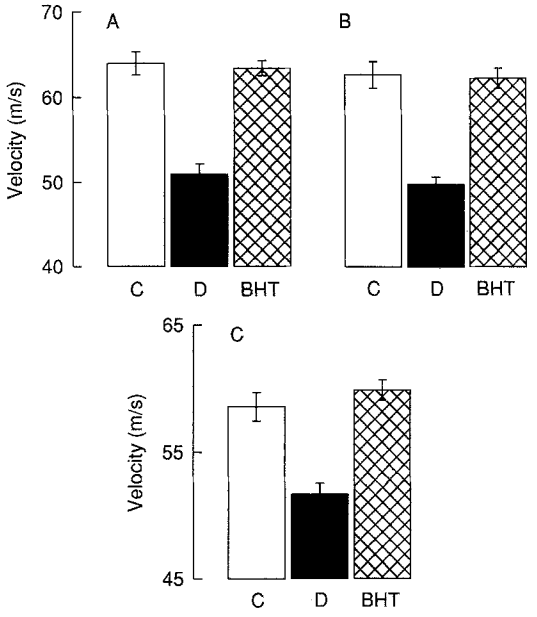

Fig. 1 A–C. Conduction velocity in sciatic motor nerves supplying **A**, gastrocnemius; **B**, tibialis anterior muscles; and **C**, sensory saphenous nerve. Bars show means ± SEM. C, Control rats, $n = 20$; D, diabetic control rats, $n = 19$; BHT, butylated hydroxytoluene-treated diabetic rats, $n = 11$. Statistical analysis: for all three NCV measurements D vs C, $p < 0.001$; D vs BHT, $p < 0.001$; BHT vs C, NS

ment of opacity in rats lenses exposed to high glucose or galactose concentrations in vitro and in vivo, with little effect on the accumulation of polyol pathway metabolites [28–31]. Cumulative cell damage due to oxidative stress could be responsible for the progressive deterioration of function in tissues other than lens in diabetes [14]. To test the applicability of this hypothesis to peripheral nerve, the effect of a BHT dietary supplement in preventing NCV and RICF abnormalities was examined during 2 months of streptozotocin-induced diabetes.

Materials and methods

All experiments were carried out on mature male Sprague-Dawley rats (Aberdeen University colony), 19 weeks old at the start of the study. One group of non-diabetic rats was used as controls. Two other groups were given 40 mg/kg i. p. streptozotocin (Sigma, Poole, Dorset, UK) in 20 mmol/l sodium citrate buffer (pH 4.5). Diabetes was verified 24 h later by estimating hyperglycaemia and glycosuria (Visidex II and Diastix; Ames, Slough, Bucks., UK), and was monitored at weekly intervals. Body weights were measured daily, and rats were rejected if blood glucose levels in the fed state were less than 20 mmol/l, or if there was a sustained increase in body weight over 3 consecutive days. Samples for non-fasted plasma glucose measurement using a standard test kit (GOD-Period method; Boehringer Mannheim, Mannheim, FRG) were taken on the day of final experiments. One group of diabetic rats was untreated for 2 months to act as diabetic controls. The other diabetic group was treated with a 1 % BHT (Sigma) dietary supplement added to the rat chow. BHT treatment proved to have a partial protective effect against induction of diabetes by streptozotocin. In a first group of 12 rats, treatment was started immediately following streptozotocin injection and, although all animals showed varying degrees of hyperglycaemia, six rats failed to reach a criterion plasma glucose of 20 mmol/l or more and were rejected from the experiment. In a second group of six rats, treatment was started 3 days after diabetes induction and only one rat was rejected as not being sufficiently diabetic, which is not exceptional. The early protective effect of BHT on diabetes induction is likely to be due to neutralisation of free radical production by streptozotocin, which is thought to underlie the destruction of beta cells [32].

In final experiments (1–1.5 g/kg i. p. urethane anaesthesia), NCV was measured in vivo between sciatic notch and knee for motor branches supplying tibialis anterior and gastrocnemius muscles. Sensory NCV was measured in the saphenous nerve between groin and ankle. Methods have been previously described in detail [13].

RICF was measured in vitro as previously described [7]. Briefly, the sciatic trunk was removed and mounted on bipolar stimulating (proximal) and recording (distal) electrodes. The nerve was equilibrated in Krebs' solution at 35 °C gassed with 95 % O_2 / 5 % CO_2 for 30 min, and then transferred to mineral oil pre-gassed with 100 % N_2 for 1 h. Nerves were stimulated with supramaximal pulses (1 Hz, 50 μs width, 10 mA) and compound action potential amplitude was monitored at 2-min intervals until it fell below 10 % of its initial value.

Nerve capillarization was estimated from 10 μm frozen sections of sciatic trunk in which the capillary endothelium was stained for alkaline phosphatase, as previously described [7]. Fascicle outlines were traced on a projection microscope and their areas were measured with a digitizing pad linked to a microcomputer to calculate capillary density.

Sciatic nerve sugars and polyols were determined by gas chromatography of trimethylsilyl derivatives prepared from aqueous deproteinized extracts [33].

Statistical analysis

Data are expressed as means ± SEM. One way analysis of variance was performed, and any significant ($p < 0.05$) differences were assigned to individual between-group comparisons using Student's *t*-tests, applying the Bonferroni correction for multiple comparisons (Instat; GraphPad, San Diego, Calif., USA).

Results

Diabetic control and BHT-treated diabetic groups had final plasma glucose levels of 32.6 ± 2.3 and 28.7 ± 1.7 mmol/l, respectively compared to a control value of 7.2 ± 0.2 mmol/l. Both diabetic groups lost weight, from a starting weight of 485 ± 9 to 370 ± 9 g for diabetic control rats, and from 489 ± 11 g to 376 ± 10 g for BHT-treated rats.

Measurements of NCV are shown in Figure 1. For motor nerves supplying tibialis anterior (Fig. 1 A) and gastrocnemius (Fig. 1 B) muscles, NCV was reduced by 20.5 % on average compared with onset controls ($p < 0.001$ for both branches). BHT treatment completely prevented this decline in NCV ($p < 0.001$), values remaining at the onset control level. Previous work has shown that no significant increase in NCV over the 2-month experimental period would be expected in controls [13]. For sensory fibres in saphenous nerve (Fig. 1 C) there was an 11.7 % reduction in NCV after 2 months diabetes ($p < 0.001$). However, with BHT treatment, sensory NCV was in the non-diabetic range, significantly improved compared to untreated diabetes ($p < 0.001$).

Data for hypoxic resistance, measured in vitro, are plotted in Figure 2. There were no significant differences in initial compound action potential amplitude between the three groups, which were 3.5 ± 0.3, 4.0 ± 0.4, and

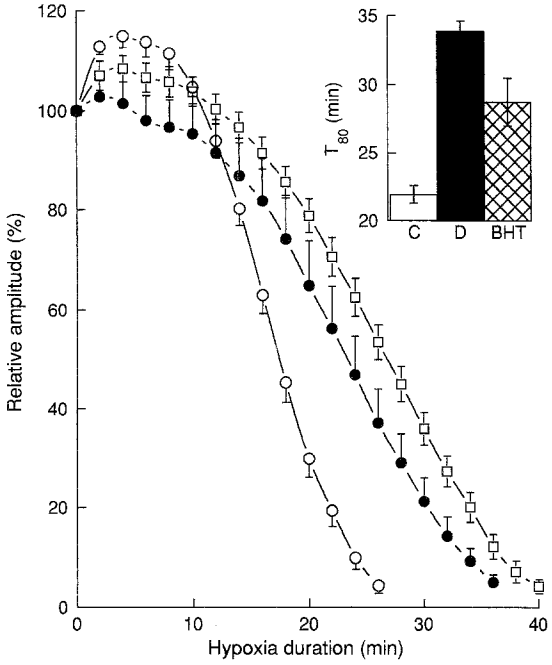

Fig. 2. Percentage change in sciatic nerve compound action potential amplitude with duration of hypoxia in vitro. Symbols and error bars show group means ± SEM. Control rats (○), $n = 20$; diabetic control rats (□), $n = 19$, butylated hydroxytoluene-treated diabetic rats (●), $n = 11$. The inset histogram shows the durations of hypoxia taken for compound action potential amplitude to decline by 80% (T_{80}) for control (C), diabetic control (D), and the butylated hydroxytoluene-treated diabetic rats (BHT). Statistical analysis: D vs C, $p < 0.001$; D vs BHT, $p < 0.01$; BHT vs C, $p < 0.001$

2.9 ± 0.2 mV, respectively for control, diabetic and BHT-treated diabetic animals. With increasing hypoxia duration, compound action potential amplitude declined after an initial period of hyperexcitability [34]. The rate of decline was much more rapid in nerves from control than diabetic rats. Relative compound action potential amplitude was significantly ($p < 0.05$) elevated by untreated diabetes at all time points sampled from 16 min. For the BHT-treated diabetic group, compound action potential amplitude was reduced at a rate between that of non-diabetic and diabetic controls. The BHT-treated diabetic group showed significant ($p < 0.05$) differences from the diabetic control group at all time points after 22 min. The inset histogram in Figure 2 shows hypoxia durations for an 80% decline in compound action potential amplitude (T_{80}), calculated by linear interpolation from the curves for individual nerves. T_{80} was 55% elevated by diabetes ($p < 0.001$) but this was restricted to 31% by BHT treatment ($p < 0.001$ vs non-diabetic control group; $p < 0.01$ vs diabetic control group).

Table 1 shows data for sciatic nerve capillary density and polyol levels. Endoneurial capillary density was unaffected by diabetes compared to control values and BHT treatment had no significant effect on capillarization. Sorbitol and fructose levels were elevated nine- and ten-fold by diabetes. BHT treatment had no significant effect. There was a 22% deficit in myo-inositol with diabetes which did not attain statistical significance and was unaffected by treatment.

Discussion

The data clearly demonstrate that anti-oxidant treatment with BHT largely prevents the development of nerve dysfunction in experimental diabetes. The effect occurred in the absence of changes in nerve polyol pathway metabolites or endoneurial capillarization, suggesting that BHT was not acting as an aldose reductase inhibitor [12, 13] or a general vasodilator [3, 4]. When treatment was instigated after the action of streptozotocin was completed, BHT had no effect on the severity of diabetes, shown by plasma glucose levels, body weight loss and sciatic nerve polyol concentrations. A recent report [35] suggests that anti-oxidant treatment with glutathione also partially prevents the development of reduced NCV.

Neurological changes in experimental diabetes are unlikely to result from a cytotoxic action of streptozotocin, for example due to free radical activity, as they are prevented by insulin treatment [36] and are also found in pancreotomised [37] or spontaneously diabetic rats [38, 39]. Streptozotocin is rapidly metabolised [32] and any direct effect would be expected to occur while it was present in the circulation. However, NCV changes in our model develop slowly over 4 weeks [13]. Furthermore, electrical stimulation and some pharmacological treatments rapidly and reversibly correct NCV deficits even after several months of streptozotocin-diabetes [6, 40].

The effects of BHT on NCV and RICF are properties shared with several other treatments, including vasodilators [3, 4], chronic electrical stimulation [6, 41], evening primrose oil [7], and aldose reductase inhibitors [42, 43]. Aminoguanidine improved NCV but not RICF [10, 11]. The mechanism(s) underlying these diabetic deficits are controversial. Lattimer and co-workers [18] suggested that both NCV and RICF abnormalities are caused by elevated polyol pathway flux, reduced myo-inositol in-

Table 1. Sciatic nerve capillary density and polyol levels in rats

Group	Capillary density (mm^{-2})	Sorbitol	Fructose	Myo-inositol
		(μmol/g nerve wet weight)		
Control ($n = 10$)	61.21 ± 1.51	0.156 ± 0.011	0.456 ± 0.039	2.298 ± 0.116
Diabetic control ($n = 10$)	56.65 ± 2.25	1.494 ± 0.200[b]	4.628 ± 0.272[b]	1.797 ± 0.064
Butylated hydroxytoluene-treated diabetic ($n = 10$)	60.52 ± 2.57	0.931 ± 0.192[a]	3.897 ± 0.683[b]	1.759 ± 0.250

[a] $p < 0.01$, [b] $p < 0.001$ compared to control group
Data are group means ± SEM.

corporation into membrane phosphoinositides, and impaired Na^+-K^+-ATPase activity. The results for BHT, in common with those for some vasodilators [3, 4], evening primrose oil [9], and aminoguanidine [11] do not support this hypothesis as no effects on polyol pathway metabolites or nerve myo-inositol were noted, and where studied, Na^+-K^+-ATPase activity was not improved [3,44]. A more plausible hypothesis is that NCV and RICF changes result from reductions in nerve blood flow leading to endoneurial hypoxia [3, 45]. Correction of blood flow by vasodilator treatment restores NCV in diabetic rats [2]. Increased RICF may reflect an adaptation to endoneurial hypoxia, which depends on greater reliance on anaerobic metabolism [3, 45]. Once established, the phenomenon is not readily reversible by vasodilator or aldose reductase inhibitor treatments [3, 43], although further progression is slowed. However, electrical stimulation to increase nerve activity, which would increase reliance on oxidative metabolism [46], does reverse RICF [41]. The data suggest that amelioration of oxidative stress could provide a common mechanism to account for the functional effects of these diverse manipulations on diabetic nerve.

There are several causes of increased oxidative stress in diabetes. Elevated polyol pathway activity decreases glutathione levels because glutathione reductase requires NADPH [47]. Aldose reductase also uses NADPH, thus, there is competition for available co-factor. Glutathione is a substrate for the glutathione peroxidase-mediated neutralization of hydroperoxides. In addition, levels of superoxide dismutase, an important anti-oxidant enzyme, are reduced by diabetes [26, 27] and this is related to changes in glutathione. Impaired blood flow would also contribute, as endoneurial hypoxia would increase production of oxygen free radicals via xanthine oxidase [24].

Nerve dysfunction in diabetes as a result of increased free radical activity could arise because of direct effects on neurons and Schwann cells, or indirectly by actions on their microenvironment and vascular supply or both. Direct effects could involve an increase in lipid peroxidation of axonal and Schwann cell membranes resulting in impaired function. Low and co-workers [10, 27] found increases in sciatic nerve conjugated dienes with streptozotocin-diabetes. Important membrane proteins could also be damaged, or modified in conjunction with hyperglycaemia, as oxidative stress increases the rate of formation of advanced glycation end-products [14]. Whilst these products have been demonstrated to accumulate in myelin [48], there is at present no evidence that they have a functional effect. However, McLean and coworkers [49] have shown non-enzymatic glycation of sciatic nerve tubulin in experimental diabetes, which could have important consequences for axonal transport and integrity.

There is substantial evidence for potentially relevant vascular effects of oxidative stress, which may act at several levels. LDL, which is elevated in diabetes, inhibits endothelium-dependent relaxation of vascular tissue [50]. When oxidised, lipoproteins are cytotoxic to many cell types including endothelial cells [51]. BHT, which is highly lipid soluble, is effective in preventing LDL oxidation and subsequent endothelial cell toxicity [52]. Oxidised LDL may also play an important role in the development of atherosclerosis in diabetes [53]. Thus, resistance vessel atherosclerosis, and endothelial damage which develops early in experimental diabetes [54], could contribute to the reduction in endothelial synthesis of local vasodilators and platelet anti-aggregants such as nitric oxide and prostacyclin [22, 55–58]. In addition, high levels of lipid hydroperoxides inhibit the cyclooxygenase-mediated synthesis of prostacyclin [59], and superoxide destroys nitric oxide [60]. The reduction in acetylcholine-stimulated endothelium-dependent relaxation in diabetic rat aorta in vitro may be partially compensated by addition of superoxide dismutase to the bathing fluid [61]. Endothelium of vessels from diabetic rats shows enhanced sensitivity to oxygen free radical damage, indicating reduced anti-oxidant protection [25, 55]. Moreover, vessel basement membrane thickening may be promoted by oxidative stress in combination with glycation because of cross linking of long-lived structural proteins [14].

Thus, it is likely that oxidative stress affects neurons and Schwann cells directly, and indirectly via vascular effects. The latter may introduce a deleterious positive feedback element because ischaemia-reperfusion [24] would cause oxidative stress to further damage small vessels.

The mechanisms of action of some therapeutic interventions in diabetic rats may be viewed against this background. Prevention and reversal of NCV deficits by vasodilators [2–5] compensate for the reduction in local endothelium-dependent vasodilation because of deficits in nitric oxide [22, 55–57, 61–63] and prostacyclin release [58], as well as counteracting increased vascular reactivity to catecholamines [64] and the vasoconstrictor effect of elevated levels of angiotensin converting enzyme [65]. Chronic electrical stimulation may also produce vascular benefits [6] as it increases nerve blood flow [66]. Evening primrose oil may provide substrate for vasodilator prostanoid synthesis [7] to compensate for the deficit in prostacyclin release [58]. Aminoguanidine inhibits the formation of advanced glycation end-products [14], and prevents their "quenching" effect on nitric oxide and nitrodilators [15]. Further beneficial vascular effects, particularly prevention of endothelial damage, would accrue from inhibition of the oxidation of LDL [67]. These actions could explain the observation that sciatic endoneurial blood flow is returned to normal by aminoguanidine treatment in diabetic rats [10].

Aldose reductase inhibitors could have mixed vascular-neuronal effects. Thus, prevention of changes in vessel contractility and relaxation [22] may improve nerve blood flow [21], giving similar benefits to those seen with vasodilators. Polyol pathway activity diverts glucose through the hexose monophosphate shunt, with a concomitant decrease in ATP synthesis [68]. Moreover, the glutathione cycle in mitochondria is NADPH dependent, and dysfunction would allow hydroxyl radical damage to cell membranes, which would also impair mitochondrial function and oxidative metabolism [42]. In addition to a deleterious effect on axons and Schwann cells, interference with ATP production also reduces endothelial synthesis of

nitric oxide [69, 70]. Thus, aldose reductase inhibitors would have a profound indirect protective effect against oxidative and metabolic stress.

In conclusion, anti-oxidant treatment prevents nerve dysfunction in experimental diabetes. The effects of many manipulations that improve nerve function can also be viewed as correcting oxidative stress by vascular and other mechanisms. It is, therefore, plausible that the short-term deleterious effects of diabetes reflect oxidative stress which may be manifested in the long-term as progressive damage [24] to neurons which could be of relevance to the aetiology of clinical neuropathy. Thus, anti-oxidant treatment may provide a potential therapeutic approach to diabetic neuropathy. The lower level of complications seen in developed compared to underdeveloped countries may relate in part to a higher dietary intake of anti-oxidants such as BHT [31].

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