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RESEARCH PAPER

Impairment of both nitric oxide-mediated and EDHF-type relaxation in small mesenteric arteries from rats with streptozotocin-induced diabetes

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BACKGROUND AND PURPOSE

To investigate whether diabetes affects either or both nitric oxide (NO)-mediated and endothelium-derived hyperpolarizing factor (EDHF)-type relaxation in endothelium-dependent relaxation of mesenteric arteries from streptozotocin-induced diabetic rats.

EXPERIMENTAL APPROACH

Wire myography was employed to examine endothelial function of mesenteric arteries. Superoxide levels were measured by L-012 and lucigenin-enhanced chemiluminescence. Western blotting was used to quantify protein expression levels.

KEY RESULTS

Superoxide levels were significantly increased in diabetic mesenteric arteries compared with normal arteries. Diabetes significantly reduced the sensitivity to the endothelium-dependent relaxant, acetylcholine (ACh) in mesenteric arteries. When the contribution of NO to relaxation was abolished by N-nitro-L-arginine (L-NNA) + a soluble guanylate cyclase inhibitor (ODQ), the sensitivity to ACh was significantly decreased in the diabetic arteries compared with normal arteries, indicating an impaired EDHF-type relaxation despite increased expression of intermediate- and small-conductance calcium-activated potassium channels. Conversely, when the contribution of EDHF was inhibited with TRAM-34 + apamin + iberiotoxin, maximum relaxations to ACh were significantly decreased in diabetic compared with normal arteries, suggesting that the contribution of NO was also impaired by diabetes. Basal levels of NO release, indicated by contraction to L-NNA, were also significantly decreased in diabetic arteries. Western blot analysis demonstrated that diabetic arteries had an increased expression of Nox2, decreased $pSer^{473}$Akt and a reduced proportion of endothelial NO synthase (eNOS) expressed as a dimer, indicating uncoupling.

CONCLUSION AND IMPLICATIONS

The contribution of both NO and EDHF-type relaxations was impaired in diabetes and was caused by increased oxidative stress, decreased $pSer^{473}$Akt and/or eNOS uncoupling.

Abbreviations

EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; $HbA1_c$, glycated haemoglobin; HXC, hydroxocobalamin; IK_{Ca}, intermediate-conductance calcium-activated K^+ channel; L-NNA, N-nitro-L-arginine; maxi K_{Ca}, large-conductance calcium-activated K^+ channel; ODQ, 1H-(1,2,4)-oxadiazolo(4,2-a)quinoxalin-1-one; R_{max}, maximum relaxation; sGC, soluble guanylate cyclase; SK_{Ca}, small-conductance calcium-activated K^+ channel; SNP, sodium nitroprusside; TRAM-34, 1-[(2-chlorophenyl)(diphenyl)methyl]-1H-pyrazole

Introduction

Endothelial cells, lining all blood vessels, can release both relaxing and contracting factors to modulate the tone of the underlying smooth muscle. Endothelium-dependent relaxation is mediated by multiple factors including nitric oxide (NO) (Furchgott and Zawadzki, 1980; Palmer *et al.*, 1988), prostacyclin (Moncada *et al.*, 1976) and an unidentified non-NO/non-prostanoid endothelium-derived hyperpolarizing factor (EDHF) (Busse *et al.*, 2002).

There are two broad categories of EDHF responses. The classical EDHF pathway involves the opening of endothelial intermediate- (IK_{Ca} or $K_{Ca}3.1$) and small-conductance (SK_{Ca} or $K_{Ca}2$) calcium-activated potassium channels (ion channel nomenclature follows Alexander *et al.*, 2009) and subsequent hyperpolarization and relaxation of smooth muscle cells by either downstream microdomain signalling pathways (Sandow *et al.*, 2006; Absi *et al.*, 2007; Dora *et al.*, 2008) or myoendothelial gap junctions (Edwards *et al.*, 1999). The second category of EDHF does not involve endothelial hyperpolarization, but involves the release of endothelium-derived factors such as NO, nitroxyl (HNO) and epoxyeicosatrienoic acids (EETs) to cause smooth muscle hyperpolarization by acting on the potassium channels located on the smooth muscle cells (Edwards *et al.*, 2010). Different vascular beds, exposed to varying local or external stimuli, exhibit a marked heterogeneity in the relative contribution of these factors to alterations in tone (Chen *et al.*, 1988; Clark and Fuchs, 1997). For example, in the rat mesenteric artery, endothelium-dependent relaxation is mediated by NO, the classical EDHF pathway and there is also a role for the non-classical EDHF pathway (McCulloch and Randall, 1998; Andrews *et al.*, 2009; Ellis *et al.*, 2009). The relative contribution of each pathway may be altered in vascular disease (Nishikawa *et al.*, 2000; Sofola *et al.*, 2002).

Diabetes mellitus is characterized by hyperglycaemia resulting from a defective secretion or action of endogenous insulin. It is well established that diabetes often causes macrovascular and microvascular complications in human and animal models of diabetes, and several lines of evidence suggest that endothelial dysfunction could play a critical and initiating role in the development of these complications (Vanhoutte *et al.*, 2009). The relative contribution of endothelium-derived factors to relaxation in the presence of diabetes-induced endothelial dysfunction remains poorly understood especially in the smaller arteries, such as the mesenteric artery, where both NO and EDHF are contributing to endothelium-dependent relaxation (McCulloch and Randall, 1998; De Vriese *et al.*, 2000; Wigg *et al.*, 2001; Shi *et al.*, 2006). For example, Shi *et al.* (2006) reported an augmented contribution of EDHF and reduced contribution of NO to endothelium-dependent relaxation in the mesenteric artery. In contrast, Wigg *et al.* (2001) reported a selective impairment of the EDHF component of relaxation and preserved NO-mediated relaxation in the mesenteric artery.

Hence, it is not established whether diabetes affects the contribution to relaxation of either or both NO and EDHF. In addition, other studies using mesenteric arteries focused on the effect of diabetes on EDHF-dependent relaxation, as experiments were conducted in the presence of NO synthase and cyclo-oxygenase inhibitors (Fukao *et al.*, 1997; Matsumoto *et al.*, 2003; 2005). Therefore, the aim of the present study is to evaluate the mechanism of endothelium-dependent relaxation of small mesenteric arteries in rats with type 1 diabetes. In particular, we sought to assess the relative contribution of NO-mediated and EDHF-type relaxation to endothelium-dependent responses in normal and diabetic vessels.

Methods

Animals

All animal care and experimental procedures were approved by the Animal Experimentation Ethics Committee of RMIT University and conformed to the Australian National Health and Medical Research Council code of practice for the care and use of animals for scientific purposes. Male 8- to 10-week-old Sprague-Dawley rats ($n = 38$, University of Melbourne animal facility, Melbourne, Vic, Australia) were randomly divided into two groups: normal and diabetic. Type 1 diabetes was induced by a single injection of streptozotocin (48 $mg \cdot kg^{-1}$) into the rat tail vein after fasting overnight. The control groups received an equivalent volume of the vehicle (0.1 M citrate buffer) alone. Ten weeks after streptozotocin or vehicle treatment, the rats were killed with pentobarbitone sodium (325 $mg \cdot kg^{-1}$, i.p., Virbac, Australia). Blood samples were obtained from the left ventricle via cardiac puncture and the glucose concentration and glycated haemoglobin ($HbA1_c$) were measured using a one touch glucometer (Roche, Sydney, NSW, Australia) and Micromat $HbA1_c$ analyser (Biorad, Sydney, NSW, Australia) respectively. Induction of diabetes was considered successful when the glucose level and $HbA1_c$ was higher than 25 mM and 6.5% respectively.

Isolation of mesenteric arteries

After the rats were killed, the mesenteric arcade was isolated and immediately placed in ice-cold Krebs

bicarbonate solution (118 mM NaCl, 4.7 mM KCl, 1.18 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11.1 mM D-glucose and 1.6 mM $CaCl_2$) containing indomethacin (10 μM), a non-selective cyclo-oxygenase (COX) inhibitor, to inhibit the synthesis of prostanoids. Our preliminary data suggested that there is no significant contribution of prostanoids to endothelium-dependent relaxation in either the normal or diabetic mesenteric arteries (data not shown). Small mesenteric arteries (third order branch of the superior mesenteric artery, internal diameter ~300 μm) were isolated, cleared of fat and connective tissue, and cut into 2-mm-long rings and mounted on a Mulvany–Halpern myograph (model 610M, Danish Myo Technology, Aarhus, Denmark). After the arteries were mounted, the vessels were allowed to stabilize at zero tension for 15 min before normalization. The passive tension–internal circumference was determined by stretching to achieve an internal circumference equivalent to 90% of that of the blood vessel under transmural pressure of 100 mmHg (Mulvany and Halpern, 1977; McPherson, 1992). All experiments were performed at 37°C and in Krebs solution bubbled with carbogen (95% O_2 and 5% CO_2).

Functional experiments

Thirty minutes after normalization, vessels were maximally contracted with an isotonic high K^+-containing physiological saline solution (123 mM KPSS, normal Δ14.9 ± 0.39 mN vs. diabetic Δ15.3 ± 0.53 mN, $P > 0.05$). After several washouts using normal Krebs solution, basal tension was regained. To assess the integrity of the endothelium, mesenteric arteries were precontracted to ~50% of the KPSS response with phenylephrine (0.1–3 μM) and a high dose of acetylcholine (ACh, 10 μM) was used to relax the artery rings. ACh-induced relaxation was greater than 80% of the precontracted tone in all cases, indicating that the endothelium was functionally intact. After further washouts, arteries were again precontracted to ~50% KPSS response using phenylephrine (0.1–3 μM) or in some cases 45 mM K^+. The effect of the treatment on relaxant responses was determined by cumulative concentration–response curves to the endothelium-dependent relaxant, ACh (0.1 nM–10 μM) and endothelium-independent relaxant, sodium nitroprusside (SNP, 0.01 nM–10 μM). In addition, responses to ACh and SNP were examined after 20 min incubation with different combinations of N-nitro-L-arginine (L-NNA, 100 μM), a non-selective nitric oxide synthase (NOS) inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM), a soluble guanylate cyclase (sGC) inhibitor, tempol (100 μM), a cell permeable superoxide dismutase (SOD) mimetic, hydroxocobalamin (HXC, 200 μM), a NO scavenger, 1-[(2-chlorophenyl)(diphenyl)methyl]-1H-pyrazole (TRAM-34, 1 μM), a selective blocker of the IK_{Ca}, iberiotoxin (Ibtx, 100 nM), a selective blocker of the large-conductance calcium-activated K^+ channel (maxi K_{Ca} or $K_{Ca}1$) and apamin (1 μM), a SK_{Ca} inhibitor.

To evaluate the constrictor reactivity, cumulative concentration–response curves to endothelin-1 (ET-1, 0.1 nM–0.1 μM) were constructed in the absence of indomethacin. In another set of experiments, the effect of diabetes on basal levels of NO release was also examined in the absence of indomethacin through the addition of L-NNA (100 μM) in endothelium-intact rings precontracted with phenylephrine (10–100 nM) to approximately 20% KPSS, as previously described (Woodman *et al.*, 2004; Judkins *et al.*, 2010; Miller *et al.*, 2010).

Western blots

Western blots were performed as described previously (Woodman *et al.*, 2004) with the following modifications. First order, second order and third order mesenteric arteries from three animals from the same treatment group were pooled, and considered as $n = 1$. The tissues were homogenized in 200 μL of ice-cold lysis buffer (100 mM NaCl, 10 mM Tris, 2 mM EDTA, 0.5% w/v sodium deoxycholate, 1% vol/vol Triton X-100, pH 7.4, protease and phosphatase inhibitor cocktails (Roche, Sydney, NSW, Australia) and the total protein concentration of the samples was quantified using a Bradford assay. Equal amounts of protein homogenate were subjected to SDS-PAGE and Western blot analysis with mouse/rabbit primary antibodies (all 1:1000, overnight, 4°C) against endothelial NO synthase (eNOS), inducible NOS, Nox2 (all BD Transduction Laboratories, Lexington, KY, USA), maxi K_{Ca} ($K_{Ca}1.1$), SK_{Ca} ($K_{Ca}2.3$), IK_{Ca} ($K_{Ca}3.1$) (all 1:500, Alomone Laboratories, Jerusalem, Israel), $pSer^{473}$Akt and Akt (Cell Signalling, Danvers, MA, USA). After the detection of $pSer^{473}$Akt, membranes were stripped with stripping buffer (Thermo Scientific, Rockford, IL, USA) following manufacturer's instructions. After stripping, the membrane was then probed with Akt antibody. To normalize for the amount of proteins, membranes were reprobed with a loading control antibody (actin). All proteins were detected by either enhanced chemiluminescence (Amersham, GE Healthcare, Sydney, NSW, Australia) or Supersignal West Femto (Thermo Scientific, Rockford, IL, USA) after incubation with anti-mouse/rabbit secondary antibody (Millipore, Billerica, MA, USA) for 1 h at room temperature (1:2000). All protein bands were quantified by densitometry (Biorad Chemidoc, Sydney, NSW, Australia) and expressed as a ratio of

the loading control. To investigate eNOS homodimer formation in the tissue, a non-boiled sample was resolved by 6% SDS-PAGE at 4°C (Klatt *et al.*, 1995), and the membranes were probed and visualized as described above.

Superoxide measurement in mesentery artery

Superoxide production in the mesenteric artery was measured by using L-012 and lucigenin-enhanced chemiluminescence (Miller *et al.*, 2005; Leo *et al.*, 2010) with the following modification. The superior mesenteric artery was isolated, cleared of fat and connective tissue and cut into 2- to 3-mm-long segments in Krebs-HEPES buffer. Mesenteric arteries were incubated at 37°C for 30 min in Krebs-HEPES buffer either alone or in the presence of tempol, a cell permeable SOD mimetic (100 μM). Krebs-HEPES buffer (300 μL), containing L-012 (100 μM, Wako Pure Chemicals, Osaka, Japan) and the appropriate treatments were placed into a 96-well Optiplate, which was loaded into a Polarstar Optima photon counter (BMG Labtech, Melbourne, VIC, Australia) to measure background photon emission at 37°C. After background counting was completed, a single ring segment of mesenteric artery was added to each well and photon emission was recounted. To evaluate NADPH oxidase-driven superoxide production (Miller *et al.*, 2005), mesenteric artery homogenates were diluted 1:40 in Krebs-HEPES buffer containing either lucigenin (5 μM) alone or with the addition of NADPH (100 μM). The NAPDH-stimulated signal was also measured in the presence of a flavoprotein inhibitor, diphenyliodonium (5 μM). The superoxide counts were measured as described above and the NADPH-stimulated signal was subtracted from background. Superoxide counts were either normalized with dry tissue weight or total protein content.

Statistical analyses

All results are expressed as the mean ± SEM, *n* represents the number of animals per group or the number of assays when tissue from animals was pooled. Concentration–response curves from rat isolated mesenteric arteries were computer fitted to a sigmoidal curve using nonlinear regression (Prism version 5.0, GraphPad Software, San Diego, CA, USA) to calculate the sensitivity of each agonist (pEC_{50}). Maximum relaxation (R_{max}) to ACh or SNP was measured as a percentage of precontraction to phenylephrine. Group pEC_{50} and R_{max} values were compared via one-way ANOVA with *post hoc* analysis using Bonferroni's test or Student's unpaired *t*-test as appropriate. $P < 0.05$ was considered statistically significant.

Materials

All drugs were purchased from Sigma-Aldrich (St Louis, MO, USA), except for acetylcholine perchlorate (BDH Chemicals, Poole, Dorset, UK) and ODQ (Cayman Chemical, Ann Arbor, MI, USA). All drugs were all dissolved in distilled water, with the exception of indomethacin, which was dissolved in 0.1 M sodium carbonate, L-NNA, which was dissolved in 0.1 M sodium bicarbonate, ODQ and TRAM-34, which were dissolved in dimethyl sulfoxide (DMSO).

Results

Body weights and blood glucose

The body weight gained, blood glucose and $HbA1_c$ levels of the rats are shown in Table 1. Ten weeks after treatment with streptozotocin or vehicle, the body weight gained in normal rats was significantly greater than in diabetic rats (Table 1). The blood glucose and $HbA1_c$ level of diabetic rats were significantly greater than normal rats (Table 1).

Superoxide production in diabetes

The superoxide level in intact mesenteric arteries from diabetic rats was significantly higher than in

Table 1

Body weight gained, blood glucose and glycated haemoglobin levels of male Sprague-Dawley rats treated with either streptozotocin (STZ) or vehicle

	10 weeks after vehicle or STZ treatment			
	n	Normal	*n*	Diabetic
Body weight gained (g)	19	324 ± 13	19	135 ± 15*
Blood glucose (mM)	19	9.5 ± 0.5	19	High (>33)
$HbA1_c$ (%)	4	4.6 ± 0.5	6	12.3 ± 1.2*

A comparison of body weight gain, blood glucose and glycated haemoglobin level ($HbA1_c$) from streptozotocin (48 $mg \cdot kg^{-1}$) or vehicle treated rats. *n* = the number of rats. Results are shown as mean ± SEM.
*Significantly different from values in normal rats (Student's unpaired *t*-test, $P < 0.05$).

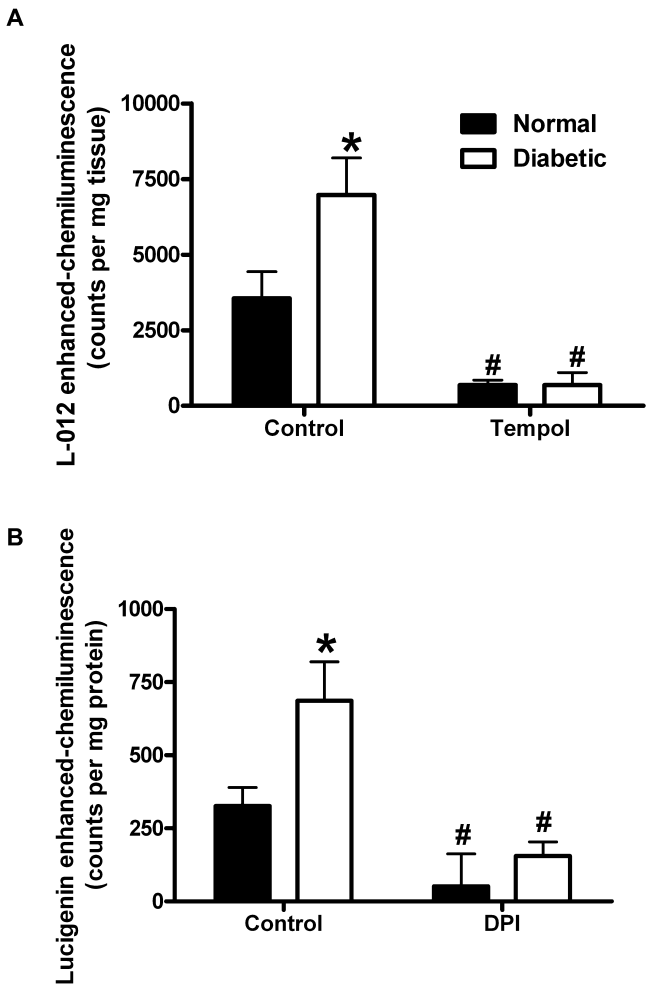

Figure 1

Superoxide levels in intact superior mesenteric arteries (A) and mesenteric homogenates (B) from normal and diabetic rats. Diabetes has a significantly higher level of superoxide production in either intact or mesenteric homogenates compared with normal rats. Superoxide levels could be attenuated either by the presence of tempol (100 μM), a cell permeable superoxide dismutase mimetic (A) or diphenyliodonium (DPI, 5 μM), a flavoprotein inhibitor that inhibits NADPH oxidase (B). $n = 5$–10 experiments. Results are shown as mean ± SEM. *Significantly different from normal rats (Student's unpaired t-test, $P < 0.05$). #Significantly different from control within respective group (Student's unpaired t-test, $P < 0.05$).

normal rats (Figure 1A). Treatment with tempol, a cell permeable SOD mimetic attenuated the generation of superoxide in diabetic arteries to levels comparable to those in normal tissues. NADPH oxidase-driven superoxide production from mesenteric homogenates was also significantly increased in diabetes and this increase was inhibited by the flavoprotein inhibitor, diphenyliodonium (Figure 1B).

Effect of diabetes on vascular function

Diabetes significantly reduced the sensitivity (as pEC_{50}), but not the maximum relaxation, to ACh in mesenteric arteries (Figure 2, Table 2), however, the sensitivity (pEC_{50}, diabetic, 8.48 ± 0.10 vs. normal, 8.18 ± 0.14, $n = 5$, $P > 0.05$) and maximum relaxation (diabetic, 97 ± 2% vs. normal, 100 ± 0%, $n = 5$, $P > 0.05$) to SNP were not affected. In addition, the diabetic arteries also showed a significant increase in sensitivity to ET-1 (pEC_{50}, diabetic, 8.46 ± 0.25 vs. normal, 7.99 ± 0.06, $n = 4$, $P < 0.05$), without affecting the maximum contraction (Figure 2).

Role of EDHF-type relaxation in diabetic mesenteric arteries

The sensitivity to ACh in normal mesenteric arteries was significantly reduced by the presence of L-NNA + ODQ (Figure 3A, Table 2). In contrast, in arteries from diabetic rats, the sensitivity to ACh was unaffected by the presence of L-NNA + ODQ (Figure 3B, Table 2). When the contribution of NO was eliminated by L-NNA + ODQ, the sensitivity to ACh was significantly lower in the diabetic, compared with the normal arteries (pEC_{50}, diabetic, 6.68 ± 0.12 vs. normal, 7.08 ± 0.11, $n = 11$–12, $P < 0.05$), indicating that diabetes impairs the contribution of a non-NO and non-prostanoid factor to endothelium-dependent relaxation. The maximum relaxation to ACh was unaffected by the presence of L-NNA + ODQ in normal or diabetic arteries.

To determine the individual roles of IK_{Ca} and SK_{Ca} in EDHF-type relaxation, we examined responses to ACh in the presence of either TRAM-34 or apamin, respectively, in addition to NOS inhibition (L-NNA + ODQ). In normal arteries, the addition of either TRAM-34 or apamin caused a further significant decrease in the sensitivity to ACh, without affecting the maximum relaxation (Table 2). In comparison, in diabetic arteries, the addition of TRAM-34 significantly decreased the sensitivity to ACh, whereas the addition of apamin caused a significant reduction in the maximum relaxation to ACh (Table 2). When the contribution of IK_{Ca} was eliminated by L-NNA + ODQ + TRAM-34, the sensitivity to ACh was significantly lower in the diabetic compared with the normal arteries (Table 2). When the contribution of SK_{Ca} was eliminated by L-NNA + ODQ + apamin, the maximum relaxation to ACh was significantly reduced in the diabetic compared with the normal arteries (Table 2), indicating that diabetes impairs both the IK_{Ca}- and SK_{Ca}-mediated relaxation.

The addition of TRAM-34 + apamin, in the presence of L-NNA + ODQ, abolished ACh-induced relaxation in diabetic arteries but in normal arteries ACh continued to cause a maximum relaxation of almost 60% (Table 2). The additional presence of Ibtx, to block maxi K_{Ca}, caused a further significant reduction in relaxation to ACh in normal arteries

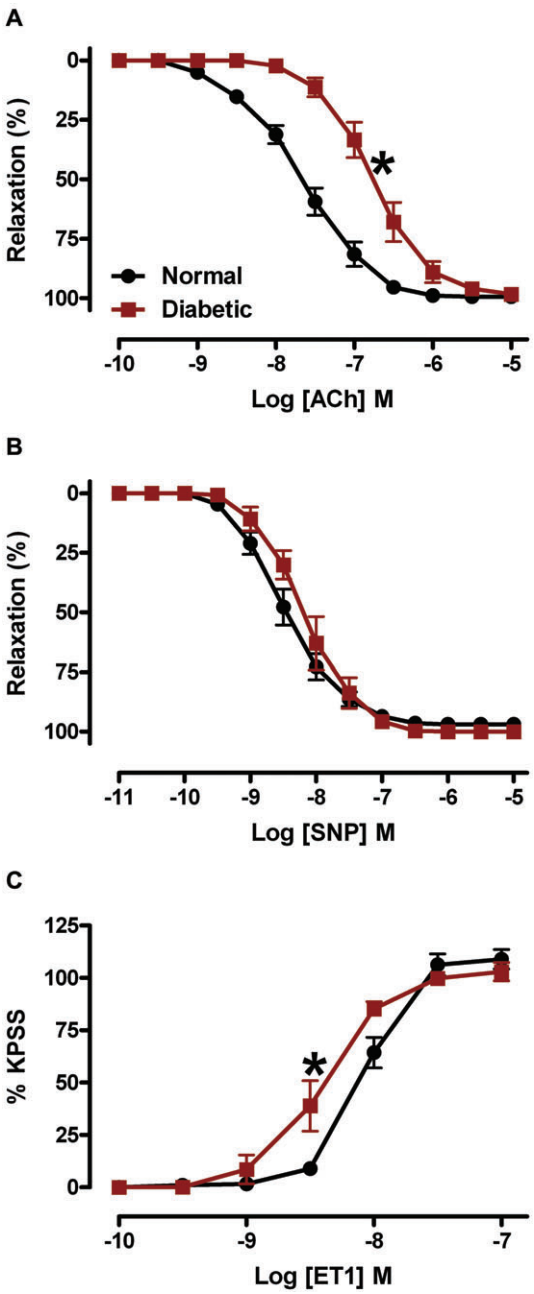

Figure 2

Cumulative concentration–response curves to ACh (A), SNP (B) or ET-1 (C) in endothelium-intact mesenteric arteries isolated from normal or diabetic rats. All experiments were conducted in the presence of indomethacin except for (C). In each group of experiments (A,B), mesenteric arteries were precontracted with phenylephrine to similar level: (A) normal 50 ± 1 versus diabetic 53 ± 4 (B) normal 44 ± 3 versus diabetic 46 ± 2%KPSS. $n = 4$–15 experiments. Results are shown as mean ± SEM. *pEC_{50} significantly different from normal rats (Student's unpaired t-test, $P < 0.05$). See Table 2 or results section for pEC_{50} and R_{max} values.

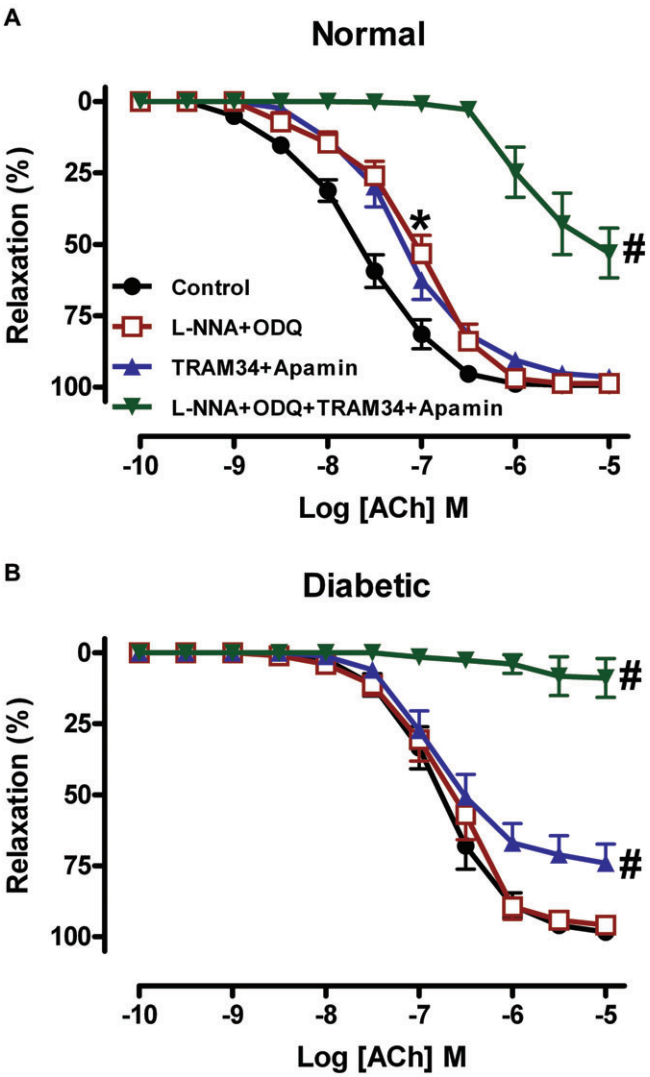

Figure 3

Cumulative concentration–response curves to ACh in the absence (control) or in the presence of L-NNA + ODQ, TRAM-34 + apamin or L-NNA + ODQ + TRAM-34 + apamin in endothelium-intact mesenteric arteries isolated from normal (A) or diabetic (B) rats. All the experiments were conducted in the presence of indomethacin. In each group of experiments, mesenteric arteries were precontracted with phenylephrine to similar level: (A) 54 ± 1 (B) 56 ± 2%KPSS. $n = 9$–15 experiments. Results are shown as mean ± SEM. *pEC_{50} significantly different from control (Bonferroni's test, $P < 0.05$). #R_{max} significantly different from control (Bonferroni's test, $P < 0.05$). See Table 2 for values.

(Figure 4A, Table 2), suggesting an additional role of other endothelium-derived factors to relaxation in normal arteries, but not in diabetes.

To investigate the contribution of NO to the maxi K_{Ca} component of relaxation, responses to ACh were tested in the presence of a NO scavenger, HXC in addition to K_{Ca} channel inhibitors (TRAM-34 + apamin or TRAM-34 + apamin + Ibtx). In normal arteries, the addition of HXC to TRAM-34

Table 2

Role of EDHF in ACh-induced relaxation of mesenteric arteries from normal and diabetic rats in the presence of indomethacin

	Normal			Diabetic		
Ach	***n***	**pEC_{50}**	**R_{max} (%)**	***n***	**pEC_{50}**	**R_{max} (%)**
Control	15	7.66 ± 0.09	99 ± 0	14	6.72 ± 0.12$^{\#}$	98 ± 1
L-NNA + ODQ	11	7.08 ± 0.11*	99 ± 1	12	6.68 ± 0.12$^{\#}$	96 ± 3
L-NNA + ODQ + apamin	4	6.19 ± 0.13*†	96 ± 2	4	ND	28 ± 6*$^{\dagger\#}$
L-NNA + ODQ + TRAM-34	4	6.28 ± 0.17*†	96 ± 3	4	5.79 ± 0.15*$^{\dagger\#}$	89 ± 9*
L-NNA + ODQ + TRAM-34 + apamin	10	ND	58 ± 8*†	9	ND	9 ± 7*$^{\dagger\#}$
L-NNA + ODQ + TRAM-34 + apamin + Ibtx	7	ND	29 ± 9*†	6	ND	0 ± 0*$^{\dagger\#}$
45 mM K^+ + L-NNA + ODQ	4	ND	0 ± 0*†	4	ND	0 ± 0*†

A comparison of the sensitivity (pEC_{50}) and maximum relaxation (R_{max}) to ACh in the absence (control), or in the presence of L-NNA + ODQ, L-NNA + ODQ + apamin, L-NNA + ODQ + TRAM-34, L-NNA + ODQ + TRAM-34 + apamin, L-NNA + ODQ + TRAM-34 + apamin + Ibtx or L-NNA + ODQ + 45 mM K^+ in endothelium-intact mesenteric arteries from normal and diabetic rats. All experiments were conducted in the presence of indomethacin. *n* = the number of experiments. Results are shown as mean ± SEM.
*Significantly different from corresponding control ACh response in the mesenteric arteries within the same treatment groups (Bonferroni's test, $P < 0.05$).
†Significantly different from corresponding 'L-NNA + ODQ' ACh response in the mesenteric arteries within the same treatment groups (Bonferroni's test, $P < 0.05$).
$^{\#}$Significantly different from normal treatment group in the mesenteric arteries within the respective inhibitor group (Student's unpaired *t*-test, $P < 0.05$).
EDHF, endothelium-derived hyperpolarizing factor; ND, not determined.

Table 3

Role of NO in ACh-induced relaxation of mesenteric arteries from normal and diabetic rats in the presence of indomethacin

	Normal			Diabetic		
ACh	***n***	**pEC_{50}**	**R_{max} (%)**	***n***	**pEC_{50}**	**R_{max} (%)**
Control	15	7.66 ± 0.09	99 ± 0	14	6.72 ± 0.12$^{\#}$	98 ± 1
Apamin	4	6.80 ± 0.09*	98 ± 1	4	6.41 ± 0.08$^{\#}$	96 ± 1†
TRAM-34	4	7.23 ± 0.22	99 ± 1	4	6.45 ± 0.15$^{\#}$	98 ± 1†
TRAM-34 + apamin	10	7.19 ± 0.11*	96 ± 1	9	6.74 ± 0.11$^{\#}$	74 ± 7*$^{\#}$
TRAM-34 + apamin + Ibtx	9	6.56 ± 0.19*†	81 ± 5*†	8	ND	17 ± 5*$^{\dagger\#}$
TRAM-34 + apamin + HXC	4	6.47 ± 0.13†	96 ± 3	4	ND	41 ± 6*$^{\dagger\#}$
TRAM-34 + apamin + Ibtx + HXC	4	ND	47 ± 8*†	4	ND	0 ± 0*$^{\#}$
45 mM K^+	7	6.61 ± 0.08*	50 ± 5*†	8	6.61 ± 0.10	27 ± 5*$^{\#}$

A comparison of the sensitivity (pEC_{50}) and maximum relaxation (R_{max}) to ACh in the absence (control), or in the presence of apamin, TRAM-34, TRAM-34 + apamin, TRAM-34 + apamin + Ibtx, TRAM-34 + apamin + HXC, TRAM-34 + apamin + Ibtx + HXC or 45 mM K^+ in endothelium-intact mesenteric arteries from normal and diabetic rats. All experiments were conducted in the presence of indomethacin. *n* = the number of experiments. Results are shown as mean ± SEM.
*Significantly different from corresponding control ACh response in the mesenteric arteries within the same treatment groups (Bonferroni's test, $P < 0.05$).
†Significantly different from corresponding 'TRAM-34 + apamin' ACh response in the mesenteric arteries within the same treatment groups (Bonferroni's test, $P < 0.05$).
$^{\#}$Significantly different from normal treatment group in the mesenteric arteries within the respective inhibitor group (Student's unpaired *t*-test, $P < 0.05$).
ND, not determined.

+ apamin significantly decreased the sensitivity to ACh without affecting the maximum relaxation (Table 3), whereas the addition of HXC to TRAM-34 + apamin + Ibtx caused a significant reduction (~50%) of the maximum relaxation to ACh. In contrast, in diabetic arteries, the addition of HXC to TRAM-34 + apamin (Table 3) caused a significant reduction (~60%) in the maximum relaxation,

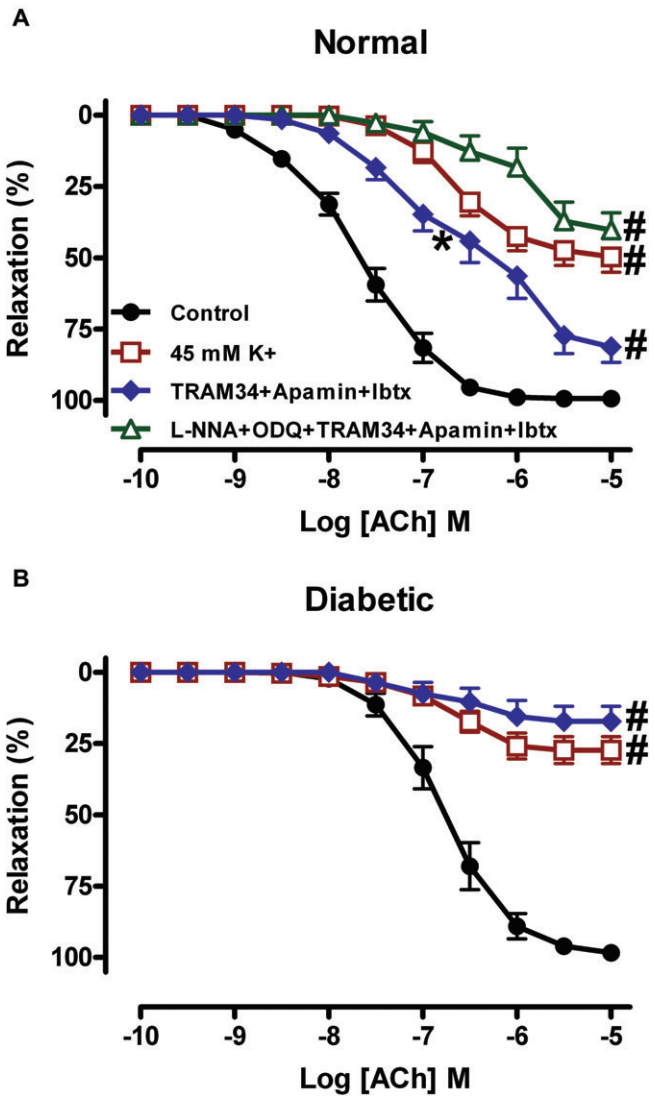

Figure 4

Cumulative concentration–response curves to ACh in the absence (control) or in the presence of 45 mM K^+, L-NNA + ODQ + TRAM-34 + apamin + Ibtx or TRAM-34 + apamin + Ibtx in endothelium-intact mesenteric arteries isolated from normal (A) or diabetic (B) rats. All the experiments were conducted in the presence of indomethacin. In each group of experiments, mesenteric arteries were precontracted with phenylephrine or depolarizing solution to similar level: (A) 53 ± 2 (B) 56 ± 2%KPSS. n = 7–15 experiments. Results are shown as mean ± SEM. *pEC_{50} significantly different from control (Bonferroni's test, $P < 0.05$). #R_{max} significantly different from control (Bonferroni's test, $P < 0.05$). See Tables 2 and 3 for values.

which was abolished by the additional presence of Ibtx (Table 3). If the responses to ACh in the presence of TRAM-34 + apamin + HXC or TRAM-34 + apamin + Ibtx + HXC are compared between arteries from normal and diabetic rats, it is apparent that diabetes also significantly reduced the remaining maximum relaxation to ACh.

Role of NO-mediated relaxation in diabetic mesenteric arteries

In normal arteries, the presence of either apamin alone or the combination of TRAM-34 + apamin, significantly reduced the sensitivity but not the maximum relaxation to ACh (Figure 3A, Table 3). The presence of TRAM-34 alone had no effect on the sensitivity or maximum relaxation to ACh in normal arteries (Table 3). In contrast, in diabetic arteries, the sensitivity to ACh was unaffected, while the maximum relaxation to ACh was significantly reduced (Figure 3B, Table 3) by the combination of the IK_{Ca} and SK_{Ca} blockers in comparison with control. In arteries from normal and diabetic rats, the addition of Ibtx, a blocker of maxi K_{Ca}, in the presence of TRAM-34 + apamin, significantly reduced the sensitivity and maximum relaxation to ACh (Figure 4, Table 3).

If the responses to ACh in the presence of TRAM-34 + apamin or TRAM-34 + apamin + Ibtx are compared between arteries from normal and diabetic rats, it is apparent that diabetes decreased both the sensitivity and maximum relaxation to ACh in the presence of TRAM-34 + apamin, and maximum relaxation in the presence of TRAM-34 + apamin + Ibtx (Table 3), suggesting that diabetes impaired the contribution of NO to endothelium-dependent relaxation. In addition, in arteries precontracted with a depolarizing solution of 45 mM K^+ to eliminate any contribution of the opening of potassium channels, ACh-induced relaxation was significantly attenuated and in comparison with normal arteries, the R_{max} was significantly lower in diabetic rats (Figure 4). The additional presence of L-NNA + ODQ in arteries that were precontracted with 45 mM K^+, abolished ACh-induced relaxation in normal and diabetic rats (Table 2).

The basal level of NO release was assessed by measuring the contraction induced by L-NNA in arteries with a low level of phenylephrine-induced tone (Woodman *et al.*, 2004; Judkins *et al.*, 2010; Miller *et al.*, 2010). The L-NNA-induced contraction was significantly greater in arteries from normal rats compared with diabetic rats (normal, 44 ± 4% vs. diabetic, 30 ± 3%, n = 4–6, $P < 0.05$), indicating that diabetes impaired the basal release of NO.

Role of oxidative stress in diabetic mesenteric arteries

In normal arteries, the presence of tempol had no effect on either the sensitivity (pEC_{50}, normal 7.64 ± 0.10 vs. normal + tempol 7.86 ± 0.09, n = 4, $P > 0.05$) or maximum relaxation to ACh. In contrast, in diabetic arteries (Figure 5), tempol significantly improved the sensitivity (pEC_{50}, diabetic 6.85 ± 0.08 vs. diabetic + tempol 7.55 ± 0.05, n = 4, $P < 0.05$) to

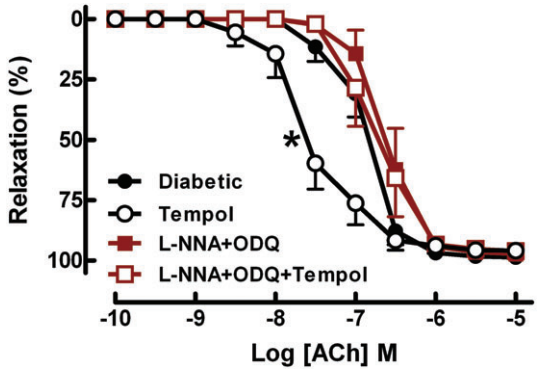

Figure 5

Cumulative concentration–response curves to ACh in the absence (control) or in the presence of tempol, L-NNA + ODQ or L-NNA + ODQ + tempol in endothelium-intact mesenteric arteries isolated from diabetic rats. All the experiments were conducted in the presence of indomethacin. $n = 4$ experiments. Results are shown as mean ± SEM. *pEC_{50} significantly different from control (Bonferroni's test, $P < 0.05$). See *Results* for pEC_{50} and R_{max} values.

ACh, but had no effect on the maximum relaxation. This effect was eliminated by the additional presence of L-NNA + ODQ (pEC_{50}, L-NNA + ODQ 6.57 ± 0.15 vs. L-NNA + ODQ + tempol 6.68 ± 0.18, $n = 4$, $P > 0.05$), indicating that tempol improves NO-dependent relaxation.

Effect of diabetes on the expression of NOS, Nox2, Akt and K_{Ca} channels

Diabetes did not change the expression of total eNOS in the mesenteric arteries but significantly decreased the proportion of eNOS expressed as a dimer (Figure 6A). The expression of total Akt was not changed but phosphorylation of the Ser^{473} residue was significantly decreased in the diabetic arteries (Figure 6B) The expressions of Nox2, IK_{Ca} ($K_{Ca}3.1$) and SK_{Ca} ($K_{Ca}2.3$) were significantly increased in diabetic arteries compared with normal arteries (Figure 6C–E). Diabetes had no effect on the expression of maxi K_{Ca} ($K_{Ca}1.1$) in the mesenteric arteries (Figure 6F) and inducible NOS expression was not detected in samples from either group (data not shown).

Discussion

This study demonstrated that, after 10 weeks of diabetes, endothelial dysfunction was evident in the rat small mesenteric artery, which was due to a decreased contribution of both NO-mediated and EDHF-type relaxation to endothelium-dependent relaxation. Diabetes also caused an increase in superoxide production in mesenteric arteries associated with an increase in Nox2 expression. Western blot analysis also indicated that diabetes significantly decreased the proportion of eNOS expressed as a dimer and decreased the activation of eNOS by decreasing the level of phosphorylation of Akt, actions that would decrease NO synthesis (Fulton *et al.*, 1999; Hink *et al.*, 2001). Uncoupled eNOS may also contribute to the increase in superoxide production (Hink *et al.*, 2001). Furthermore, the basal level of NO release was also decreased. In contrast, diabetes caused a significant increase in the expression of the endothelial K_{Ca} channels, suggesting that, rather than a decrease in channel expression, the impairment of the EDHF-type relaxation may be attributed to defective downstream processes such as microdomain signalling and/or activity of myo-endothelial gap junctions (Edwards *et al.*, 2010).

Endothelial dysfunction in diabetes

In the present study, diabetes increased the level of vascular superoxide production, associated with an increase in Nox2 expression, and endothelial dysfunction, as observed by an impairment of endothelium-dependent relaxation, without affecting endothelium-independent relaxation. Endothelial dysfunction could be acutely reversed by decreasing the activity of superoxide with the cell permeable SOD mimetic, tempol that restored the NO-mediated responses. These observations are consistent with several other studies that have demonstrated that diabetes causes vascular oxidant stress and causes endothelial dysfunction in either large conduit or resistance vasculature (De Vriese *et al.*, 2000; Xu and Zou, 2009). Despite the many reports of endothelial dysfunction induced by diabetes, the mechanism(s) of that effect remain poorly understood (Wigg *et al.*, 2001; Shi *et al.*, 2006), in particular the relative contribution of NO and EDHF in the presence of diabetes-induced endothelial dysfunction of resistance vessels.

Role of EDHF-type relaxation in diabetic mesenteric arteries

To investigate the role of EDHF, we assessed endothelium-dependent relaxation in the presence of L-NNA and ODQ to inhibit NO synthesis and sGC activity respectively. In the presence of L-NNA + ODQ (and indomethacin), the sensitivity to ACh was decreased in diabetic arteries when compared with normal arteries, indicating an impairment of the contribution of a EDHF-type relaxation, although it is important to note that the non-NO, non-prostanoid effect was able to maintain the maximal response to ACh. These observations were consistent with several other studies (Fukao *et al.*, 1997; Matsumoto *et al.*, 2003; 2005). To further

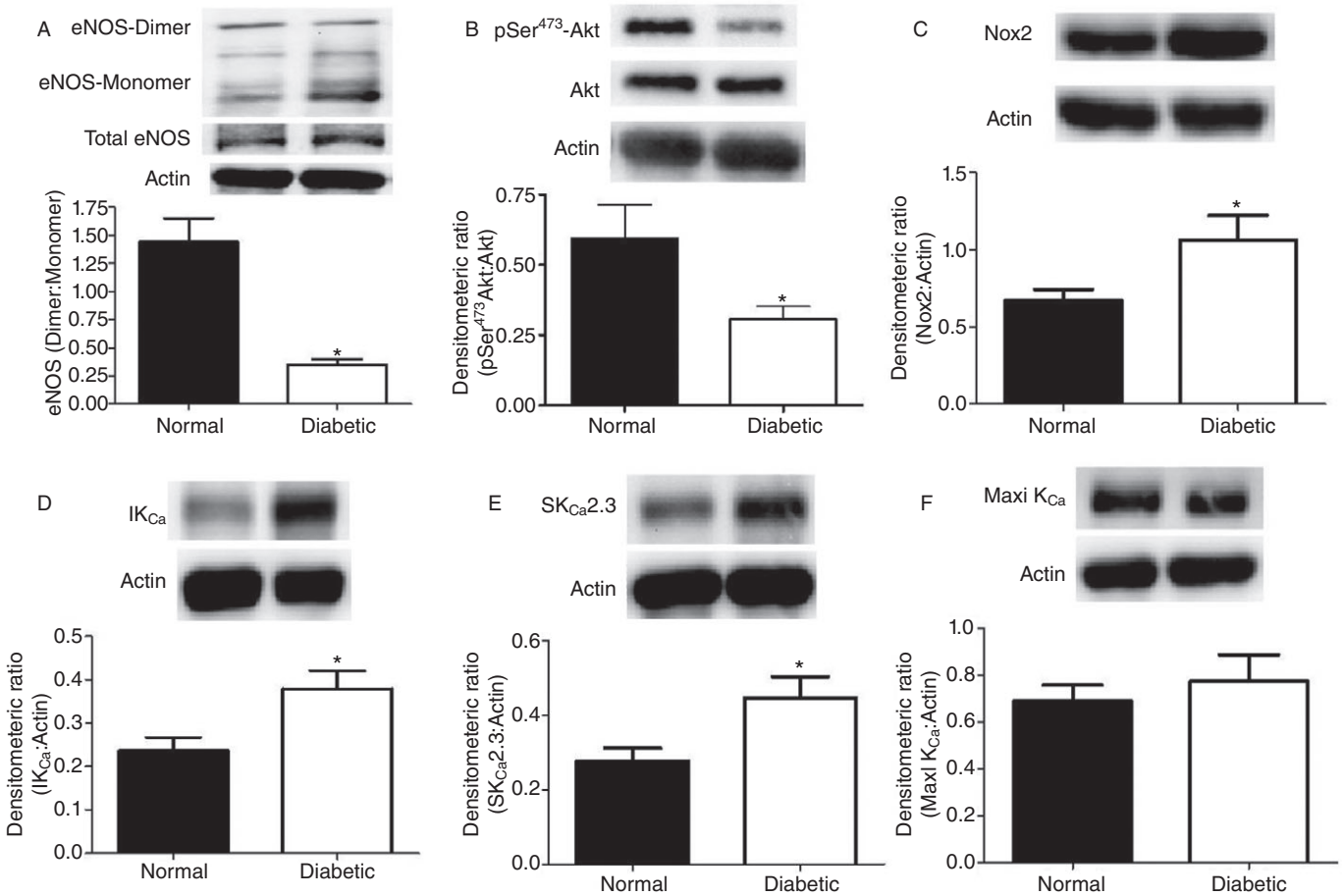

Figure 6

Protein expressions of eNOS (130 kDa) and homodimer (260 kDa) formation (A), pAkt/Akt (60 kDa) (B), Nox2 (58 kDa) (C), IK_{Ca} (75 kDa) (D), $SK_{Ca}2.3$ (80 kDa) (E) and maxi K_{Ca} (125 kDa) (F) in the normal and diabetic mesenteric arteries determined by Western blot analysis. Diabetes significantly reduced the proportion of eNOS expressed as the dimer, decreased the phosphorylation of Akt Ser^{473} and increased the expression of Nox2, IK_{Ca} and $SK_{Ca}2.3$. $n = 5–7$ experiments. Representative blots were shown on each corresponding graphs. Results are shown as mean ± SEM. *Significantly different from normal rats (Student's unpaired *t*-test, $P < 0.05$).

investigate the individual K_{Ca} channels (IK_{Ca} and SK_{Ca}) involved in the classical type of EDHF-type relaxation in the diabetic mesenteric arteries, responses to ACh were assessed in the presence of either TRAM-34 or apamin, blockers of IK_{Ca} or SK_{Ca} respectively. Addition of either TRAM-34 or apamin to L-NNA + ODQ, significantly attenuated the remaining ACh-induced relaxation in diabetic arteries in comparison with normal arteries, indicating that diabetes impairs both IK_{Ca}- and SK_{Ca}-mediated EDHF-type relaxation. Consistent with this observation, when the combination of IK_{Ca} and SK_{Ca} blockers were added to L-NNA + ODQ, endothelium-dependent relaxation was abolished in diabetic arteries, but only significantly attenuated in normal arteries. This suggests that in normal mesenteric artery, there is a role of a non-classical type of EDHF-type relaxation, that is, an endothelium-derived factor that caused smooth muscle cell hyperpolarization and relaxation that is independent of IK_{Ca} and SK_{Ca} (Edwards *et al.*, 2010). Endothelium-derived factors such as EETs, HNO or NO could contribute to the non-classical EDHF-type relaxation observed in normal mesenteric arteries. In the diabetic mesenteric arteries, however, the role of the non-classical EDHF-type relaxation was also abolished.

In the diabetic arteries, the portion of relaxation resistant to TRAM-34 + apamin was abolished by Ibtx suggesting a role of maxi K_{Ca} perhaps through NO, HNO or EETs as previously described (Andrews *et al.*, 2009; Ellis *et al.*, 2009; Edwards *et al.*, 2010; Yuill *et al.*, 2010). We examined the role for NO by the addition of a NO scavenger HXC, which in the presence of TRAM-34 + apamin, significantly reduced the maximum relax-

ation in diabetic arteries but not in normal arteries. The remaining relaxation was partially inhibited in normal and abolished in diabetic arteries by the addition of Ibtx. The remaining Ibtx-sensitive component to the endothelium-dependent relaxation, which is not mediated by NO, may be acting through the release of HNO and/or EETs but this requires further investigation.

While our functional experiments demonstrated an impairment of IK_{Ca}- and SK_{Ca}-mediated EDHF-type relaxation, the results from our Western blot analysis have shown an increased expression of IK_{Ca} and SK_{Ca} in diabetes. This phenomenon could be a compensatory response to diabetes in order to maintain both EDHF and NO-mediated relaxation (Sheng *et al.*, 2009). Indeed, many studies have shown that diabetes causes compensatory changes in endothelial function. For example, we and others (Hink *et al.*, 2001; Cai *et al.*, 2005; Leo *et al.*, 2010) have demonstrated that in the large vessels, NO synthesis was significantly impaired in diabetes but eNOS expression was significantly increased. Therefore, in this study, despite the increased expression of IK_{Ca} and SK_{Ca}, the impairment of IK_{Ca}- and SK_{Ca}-mediated EDHF-type relaxation may be due to the impairment of downstream microdomain signalling and/or myoendothelial gap junction pathways (Young *et al.*, 2008; Weston *et al.*, 2010).

Role of NO-mediated relaxation in diabetic mesenteric arteries

In order to evaluate NO-mediated relaxation, the EDHF-type relaxation was inhibited either by the endothelial K_{Ca} channel blockers or by the presence of a depolarizing high K^+ solution. This confirmed an impaired release of NO as maximum relaxation to ACh was decreased in the diabetic mesenteric arteries in comparison with normal arteries. In addition to stimulated NO release, basal release of NO release was also decreased in diabetic mesenteric arteries, as demonstrated by impaired contraction in response to NOS inhibition. Hence, endothelial dysfunction in the diabetic mesenteric arteries was partly attributed to the impairment of NO activity.

Our functional results demonstrated an impairment of NO-mediated relaxation in diabetic mesenteric arteries. We found that the expression of total eNOS was unaffected by diabetes, but we observed eNOS uncoupling demonstrated by the decreased proportion of eNOS expressed as a dimer in the diabetic mesenteric vasculature without affecting the total expression of eNOS, suggesting that in the diabetic rats eNOS is producing more superoxide and less NO (Hink *et al.*, 2001; Cai *et al.*, 2005; Leo *et al.*, 2010), than the normal rats. The activation of eNOS is regulated by several protein kinases (Dudzinski and Michel, 2007), including the protein kinase Akt (Fulton *et al.*, 1999), hence we investigated the level of phosphorylation of Akt in the diabetic mesenteric arteries. We observed that the level of phosphorylation of Akt on Ser^{473} was decreased in diabetes, without affecting the total expression of Akt. This observation is consistent with a recent study where it has been demonstrated that diabetes was able to decrease the phosphorylation of Akt on Ser^{473} (Hamamdzic *et al.*, 2010). A further possible explanation for decreased NO activity could be the increased expression of Nox2, which could contribute to the elevated vascular superoxide production evident in diabetic mesenteric arteries, which could then promote an increased degradation of NO by superoxide resulting in the formation of peroxynitrite. Indeed, endothelium-dependent relaxation was restored with the antioxidant, tempol, presumably due to acute enhancement of NO activity resulting from decreased inactivation by superoxide. Taken together, we have shown that several diabetes-induced changes could contribute to the decrease in NO activity, such as the decrease in phosphorylation of Akt, the uncoupling of eNOS and the increased vascular superoxide levels, at least in part, due to increased NADPH oxidase-driven superoxide production.

In conclusion, after 10 weeks of diabetes in rats, hyperglycaemia increased superoxide production, causing endothelial dysfunction, which was due to the impairment of both NO and EDHF-type relaxation. The impairment of NO-mediated relaxation could be, at least in part, due to increased superoxide production, eNOS uncoupling and decreased activation of eNOS via the protein kinase Akt. The impairment of EDHF-type relaxation did not involve a decrease in channel expression, rather it may arise from the disruption of downstream pathways of IK_{Ca} and SK_{Ca} channels. The degree of impairment in NO activity appears to be greater than the reduction in EDHF activity as EDHF is able to elicit maximum relaxation when NO is inhibited, suggesting that NO is more susceptible to impairment by diabetes. Therefore, pharmacological intervention that aimed to improve the bioavailability of NO could be useful in the prevention of diabetic microvascular diseases.

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Conflicts of interest

None.

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