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Effects of Trientine, a Metal Chelator, on Defective Endothelium-dependent Relaxation in the Mesenteric Vasculature of Diabetic Rats

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Diabetes mellitus compromises endothelium-dependent relaxation of blood vessels. This has been linked to the generation of reactive oxygen species (ROS), which neutralise nitric oxide (NO) and interfere with vasodilator function. Experiments using chelators have emphasised the importance of ROS produced by transition metal catalysed reactions. However, particularly for the small arteries and arterioles that control microcirculatory blood flow, NO is not the only endothelium-derived mediator; endothelium-derived hyperpolarizing factor (EDHF) has a major role. EDHF-mediated vasodilation is severely curtailed by diabetes; however, little information exists on the underlying pathophysiology. Deficits in the EDHF system, alone or in combination with the NO system, are crucial for the development of diabetic microvascular complications. To further elucidate the mechanisms involved, the aim was to examine the effects of diabetes and preventive and intervention chelator therapy with trientine on a preparation that has well-defined NO and EDHF-mediated responses, the rat mesenteric vascular bed.

In phenylephrine-preconstricted preparations, maximum vasodilation to acetylcholine was reduced by 35 and 44% after 4 and 8 weeks of streptozotocin-induced diabetes, respectively. Trientine treatment over the first 4 weeks gave 72% protection; intervention therapy over the final 4 weeks prevented deterioration and corrected the initial deficit by 68%. These responses depend on both NO and EDHF. When the latter mechanism was isolated by NO synthase inhibition, diabetic deficits of 53.4 (4 weeks) and 65.4% (8 weeks) were revealed, that were 65% prevented and 50% corrected by trientine treatment. Neither diabetes nor trientine altered vascular smooth muscle responses to the NO donor, sodium nitroprusside (SNP). Thus, the data suggest that metal catalysed ROS production makes a substantial contribution to defects in both the EDHF and NO endothelial mechanisms in diabetes, which has therapeutic implications for microvascular complications.

Keywords: Diabetes mellitus; Rat; Nitric oxide; Endothelium-derived hyperpolarizing factor (EDHF); Diabetic complications; Microangiopathy

Abbreviations: ACh, acetylcholine; EDHF, endothelium-derived hyperpolarizing factor; L-NNA, N^G-nitro-L-arginine; NO, nitric oxide; PE, phenylephrine; ROS, reactive oxygen species; SNP, sodium nitroprusside

INTRODUCTION

Vascular alterations in diabetes mellitus cause or contribute to the aetiology of complications such as nephropathy, neuropathy and retinopathy.[1–3] A consistent finding in diabetic animal models is the presence of vascular endothelial dysfunction. This has been linked to elevated oxidative stress and is characterised by impaired nitric oxide (NO) mediated endothelium-dependent relaxation, as noted for numerous individual vessels and vascular beds, including aorta, basilar and cerebral arteries, corpus cavernosum and renal and mesenteric vascular beds.[4–11] Several studies in diabetic patients have shown comparable deficits.[12–15]

While a major focus has been on the NO system in diabetes, there are other important endothelium-derived vasodilators. In the majority of resistance

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vessels including those in the mesenteric vascular bed, vasodilation to an agonist such as acetylcholine (ACh) persists after blockade of NO synthase and cyclooxygenase. This is due to the release of an agent that causes hyperpolarisation of vascular smooth muscle, an endothelium-derived hyperpolarizing factor (EDHF).[16] The precise chemical identity of EDHF is not known; indeed, there may be several different EDHFs.[17] Studies have emphasised the relative importance of EDHF compared to NO in the microcirculation and the local control of tissue perfusion. Thus, EDHF expression is greater in microvessels than in large blood vessels and may be the major determinant of vascular tone in small resistance arteries, where a change in membrane potential of a few millivolts causes a substantial change in vessel diameter and vascular conductance.[18,19] Recent studies have noted EDHF deficits in experimental diabetes even more profound than those for the NO system,[9,11] which should be highly relevant for the pathophysiology of diabetic microvascular complications.

Elevated levels of reactive oxygen species (ROS), which react with NO to neutralise vasodilator activity, are one of the potential causes for the diabetic endothelial deficit.[1,2] Chronic treatment with high doses of free radical scavengers, such as vitamin E, attenuates the development of deficits in endothelium-dependent relaxation[20–22] in vessels of diabetic rats, as well as preventing some diabetic complications such as impaired peripheral nerve function and blood flow.[23] The disadvantage of this approach is that extremely high pharmacological doses of antioxidant are necessary for beneficial effects.[24] An alternative approach, targeting the processes responsible for ROS production as opposed to scavenging them once formed, may therefore be more suitable for therapeutic application in diabetes.

Transition metal ions, such as iron and copper, are normally in a bound homeostasis, which may be compromised by diabetes.[25] These can enhance ROS-related processes, such as glucose autoxidation, glycoxidation and the production of cytotoxic hydroxyl radicals from hydrogen peroxide.[26] Recently it has been shown that transition metal chelator treatment with hydroxyethyl starch deferoxamine or trientine can prevent the development of impaired NO-mediated endothelium-dependent relaxation in rat aorta and corpus cavernosum, as well as reversing deficits in sciatic nerve blood flow and function in diabetic rats.[27–30] It is not known whether a chelator treatment approach would have an impact on the EDHF defect in experimental diabetes, or whether such dysfunction is reversible. Thus, the aim of this study was to examine the effects of both prevention and intervention treatment with trientine on the diabetic deficits of NO and particularly EDHF mediated vascular responses in the rat mesenteric vascular bed.

MATERIALS AND METHODS

The experiments were performed in accordance with regulations specified by the United Kingdom "Animal Procedures Act, 1986," and the National Institutes of Health "Principles of Laboratory Animal Care, 1985 revised version."

Experimental Groups and Diabetes Induction

Male Sprague–Dawley rats (University of Aberdeen breeding colony), 19 weeks old at the start of the study were used. Diabetes was induced by intraperitoneal injection of Streptozotocin (STZ) (AstraZeneca, Macclesfield. Cheshire, UK) freshly made up in sterile saline solution, at a dose of 40–45 mg/kg. Diabetes was verified after 24 h by the presence of hyperglycaemia and glycosuria (Visidex II and Diastix; Ames, Slough, UK) in non-fasted rats. In final experiments blood samples were taken by cardiac puncture to measure plasma glucose levels (GOD-Perid method, Boehringer Mannheim, Mannheim, Germany).

Groups of diabetic rats were untreated for 4 or 8 weeks. Other diabetic groups were treated with trientine (triethylenetetramine dihydrochloride; Sigma, Poole, Dorset, UK) added to the drinking water (26.7 mg/l) such that rats received a daily dose of approximately 20 (15–25) mg/kg body weight. This dose was used in previous studies on nerve perfusion and function, and vascular responses of aorta and corpus cavernosum.[27,29] Trientine has been used successfully in rat models of copper overload disease, and is also employed clinically for initial de-coppering treatment in Wilson's disease.[31,32] There were two treated diabetic groups: a preventative group where trientine was given for 4 weeks, starting 2 days after diabetes induction and an intervention group where treatment was given for the final 4 weeks of an 8-week period of diabetes.

Mesenteric Vascular Preparation

In final experiments, rats were anaesthetised (4% halothane in air), and the mesenteric vascular bed was prepared as previously described.[9] Briefly, the mesentery was exposed, the superior mesenteric artery was cannulated, and the mesenteric bed was cut away close to the intestine. The mesenteric bed was then mounted on a water filled bulb maintained at 37°C, with free drainage, and was covered with plastic film. The cannula was connected to a peristaltic pump which intraluminally perfused the preparation at a constant rate (5 ml/min) with

modified Krebs–Ringer solution (144.0 Na^+, 5.0 K^+, 1.25 Ca^{2+}, 1.1 Mg^{2+}, 25 HCO^-, 1.1 PO_4^{3-}, 1.1 SO_4^{2-}, 5.5 glucose; in mM) at 37°C, which was gassed continuously with 95% O_2: 5% CO_2 (pH 7.35). Perfusion pressure was monitored by a pressure transducer connected close to the mesenteric artery cannulation point, with drug responses being measured as the pressure changes. The peristaltic pump was set up so that three inputs were available for the delivery of Krebs–Ringer and/or drugs dissolved in Krebs–Ringer solution.

The mesenteric bed was equilibrated for 40 min, after which cumulative concentration-response relationships were determined for endothelium-dependent relaxation to ACh, following precontraction with phenylephrine (PE) at a concentration (3–100 μM) selected to give an approximately equivalent pressor response for all groups under all conditions studied. After recovery, pressor concentration-response curves to PE were recorded followed by relaxation, and redetermination of the PE concentration-response relationship in the presence of 3 mM of the NO synthase inhibitor, N^G-nitro-L-arginine (L-NNA). The concentration of this potent antagonist[33] exceeded that required to completely block NO synthase-mediated responses in rat aorta, corpus cavernosum and mesenteric vasculature.[5,8,9,34,35] ACh concentration-responses were also determined in the presence of L-NNA, followed by the addition of 5 μM of the cyclooxygenase inhibitor, flurbiprofen, to the perfusate to isolate the EDHF component. The flurbiprofen dose used would be expected to cause >95% blockade of cycloxygenase-derived prostanoid production by inhibiting both the constitutive and inducible isoforms of the enzyme.[36] Cumulative concentration response curves were also determined for the NO donor, sodium nitroprusside (SNP) following precontraction with PE, in the absence and presence of L-NNA.

Statistical Analysis

Results are presented as group means ± SEM. The data were analysed using a standard statistical software package (Prism, Graphpad, San Diego, CA, USA). Data were subjected to Bartlett's test for homogeneity of variances, followed by log transformation if necessary before one-way analysis of variance. Where significance ($p < 0.05$) was reached, between-group differences were established using the Student–Neuman–Keuls multiple comparison test. If variances could not be homogenized by mathematical transformation, data were analysed by an appropriate nonparametric test, such as Kruskal–Wallis' one-way analysis of variance followed by Dunn's multiple comparison test. Within-group comparisons (effects before and after L-NNA) were made using paired Student's *t*-tests. Concentration-response curves were fitted by sigmoid curves using the least squares method to estimate EC_{50}.

RESULTS

Untreated and trientine-treated diabetic rats had elevated unfasted plasma glucose levels compared to non-diabetic rats and showed a similar progressive decline in body weight over the 8-week experimental period (Table I).

Concentration-response curves for endothelium-dependent relaxation to ACh in the PE-precontracted mesenteric vascular bed are shown in the absence (Fig. 1 (a,b)) and presence (Fig. 1 (c,d)) of the NO synthase inhibitor, L-NNA. Maximum endothelium-dependent relaxation to ACh (Fig.1 (a)) for the non-diabetic group was 92.4 ± 2.8%. This was reduced by 34.6% to 59.7 ± 3.6% ($p < 0.001$), and by 43.8% to 52.0 ± 1.2% ($p < 0.001$), after 4 and 8 weeks of diabetes, respectively. Preventative trientine-treated diabetic rats had a maximum relaxation of 83.4 ± 2.1%, which was significantly greater ($p < 0.001$) than that for untreated diabetics and did not significantly different from the non-diabetic group value. In the intervention trientine group (Fig. 1 (b)), the maximum relaxation value of 81.8 ± 0.6% was not significantly different from those of the non-diabetic and trientine prevention groups, but was significantly greater than both the 4 week and 8 week diabetic group values ($p < 0.001$).

Pre-incubation and co-perfusion with L-NNA depressed ACh responses ($p < 0.001$) in all groups (Fig.1(c,d)). Maximum endothelium-dependent relaxation for the non-diabetic group was reduced to 63.4 ± 3.9%. This was attenuated by 53.4% ($p < 0.001$) to 28.2 ± 3.0% and by 65.4% ($p < 0.001$) to 21.9 ± 2.3%, for 4 weeks and 8 weeks of diabetes, respectively. Preventative trientine treated diabetic rat mesenteric beds had a maximum relaxation of 51.1 ± 2.6%, which was significantly greater ($p < 0.01$) than that for untreated diabetics. Trientine intervention caused a maximum relaxation of 44.8 ± 1.9%, which was greater than the values for

TABLE I Non-fasted plasma glucose concentrations and body weights for the groups of rats used in the studies

Group	*n*	Plasma glucose (mM)	Body weight (g)
Non-diabetic	10	12.8 ± 0.9	458 ± 4
Diabetic control			
4-week	10	51.8 ± 3.7*	394 ± 14*
8-week	10	47.6 ± 1.0*	328 ± 7*
Trientine-treated diabetic			
Prevention	7	48.3 ± 0.9*	385 ± 15*
Intervention	8	46.5 ± 2.2*	338 ± 10*

Data are mean ± SEM; *$p < 0.001$ vs. non-diabetic group.

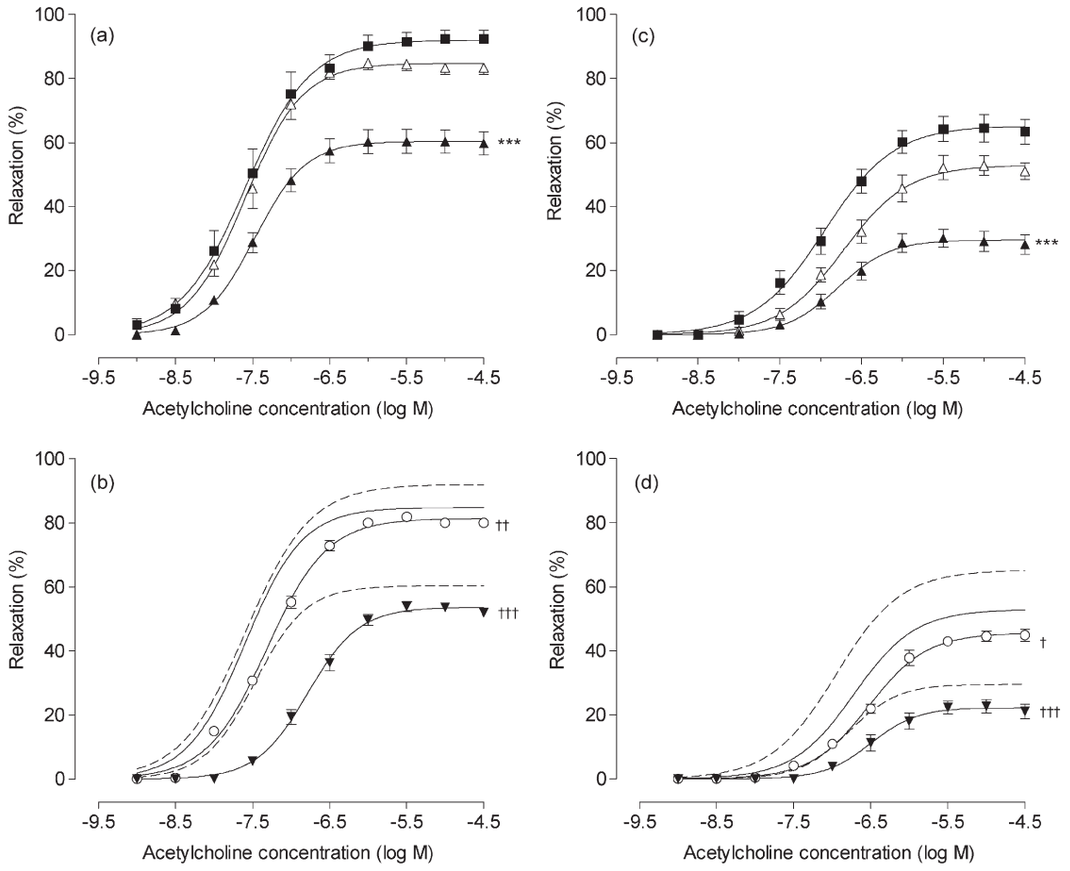

FIGURE 1 (a–d) Effects of diabetes and trientine treatment on ACh concentration-response curves for endothelium-dependent vasodilatation of the PE-preconstricted (3–100 μM) mesenteric vascular bed in the absence, (a,b), and presence, (c,d), of nitric oxide synthase inhibition by L-NNA. Non-diabetic control group (■, solid line on a and c; $n = 10$; reproduced as dashed line without symbols on b and d for comparison), 4-week diabetic control group (▲, solid line on a and c; $n = 10$; reproduced as dashed line without symbols on b and d for comparison); 8-week diabetic control group (▼, solid line, $n = 10$); 20 mg/kg trientine-treated prevention group (Δ, solid line on a and c; $n = 7$; reproduced as solid line, no symbols on c and d for comparison), 20 mg/kg trientine-treated intervention group (○, solid line, $n = 8$). Data are group means ± SEM. $^{***}p < 0.001$, 4-week diabetic control vs. non-diabetic control and trientine-treated prevention groups; $^{+}p < 0.05$, trientine-treated intervention vs. non-diabetic control group; $^{++}p < 0.01$, trientine-treated intervention vs. non-diabetic control group; $^{+++}p < 0.001$, 8-week diabetic control vs. trientine-treated prevention and intervention groups.

both diabetic groups ($p < 0.001$) and did not differ significantly from the non-diabetic and preventative trientine treatment group values.

Sensitivity to ACh, estimated from $(-\log)EC_{50}$ values (Fig. 2), was not significantly affected by 4 weeks of diabetes, however, there was an approximately 0.8 log units (or 6.7-fold) decline by 8 weeks ($p < 0.001$). Intervention trientine treatment attenuated this sensitivity loss by 73%, although there remained a 0.25 log unit deficit compared to the non-diabetic control group ($p < 0.01$). Following incubation with L-NNA, there was a marked decline in sensitivity to ACh in all groups ($p < 0.001$), the smallest differential effect being seen for the 8-week diabetic group. There were no significant between group differences in the sensitivity to ACh in the presence of L-NNA. Pre-incubation and co-perfusion with the cyclooxygenase inhibitor, flurbiprofen (5 μM for 30 min) and L-NNA did not significantly alter maximum responses or sensitivity to ACh, compared to L-NNA alone (Table II), indicating that responses were mediated by EDHF rather than a cyclooxygenase product such as prostacyclin.

Endothelium-independent relaxation (Table III) to the NO donor, SNP, was unaffected by diabetes or trientine treatment in terms of both maximum relaxation and $(-\log)EC_{50}$. Incubation with L-NNA caused a 25–30% increase in maximum relaxation and a 1–1.5 log unit elevation of sensitivity to SNP compared to pre-L-NNA values ($p < 0.0001$), the magnitude of change being similar in all groups.

Data for contraction by PE (Fig. 3 (a)) revealed that diabetes caused a reduction in maximum pressure development; 30.9 ± 4.5 mmHg at 4 weeks ($p < 0.001$) and 60.5 ± 9.8 mmHg at 8 weeks ($p < 0.01$) compared to mesentery from non-diabetic rats (102 ± 8.3 mmHg). Preventative trientine treatment values (35.6 ± 6.1 mmHg) did not differ significantly from the 4-week diabetic control group. In the trientine intervention group, however, pressure

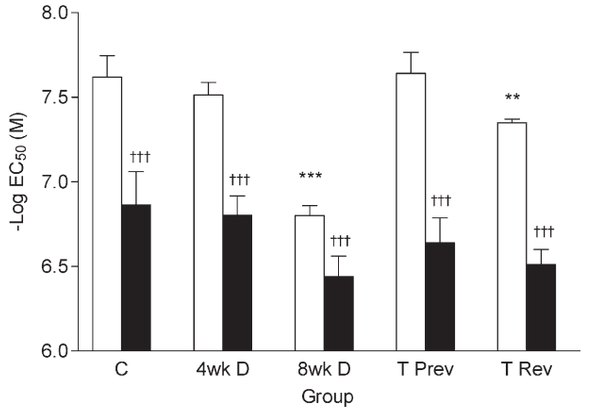

FIGURE 2 Effects of diabetes and trientine treatment on ACh ($-\log$)EC_{50} values for endothelium-dependent vasodilation of the PE-preconstricted mesenteric vascular bed in the absence (open bars) and presence (filled bars) of the nitric oxide synthase inhibitor, L-NNA. C, non-diabetic control group; 4wk D, 4-week diabetic control group; 8wk D, 8-week diabetic control group; T Prev, 4-week diabetic group given preventive trientine treatment; T Rev, 8-week diabetic group treated with trientine for the final 4 weeks. Data are group means ± SEM; group n values as for Fig.1. ***$p < 0.001$, 8-week diabetic control vs. non-diabetic control, 4-week diabetic control, trientine-prevention and trientine-intervention groups; **$p < 0.01$, trientine-intervention vs. non-diabetic control group; $^{\dagger\dagger\dagger}p < 0.001$, within-group comparison for absence vs. presence of L-NNA.

development was greater (85.9 ± 9.1 mmHg) than that of the 4- ($p < 0.001$) and 8-week ($p < 0.05$) diabetic control groups.

In the presence of L-NNA, pressor responses to PE (Fig. 3 (b)) were elevated ($p < 0.001$) in all groups. However, maximum pressure development in the 4-week diabetic groups, without (82.8 ± 13.2 mmHg) or with preventive trientine treatment (114.4 ± 12.1 mmHg) remained lower ($p < 0.05$) than for all other groups (non-diabetic 175.3 ± 18.9; 8-week diabetic 159.3 ± 10.7 mmHg; trientine intervention 160.4 ± 6.4 mmHg).

Sensitivity to PE, evidenced by ($-\log$)EC_{50} values (Fig. 4), tended to be modestly reduced by diabetes, although this did not reach statistical significance. For the trientine treated diabetic groups, however, ($-\log$)EC_{50} was depressed by approximately 0.5 log units (3.3-fold) compared to non-diabetic controls ($p < 0.01$). L-NNA co-perfusion did not significantly alter ($-\log$)EC_{50} values for PE in non-diabetic or diabetic control groups, the lowest value being found in the 4-week diabetic group. In contrast, however, for both trientine treated groups there was a marked elevation of sensitivity ($p < 0.01$), into the non-diabetic range.

DISCUSSION

The data demonstrate that chronic diabetes causes deficits in endothelium-dependent relaxation of the mesenteric vascular bed, which is in agreement with several previous studies using this tissue[9,11,35,37] as well as other vessels and vascular beds.[4–7,20–22,38] These defects, which are attributable to malfunctions of both the NO and EDHF systems, were largely prevented or corrected by treatment with the transition metal chelator, trientine. There were no statistically significant differences between the efficacy of intervention and prevention treatments.

The diabetic effect on the NO-mediated component can be gauged by the changes in sensitivity to ACh in the absence of L-NNA, and were progressive over the 8-week period. Trientine effectively prevented this progression. This defect was not caused by reduced smooth muscle NO sensitivity. Relaxation responses to the NO donor, SNP, in the mesenteric beds of all groups were not significantly different. As NO donors bypass the endothelium and act directly on vascular smooth muscle to promote relaxation, this indicates that the functional diabetic NO-related deficiency resides upstream of the smooth muscle cGMP mechanism. Normal responses to nitrodilators have been reported in the majority of studies of conduit and resistance arteries in experimental diabetes[9,21,22,29,34,35] and for some patient[12,13,15] studies. The increased response to SNP in the presence of L-NNA was probably due to inhibition of endogenous basal and flow-induced NO release, which no longer has to be surmounted by the exogenous NO supplied by SNP, as previously

TABLE II Effects of diabetes and trientine treatment on maximum relaxation and sensitivity to ACh of PE-precontracted rat mesenteric vascular bed in the presence of L-NNA, without or with flurbiprofen (FBN)

	Maximum relaxation (%)		Sensitivity ($-\log(EC_{50})$)	
Group	L-NNA	L-NNA + FBN	L-NNA	L-NNA + FBN
Non-diabetic	64.5 ± 4.3	56.0 ± 6.4	6.86 ± 0.19	6.93 ± 0.06
Diabetic control				
4-week	30.1 ± 2.7	36.0 ± 2.7	6.80 ± 0.11	6.86 ± 0.10
8-week	22.7 ± 2.1	24.2 ± 2.9	6.44 ± 0.12	6.48 ± 0.16
Trientine-treated diabetic				
Prevention	52.1 ± 3.8	55.7 ± 2.8	6.64 ± 0.14	6.70 ± 0.08
Intervention	44.8 ± 1.9	45.7 ± 2.1	6.51 ± 0.09	6.47 ± 0.08

Data are mean ± SEM; n values for L-NNA are given in the legend for Fig. 1; $n = 6$ for L-NNA + FBN for all groups. There were no statistically significant effects of L-NNA + FBN compared to L-NNA alone in any of the experimental groups.

TABLE III Effects of diabetes and trientine treatment on maximum relaxation and sensitivity to SNP of the PE-precontracted rat mesenteric vascular bed in the absence and presence of L-NNA

		Maximum relaxation (%)		Sensitivity ($-\log(EC_{50})$)	
Group	*n*	Pre L-NNA	L-NNA	Pre L-NNA	L-NNA
Non-diabetic	10	73.6 ± 2.8	97.3 ± 0.4	6.31 ± 0.10	7.81 ± 0.07
Diabetic control					
4-week	10	68.3 ± 3.5	95.5 ± 0.9	6.59 ± 0.05	7.71 ± 0.07
8-week	10	65.4 ± 1.0	95.5 ± 0.7	6.48 ± 0.12	7.68 ± 0.11
Trientine-treated diabetic					
Prevention	7	72.8 ± 1.7	97.7 ± 1.1	6.55 ± 0.08	7.75 ± 0.11
Intervention	8	65.3 ± 0.7	95.6 ± 0.9	6.40 ± 0.10	7.46 ± 0.07

Data are mean ± SEM. L-NNA increased maximum relaxation and $-\log(EC_{50})$ in all groups ($p < 0.0001$). There were no significant between group differences for these measures pre- or post-L-NNA.

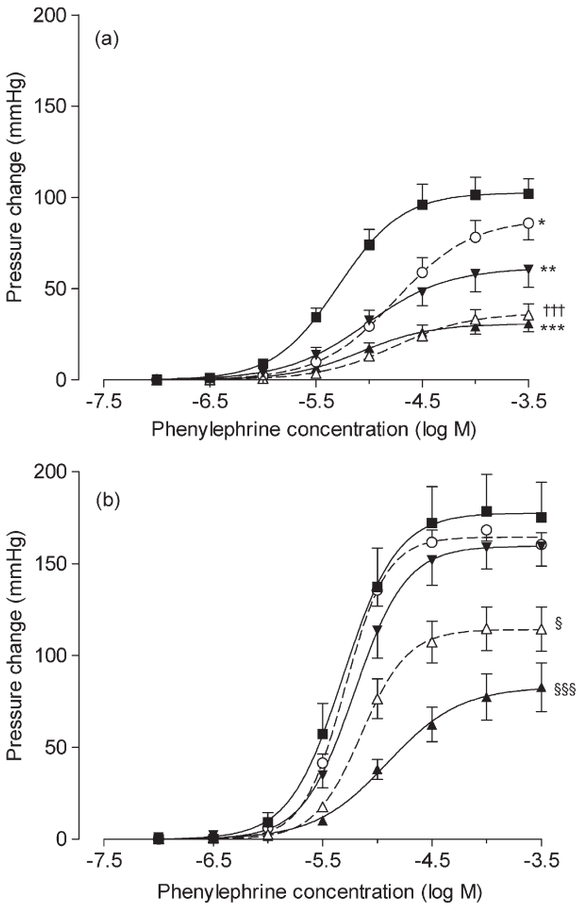

FIGURE 3 (a,b) Effects of diabetes and trientine treatment on PE pressor concentration-response curves for the mesenteric vascular bed in the absence (a) and presence (b) of nitric oxide synthase inhibition. Non-diabetic control group (■, solid line; $n = 10$); 4-week diabetic control group (▲, solid line; $n = 10$); 8-week diabetic control group (▼, solid line; $n = 10$); 20 mg/kg trientine-treated prevention group (Δ, dashed line; $n = 7$); 20 mg/kg trientine-treated intervention group (○, dashed line, $n = 8$). Data are group means ± SEM. $^{***}p < 0.001$, 4-week diabetic control vs. non-diabetic control and trientine intervention groups; $^{**}p < 0.01$, 8-week diabetic control vs. non-diabetic control; $^{*}p < 0.05$, trientine intervention vs. 8-week diabetic control group; $^{\dagger\dagger\dagger}p < 0.001$, trientine prevention vs. non-diabetic control and trientine intervention groups; $^{\S\S\S}p < 0.001$, 4-week diabetic control vs. non-diabetic control, 8-week diabetic control and trientine intervention groups; $^{\S}p < 0.05$ trientine prevention vs. non-diabetic control group.

suggested.[39] If that is the case then, given the similarity of changes in SNP responses before and after L-NNA in mesentery from diabetic and non-diabetic rats, it is likely that flow-induced release is more resistant to diabetes than agonist-stimulated NO release. A similar conclusion was reached for *in vivo* experiments on basilar artery after 6 months of diabetes,[40] although in another report a profound depression of flow-induced vasodilation was also noted for isolated mesenteric arteries after 4 weeks of diabetes.[41] The reason for this discrepancy is unknown, but may possibly relate to diabetes severity.

The most likely mechanism of trientine action in improving NO production or action is the reduction of ROS production. NO reacts with superoxide radical to form peroxynitrite,[42] which will diminish

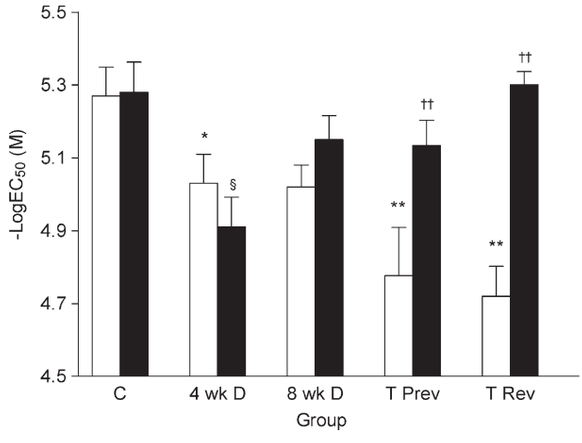

FIGURE 4 Effects of diabetes and trientine treatment on PE pressor ($-\log EC_{50}$ values for the mesenteric vascular bed in the absence (open bars) and presence (filled bars) of the nitric oxide synthase inhibitor, L-NNA. C, non-diabetic control group; 4wk D, 4-week diabetic control group; 8wk D, 8-week diabetic control group; T Prev, 4-week diabetic group given preventive trientine treatment; T Rev, 8-week diabetic group treated with trientine for the final 4 weeks. Data are group means ± SEM; group *n* values as for Fig. 3. $^{*}p < 0.05$, 4-week diabetic control vs. trientine intervention group; $^{**}p < 0.01$, non-diabetic controls vs. trientine prevention and intervention groups; $^{\S}p < 0.05$, 4-week diabetic control vs. all other groups; $^{\dagger\dagger}p < 0.01$, within-group comparison for absence vs. presence of L-NNA.

vasodilation. Peroxynitrite also nitrates and nitrosylates proteins, potentially altering their function. Increased concentrations of the nitration product, nitrotyrosine, have been observed in vessels from diabetic patients and endothelial cells cultured under high glucose conditions.[43,44]

However, several further potential explanations remain which cannot be ruled out by the present experimental findings alone, including abnormalities of ACh receptor or transduction mechanisms and reduced NO synthesis due to substrate deficiency. The impaired relaxation is not specific for muscarinic receptor mediated NO release, as relaxation to histamine and ADP, both of which are non-muscarinic endothelium-dependent relaxing agents, is reduced by diabetes.[45,46] Moreover, bypassing receptor mechanisms completely by using Ca^{2+} ionophore A23187, also reveals impaired NO-mediated vasorelaxation.[4,5] A further possibility is that depletion of the substrate for NO synthase, L-arginine, may be responsible for abnormal NO synthesis in diabetes. Thus, addition of L-arginine to the bathing fluid partially reversed the effects of glucose exposure or short-term diabetes in the rat aorta.[47] In other experiments, however, acute administration of L-arginine did not restore impaired ACh-induced vasorelaxation in diabetic rat mesenteric or basilar arteries, aorta or corpus cavernosum.[6,8,22,35,38]

The endothelium-dependent relaxation produced by ACh in the presence of the NO synthase inhibitor, L-NNA, with or without flurbiprofen, was markedly attenuated by diabetes in agreement with previous studies.[9,11,35,37] This combination of NO synthase and cyclooxygenase blockers defines EDHF as neither NO nor a prostanoid.[11,16–18] The chemical identity of EDHF is not known; several mediators probably exist, those proposed including one or more cytochrome P450-derived arachidonic acid metabolites, an endocannabinoid, and K^+.[16,17,48–50] It is generally agreed that EDHF relaxes vessels by increasing vascular smooth muscle K^+ conductance, with the subsequent hyperpolarisation leading to decreased Ca^{2+} influx due to reduced opening of voltage-gated Ca^{2+} channels.[17] It has also been noted that hyperpolarisation of the vascular smooth muscle cells may occur through electronic coupling between the endothelium and the vascular smooth muscle cells, EDHF action being disrupted by gap-junction uncoupling agents.[51] The involvement of endothelial cell charybdotoxin- and apamin-sensitive K^+ channels in this process has been noted for several tissues, including mesenteric resistance vessels. As with the NO response, it could be argued that the diabetic EDHF deficit exists at the level of the ACh receptor or transduction mechanism. However, this is unlikely because when receptor mechanisms are bypassed using Ca^{2+} ionophore A23187 to stimulate EDHF, a diminished hyperpolarising response remains in mesenteric arteries from diabetic rats.[11]

The data for trientine treatment show good protection and reversal of the diabetic deficit. This links oxidative stress with EDHF deficits although the mechanisms of this effect are not clear, in part because the precise nature of EDHF is unknown. One basis for trientine's protective action is the sequestration of the trace amounts of transition metals. These are required for catalysis of the auto-oxidation of glucose and its metabolites,[26] or the formation of hydroxyl radicals from hydrogen peroxide by the Fenton reaction, and also enhance the rate of the advanced glycosylation–glycoxidation process. Trientine has similar binding constants for both Fe^{3+} ($10^{21.9}$) and Cu^{2+} ($10^{20.4}$).[52] The ROS produced by such metal catalysed reactions, particularly hydroxyl radicals, are cytotoxic. Therefore, it is possible that generalized endothelial damage is responsible for dysfunction of both the EDHF and NO systems. Dimethylthiourea, which scavenges hydroxyl radicals as well as several other potentially relevant reactive species[53] (hydrogen peroxide, peroxynitrite and hypochlorous acid), prevented the development of deficits in NO-mediated vasorelaxation in aorta from diabetic rats.[54] However, the NO and EDHF systems are not extricably linked. Thus, the magnitude of the EDHF defect is somewhat larger than that for the NO system,[9] and the EDHF deficit can be completely prevented by ω-6 essential fatty acid treatment, while the NO deficit remains.[55] It is plausible that the necessity of transmission via gap-junctions makes the EDHF system relatively susceptible to diabetes/ROS. This may be a direct effect; in tissue culture, hydroxyl radicals derived from peroxynitrite inhibit gap junction permeability in astrocytes, protection being given by hydroxyl scavengers.[56] The action may also be indirect; antioxidants suppress the elevated vascular endothelial growth factor levels in diabetes, which otherwise increases vascular permeability and disrupts gap junction communication by endothelial cells.[57,58]

Effects of diabetes on the vascular smooth muscle contractile response were also noted. Thus, there was a transient decrease in smooth muscle contraction and pressor responses at 4 weeks of diabetes with partial recovery by 8 weeks. The mechanism is not known, but it appears to be largely independent of ROS generation by transition metal catalysed reactions; a similar time-dependent pattern of contractile responses was also noted in the trientine treated diabetic groups. There was, however, some evidence of endothelial modulation of the PE-mediated pressor effects. Thus, comparing pressor responses in the absence and presence of L-NNA, there was a greater increase in the trientine treated

preventive than in the 4-week diabetic group, indicating greater basal NO release in the former. This is in keeping with effects noted for endothelium-dependent relaxation to ACh. There is also evidence that trientine treatment promoted enhanced endothelial responses from the EC_{50} data, particularly for the intervention group. Thus, in the absence of L-NNA, sensitivity to PE was depressed below that of diabetic and non-diabetic control groups, whereas in the presence of L-NNA, sensitivity was in the non-diabetic range. The other groups did not show this differential effect of L-NNA on contractile responses.

In conclusion, treatment of diabetic rats with the transition metal chelator, trientine, had marked beneficial effects on both the NO- and EDHF-mediated endothelium-dependent relaxation of the mesenteric vasculature. This is the first report of chelator effects on the EDHF system. The actions of trientine may be attributable to protection against the deleterious effects of oxidative stress and could suggest a potential therapeutic approach to diabetic vasculopathy and microangiopathy.

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