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The potential contribution of endothelin-1 to neurovascular abnormalities in streptozotocin-diabetic rats

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Summary Abnormal vascular endothelium function may contribute to the reduced nerve perfusion implicated in the aetiology of neuropathy in diabetes mellitus. The aim was to test the hypothesis that a powerful vasoconstrictor, endothelin-1, could be involved in nerve dysfunction in streptozotocin-diabetic rats. After 6 weeks of untreated diabetes, rats were implanted with osmotic minipumps which continuously delivered the endothelin-1 antagonist, BQ-123, to the circulation via a jugular vein cannula. Sciatic motor conduction velocity, monitored serially, was increased after 4 days, treatment ($p = 0.028$), and reached asymptote by 9–11 days ($p = 0.0001$), when the degree of amelioration was approximately 60 % of the initial diabetic deficit. Treatment of non-diabetic rats for 13 days with BQ-123 had no significant effect on motor conduction velocity. Sensory saphenous nerve conduction velocity was measured acutely after 20 days, BQ-123 treatment. The amelioration of a sensory deficit was approximately 80 % ($p < 0.001$); the resultant conduction velocity value was not significantly different from that of a non-diabetic control group. After 20 days, treatment, sciatic nutritive endoneurial blood flow was measured by microelectrode polarography and hydrogen clearance. A 48 % deficit with untreated diabetes ($p < 0.001$) was 64 % ameliorated by BQ-123 treatment ($p < 0.001$). In non-diabetic rats, BQ-123 treatment had no effect on blood flow. We conclude that endothelin-1 does not seem to be involved in the control of nerve blood flow in non-diabetic rats; however, it makes a major contribution to the perfusion deficit in experimental diabetes. This has deleterious consequences for nerve conduction, and it is possible that endothelin-1 receptor blockade may have therapeutic potential in diabetic patients. [Diabetologia (1994) 37: 1209–1215]

Key words Neuropathy, nerve conduction, nerve blood flow, endothelin-1, vascular endothelium, hypoxia, streptozotocin, diabetic rat.

An early reduction in peripheral nerve blood flow and consequent endoneurial hypoxia [1, 2] in experimental diabetes mellitus leads to the rapid development of diminished nerve conduction velocity (NCV) and increased resistance to ischaemic conduction failure [3, 4]. Similar subclinical functional indicators are found in newly-diagnosed diabetic patients [5, 6]. Endoneurial hypoxia and reduced blood flow are also present in patients with established neuropathy [7–9]; therefore it is likely that neurovascular effects play a major role in the aetiology of this diabetic complication.

Abnormalities of and damage to vascular endothelium contribute to diabetic microangiopathy [10–16]. Thus, prostacyclin release is decreased in experimental diabetes [17] and in patients [18] because of reduced substrate availability as a consequence of impaired ω-6 essential fatty acid metabolism [19, 20]. In addition, the synthesis or action of nitric oxide (NO) is diminished [10–14], and activity in the

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Abbreviations: ED_{50}, 50 % effective dose; EMG, electromyogram; ET, endothelin-1; NCV, nerve conduction velocity; NO, nitric oxide.

coagulatory system is increased [21]. Together, these result in loss of local vasodilation, and increased thrombosis formation which contribute to nerve ischaemia [22]. Increased LDL and particularly its oxidised and glycated forms may play a major role in damaging the endothelium [23–25]. Endothelial damage and tissue hypoxia cause increased release of a third factor, the potent vasoconstrictor peptide, endothelin-1 (ET) [26, 27]. Plasma ET levels are elevated in several vascular disease states, including those in diabetic patients and rats [28–30]. Although the cardiovascular significance of elevated plasma ET is unknown, levels are probably too low to exert a profound general vascular effect. However, plasma ET is derived from an "overflow" effect, reflecting greatly enhanced synthesis at the local tissue level [27].

There is increased ET release by the endothelium of mesenteric vessels in diabetic rats [29]. If this also occurs in nerve vascular supply, the likely effect is vasoconstriction and reduced blood flow. Thus, the aim was to examine whether ET was involved in neurovascular deficits in experimental diabetes. To this end, the effect of a specific ET_A receptor antagonist on NCV and sciatic endoneurial blood flow was examined in streptozotocin-diabetic and non-diabetic rats.

Materials and methods

Male Sprague-Dawley rats (Aberdeen University breeding colony), 19 weeks old at the start of the study were used. Diabetes was induced by streptozotocin freshly dissolved in sterile 154 mmol l^{-1} NaCl solution (40–45 mg · kg^{-1} i.p.), and was verified 24 h later by estimating hyperglycaemia and glycosuria (Visidex II and Diastix; Ames, Slough, Bucks., UK). Animals were tested weekly, and weighed daily. They were rejected if blood glucose was less than 20 mmol l^{-1} or if they showed a consistent increase in body weight over 3 days. Samples for plasma glucose measurement using a standard test kit (GOD-Perid method; Boehringer Mannheim, Mannheim, Germany) were taken on the day of final experiments.

After 6 weeks of untreated diabetes, rats were implanted subcutaneously with osmotic minipumps (Alzet 2ML2, Alza Corp., Palo Alto, Calif., USA) filled with the selective ET_A antagonist BQ-123 (Berlex, Richmond, Calif., USA), cyclo (-D-Trp-D-Asp-Pro-D-Val-Leu), dissolved in sterile phosphate buffered NaCl solution (154 mmol · l^{-1}, pH 7.35). Output of the pump was fed to a cannula inserted in the jugular vein. Rats were treated for up to 20 days with a dose of BQ-123 of approximately 0.7 mg · kg^{-1} day^{-1}. The dose was governed by the pump delivery rate and the maximum solubility of BQ-123. In acute experiments on anaesthetized rats, infusion of BQ-123 at a dose 10–20 times less than that used in this investigation substantially (67 %) attenuated the pressor response to ET infusion [31]. Thus, a high level of chronic ET_A receptor blockade would be expected at the dose employed. Osmotic minipumps were replaced at 14 days. A separate group of non-diabetic rats was also implanted with BQ-123-containing osmotic minipumps, delivering a similar dose to that used for diabetic rats.

The day before pump implantation in the diabetic group, NCV in the sciatic branch to tibialis anterior muscle was measured to establish baseline values using a previously described method [32]. Briefly, under halothane anaesthesia (2–5 % in air), sterile bipolar needle stimulating electrodes (2 mm separation of tips) were inserted through the skin to stimulate sciatic nerve at the sciatic notch and popliteal fossa. A sterile concentric bipolar recording electrode was inserted into tibialis anterior muscle. Leg skin temperature was kept in the range 36–38 °C by radiant heat. EMGs evoked from both stimulation sites were averaged eight times and latencies of the first inflections were measured. The sciatic nerve between the two stimulating electrodes takes a fairly straight course, and inter-electrode distances were used to calculate NCV.

Serial measures of NCV were made in this treated diabetic group every 2 or 3 days for 18 days to examine the time course of NCV correction. In final experiments (day 20), saphenous sensory NCV was measured as previously described [33]. Patency of the jugular cannulae was also tested in final experiments, by connecting a syringe, filled with sterile NaCl solution (154 mmol · l^{-1}), in place of the osmotic minipump. If moderate pressure applied to the syringe plunger did not result in free flow into the jugular vein, the cannula was considered blocked. This was an a priori criterion for acceptance of data. Thirteen rats were implanted at the start of the NCV study. Of these, 1 died and of the remaining 12 rats surviving the full experimental period, 2 proved to have blocked cannulae and NCV results from these rats were excluded.

For groups of diabetic ($n = 9$) and non-diabetic ($n = 12$) rats given BQ-123 treatment, blood flow was determined after 20 and 12–14 days, respectively. There were no losses due to blocked cannulae in these groups. Before blood flow measurement, for the non-diabetic group, sciatic NCV to tibialis anterior muscle in the contralateral leg was determined. In addition to these groups, separate untreated non-diabetic and diabetic rats were used to give reference values for NCV and blood flow.

Nerve blood flow was measured by microelectrode hydrogen polarography as previously described [1, 2]. In final experiments, rats were anaesthetized with inactin (50–100 mg kg^{-1} i.p.), the trachea was cannulated for artificial ventilation and a carotid cannula was used to monitor mean systemic blood pressure. Core temperature of the animal 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 liquid paraffin at 37 °C to a depth of at least 1 cm to minimise diffusion of gases directly to or from the nerve. Rats were given neuromuscular blockade using d-tubocurarine (Sigma, 2 mg · kg^{-1} via the carotid cannula) and artificially ventilated. The level of anaesthesia was monitored by observing any reaction of blood pressure to manipulation, and supplementary inactin was given as necessary. Briefly, a glass-insulated platinum microelectrode (tip diameter 2–8 μm) was inserted into the middle portion of the sciatic nerve, above its trifurcation, and polarized at 0.25 V with respect to a reference electrode inserted subcutaneously in the flank of the rat. 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 (20–30 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 (30 min – 1 h), 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 mono- or bi-exponential curves were fitted to the

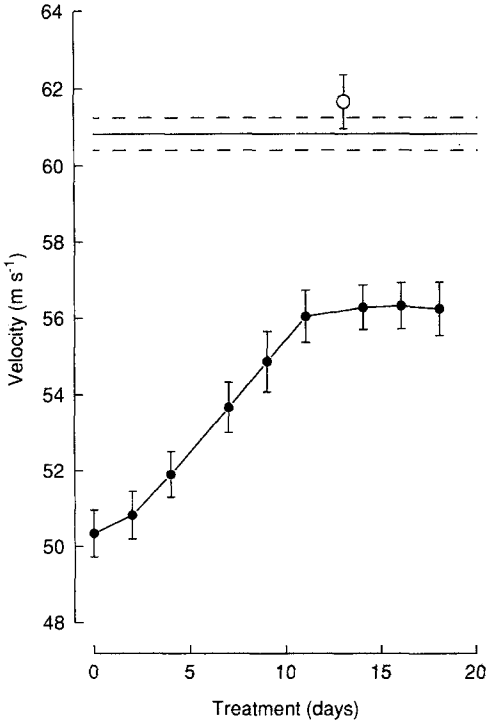

Fig. 1. Serial measurements of the effects of ET antagonist treatment on sciatic motor conduction velocity following 6 weeks of untreated diabetes. BQ-123 treated diabetic rats (●), $n = 10$; non-diabetic rats treated for 13 days (○), $n = 12$. Horizontal solid line flanked by dashed lines, mean ± SEM for a group of untreated non-diabetic rats, $n = 10$. All data are mean ± SEM. Statistics: NCV in diabetic rats was significantly improved by treatment from day 4 ($p = 0.028$ vs baseline value on day 0) onwards. Asymptote was reached by day 11 ($p < 0.0001$ vs baseline), but NCV was reduced compared to the untreated non-diabetic group ($p = 0.001$)

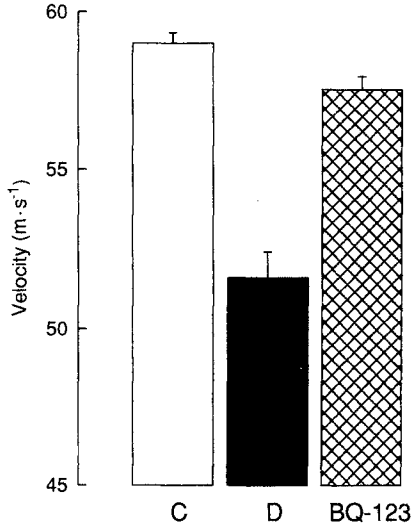

Fig. 2. Effects of ET antagonist treatment on sensory saphenous nerve conduction velocity following 6 weeks of untreated diabetes. C, non-diabetic control group, $n = 12$; D, untreated 2-month diabetic group, $n = 11$; BQ-123, 20-day ET antagonist-treated diabetic group, $n = 10$. All data are mean + SEM. Statistics: C vs D, $p < 0.001$; C vs BQ-123, NS; D vs BQ-123, $p < 0.001$

data by computer using appropriate non-linear regression software that employed the Marquardt algorithm and the least squares method for optimising goodness-of-fit (Inplot; Graphpad, San Diego, Calif., USA). The slow exponent, representing nutritive flow [34], was accepted. The average of the two determinations was taken to represent sciatic endoneurial blood flow. Vascular conductance was calculated by dividing blood flow by mean arterial blood pressure during the recording period.

Statistical analysis

Data are expressed as mean ± SEM. One-way analysis of variance was performed, followed by Student-Newman-Keuls tests to correct for multiple comparisons and assign differences to individual between-group comparisons when overall significance ($p < 0.05$) was attained. Paired Student's *t*-tests were used to assess the significance of within-rat changes for serial measurement of motor NCV and unpaired Student's *t*-tests were used for comparison with a non-diabetic control group.

Results

Diabetic rats were hyperglycaemic, with a plasma glucose concentration of 41.7 ± 2.3 mmol · l^{-1} and showed a weight loss from 473 ± 14 to 333 ± 13 g. There was no effect of BQ-123 treatment on these parameters (plasma glucose 44.1 ± 1.6 mmol · l^{-1}, weight loss from 476 ± 8 to 338 ± 18 g). BQ-123 also had no effect on non-diabetic rats; plasma glucose was 8.8 ± 0.6 mmol · l^{-1} and weight changed from 466 ± 9 to 477 ± 9 g over the 13-day treatment period.

Figure 1 shows the motor NCV changes with increasing duration of BQ-123 treatment. Compared to the baseline value following 6 weeks of untreated diabetes (day 0), there was a progressive increase in NCV which reached asymptote after 9–11 days. The amelioration for days 14–18 was approximately 60 % ($p < 0.0001$). BQ-123 treatment for 13 days had no significant effect on NCV in non-diabetic rats.

Data from sensory saphenous NCV determinations are shown in Figure 2. There was a 12.5 ± 1.4 % deficit after 2 months of untreated diabetes. BQ-123 treatment for the last 20 days reduced the deficit to 2.5 ± 0.7 % ($p < 0.001$), which was not significantly different from the non-diabetic control value.

Endoneurial blood flow (Fig. 3A) was 48 % reduced by 2 months of untreated diabetes ($p < 0.001$). Treatment with BQ-123 had no significant effect in non-diabetic rats; however, with diabetes there was a significant ($p < 0.001$) improvement in blood flow which corresponded to a 63.5 ± 9.1 % correction of the deficit. There were variations in blood pressure (Fig. 3B) between groups, with pressures generally being lower for diabetes. This was not statistically significant for untreated diabetes; however, the lowest pressure was seen in the BQ-123 treated diabetic group ($p < 0.001$). BQ-123 treatment did not have a

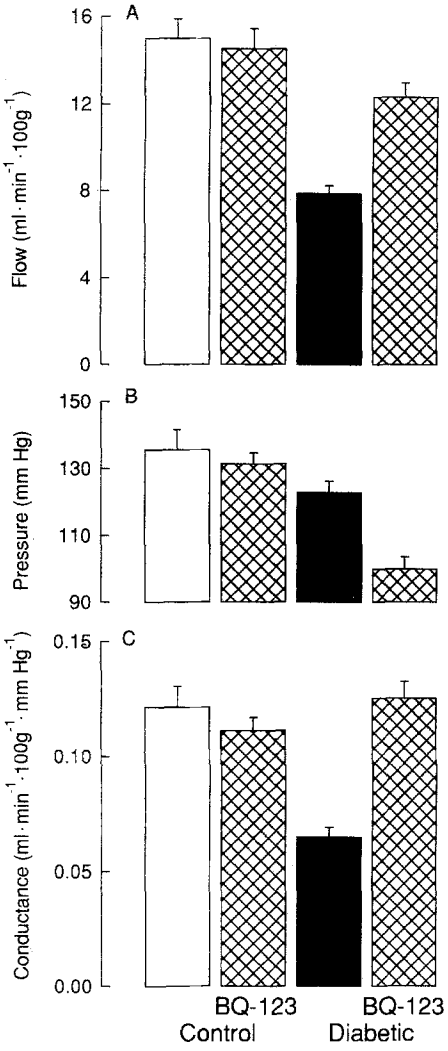

Fig. 3. (**A–C**) Nutritive endoneurial blood flow (**A**), mean systemic arterial pressure (**B**) and nutritive endoneurial vascular conductance (**C**) in non-diabetic and diabetic rats with and without ET antagonist treatment. All data are group means + SEM. Control groups, untreated, $n = 10$; BQ-123 treated for 13 days, $n = 12$. Diabetic groups, 2-month untreated, $n = 16$; BQ-123 treated for 20 days following 6 weeks of untreated diabetes, $n = 9$. Statistics: Control vs Control + BQ-123, NS for all measurements; blood flow, Control vs Diabetic untreated, $p < 0.001$; Control vs Diabetic + BQ-123, $p < 0.01$; Diabetic vs Diabetic + BQ-123, $p < 0.001$; blood pressure; all comparisons NS except for Diabetic + BQ-123 vs Control, Control + BQ-123 or Diabetic groups, $p < 0.001$; vascular conductance, Control vs Diabetic untreated, $p < 0.001$; Control vs Diabetic + BQ-123, NS; Diabetic vs Diabetic + BQ-123, $p < 0.001$; Control + BQ-123 vs Diabetic + BQ-123, NS

hypotensive effect in non-diabetic rats. When the data were expressed in terms of vascular conductance (Fig. 3C), thus compensating for blood pressure differences, there was complete normalization of conductance with BQ-123 treatment in diabetic rats ($p < 0.001$ vs untreated diabetic group, NS vs control group). In contrast, BQ-123 had no significant effect on sciatic vascular conductance in non-diabetic rats.

Discussion

These data provide the first demonstration that blockade of ET_A receptors in streptozotocin-diabetic rats produces a marked improvement in motor and sensory nerve function. In common with other vasoactive treatments that return NCV to normal, the functional improvement parallels an increase in nutritive blood flow [2, 35]. In addition to conventional vasodilators, similar findings apply to agents that act on some of the metabolic consequences of diabetes and hyperglycaemia, such as aldose reductase inhibitors [36, 37], aminoguanidine [38], anti-oxidants [39] ω-6 essential fatty acids [40] and insulin [41]. Thus, it is likely that hyperglycaemia causes vascular dysfunction which in turn leads to nerve abnormalities.

Previous work on vessel function has highlighted the importance of endothelial abnormalities in the aetiology of diabetic microvascular disease. Thus, endothelium-dependent relaxation by NO is reduced [10–14] and there may be increases in endothelium-dependent contracting factors [14, 15]. Prostacyclin synthesis is attenuated [17, 18] and the thrombolytic system is also impaired [21]. These alterations favour vasoconstriction, platelet aggregation, adhesion to the endothelium and thrombosis. Other changes, such as increased local production of angiotensin II, would further favour vasoconstriction [35] and the smooth muscle proliferation involved in atherogenesis [42].

The data for BQ-123 treatment are in accord with this generalized view of endothelial dysfunction. An increase in plasma ET levels and evidence of local enhancements of tissue activity are associated with some cardiovascular disease states including diabetes [26–29]. ET gene expression is also increased by diabetes, particularly in patients with peripheral vasculopathy [30]. There are several potential stimuli for increased ET synthesis and release, such as endothelial damage and hypoxia [27]. In addition, at least in cultured endothelial cells, high glucose levels enhance ET secretion [43]. Diabetic vessels have reduced ET sensitivity [44–46], perhaps caused by a down-regulation of receptors [47,48] or transduction mechanisms [49] in response to chronic exposure. Regional blood flow patterns for bolus ET injection are altered by diabetes in conscious rats [10, 50] but the changes are complex, suggesting tissue heterogeneity. Thus, for renal and mesenteric vascular beds, where flow is increased by diabetes, ET-mediated vasoconstriction was increased. However, in the hindlimb circulation, where flow decreases with diabetes, the vasoconstrictor response was relatively muted [50]. The data for sciatic nerve agree with the latter finding, as the effects of BQ-123 suggest that there would already be much greater local ET production and vasoconstriction with diabetes.

In non-diabetic rats, sciatic nerve segmental vascular supply is sensitive to acute exogenous ET adminis-

tration, which causes a sufficiently powerful vasoconstriction to transiently impair neuronal function [51]. The present data are the first to show that chronic BQ-123 treatment improved the reduced nutritive blood flow and vascular conductance associated with experimental diabetes. Vascular conductance was within the non-diabetic range; however, blood flow remained somewhat subnormal because blood pressure was lower in treated than untreated diabetic rats, which would be expected as vasa nervorum shows minimal pressure autoregulatory responses [52]. In marked contrast, BQ-123 had no effect on blood flow, vascular conductance or blood pressure in non-diabetic rats. This is contrary to the findings for some other vasodilators, such as those blocking the sympathetic noradrenergic system [53] or nitrovasodilators [54], which increase nerve blood flow in non-diabetic rats, although a similar lack of effect on nerve perfusion was found for an angiotensin II antagonist [35]. Thus, the data suggest that ET_A-receptor-mediated effects do not normally play a major role in the control of (resting) vasa nervorum blood flow, and probably blood pressure. However, with diabetes, basal ET release had a significant influence on both nerve perfusion and blood pressure. While this data provides evidence for the "classic" vasoconstriction effect of ET_A receptor activation [10, 27] on vasa nervorum, it is not known if there are additional effects mediated by ET_B receptors in diabetes. A test of this notion awaits the development of specific ET_B antagonists.

The sciatic motor NCV amelioration by BQ-123, in percentage terms, matched that for blood flow rather than vascular conductance. Treatments that completely return nutritive blood flow to the non-diabetic range, such as guanethidine or aldose reductase inhibition, also completely restore sciatic motor NCV [2, 36]. This suggests that the hypotensive effect of BQ-123 seen in anaesthetized diabetic rats was probably also present when they were conscious. Otherwise, based on the conductance data, a greater NCV amelioration would be expected. Compared to sciatic motor fibres, saphenous sensory fibres showed a greater NCV response to BQ-123. We have previously noted this trend from other studies, for example using aldose reductase inhibitors, where the degree of amelioration was greater for a given dose [33] and the ED_{50} for saphenous was lower than for sciatic nerve [36]. Thus, it may be that the vascular supply of the relatively small saphenous nerve has a greater safety margin than that of the major sciatic trunk.

Sciatic nerve endoneurium is hypoxic in experimental diabetes [1, 36, 39], as is the sural nerve in neuropathic patients [7]. This provides one potential stimulus for endothelial ET synthesis and release [27]. Hypoxia enhances oxidative stress which directly affects neurons [55]. A high level of oxygen free radicals causes lipid peroxidation which inhibits prostacyclin synthesis [56]. The ability of resistance vessels to relax is further impaired as oxygen free radicals react with NO [57], preventing the stimulation of smooth muscle guanylate cyclase. Reaction products, such as the hydroxyl radical [58] can damage endothelial cells directly. Cytotoxic effects of oxidised LDL on the endothelium have also been noted [24, 25]. Damage to endothelial cells is a further stimulus for ET release [27]. Thus, there may be an element of positive feedback, involving a cycle of oxidative stress, hypoxic damage, endothelial dysfunction and vasoconstriction which maintains reduced nerve perfusion in experimental diabetes. Blocking any element of this self-reinforcing cycle could potentially restore blood flow. It is of interest that treatment with oxygen free radical scavengers improves nerve conduction [39, 59], nutritive blood flow [39], endothelium-dependent relaxation [60] and protects against the development of markers of endothelial damage such as increased plasma angiotensin converting enzyme concentration [39]. Thus, it is plausible that anti-oxidant treatment would also reduce local and circulating ET levels.

Elevated ET production in diabetes may also be important for other aspects of neurovascular function. Thus, in non-diabetic rats ET causes increased vascular permeability [61], which is greatly exacerbated when NO synthase activity is also blocked [62]. Many treatments that normalise NCV in diabetic rats also improve blood flow, which would reduce nerve hypoxia and, therefore, minimise at least one potential stimulus for local ET production. Such agents include aldose reductase inhibitors, anti-oxidants and aminoguanidine [36–39, 63]. They also prevent or correct increases in vascular albumin permeation [64], therefore, it is plausible that their common actions on nerve function, blood flow and albumin permeation arise because they all protect against endothelial dysfunction, damage and consequent ET release [12–14, 16, 38, 39, 60]. The efficacy of these treatments would be further increased by their ability to counteract impaired NO action [13, 14, 35, 59, 65].

In conclusion, evidence increasingly identifies abnormalities of the vascular endothelium as an important cause of impaired nerve perfusion and neuronal dysfunction in experimental diabetes. Parallels may also be drawn with neurovascular changes in diabetic patients. Thus, ET is probably one of the important contributory factors to the multiple aetiology of diabetic microangiopathy and ET_A antagonists may have a potential therapeutic role for complications like neuropathy.

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