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Endothelial dysfunction in the streptozotocin-induced diabetic apoE-deficient mouse

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1 Endothelial dysfunction plays a role in the development of atherosclerosis and diabetes-associated vascular disease and, in the streptozotocin (STZ)-induced apoE-deficient diabetic mouse, we report that, when compared to the citrate (CIT)-treated nondiabetic apoE-deficient control, acetylcholine (Ach)-mediated endothelium-dependent relaxation was reduced in the small mesenteric arteries (SMA) and the plaque-prone regions of the aorta from the STZ-diabetic mouse.

2 In the SMA the component of Ach-mediated relaxation that was attributed to nitric oxide (NO) from STZ-treated diabetic apoE-deficient mice was enhanced; however, the endothelium-derived hyperpolarizing factor (EDHF)-mediated component was reduced. The EDHF component was assessed by determining the component of the Ach-mediated response that was resistant to the combination of the NO synthase (NOS) inhibitor N_{ω}-nitro-L-arginine methyl ester, cyclooxygenase inhibitor, indomethacin, and soluble guanylate cyclase inhibitor, ODQ, and inhibited by the combination of the intermediate conductance K_{Ca} (IK_{Ca}) inhibitor TRAM-34 and the small-conductance K_{Ca} (SK_{Ca}) inhibitor apamin.

3 Endothelial NOS was increased but SK2, SK3 and connexin (Cx) 37 mRNA expressions were significantly ($P<0.05$) decreased in the SMA from STZ-treated apoE-deficient mice compared to the CIT-treated controls. There was no difference in the IK_{Ca} expression or in Cx 40, 43 and 45 mRNA levels between STZ- and CIT-treated mice.

4 The microvasculature of STZ-induced apoE-deficient mice developed endothelial dysfunction, which may be linked to a decrease in the contribution of the EDHF component due to a decrease in SK2 and 3 and Cx 37 expression.
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Abbreviations: Ach, acetylcholine; apoE, apolipoprotein E; CIT, vehicle (citrate buffer); Cx, connexin; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; IK_{Ca}, intermediate-conductance calcium-activated potassium channels; Indo, indomethacin; L-NAME, N_{ω}-nitro-L-arginine methyl ester hydrochloride; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; PE, phenylephrine; SK_{Ca}, small-conductance calcium-activated potassium channels; SMA, small mesenteric arteries; STZ, streptozotocin; TRAM-34, 1-[(2-chlorophenyl)(diphenyl)methyl]-1*H*-pyrazole

Introduction

Diabetes-associated vascular complications are a major clinical problem and patients with diabetes have a mortality rate 3–4 times that of the general population (Haffner *et al*., 1998). Furthermore, diabetes is associated with accelerated atherogenesis, a two- to four-fold increase in the incidence of coronary artery disease, as well as a 10-fold increase in peripheral vascular diseases and up to 75% of patients diagnosed with diabetes ultimately dying from vascular disease (Haffner *et al*., 1998; Grundy *et al*., 1999; 2002; Laakso, 1999). Damage to the endothelium as well as endothelial dysfunction has long been recognized as initiating events in the development of atherosclerosis (Duncan, 1963; Ross & Glomset, 1973; Ross, 1993). Dysfunction of the endothelium plays an early prominent role in the development of cardiovascular disease and possibly precedes the development of diabetes itself (Vita *et al*., 1990; Haffner *et al*., 1998).

Endothelial dysfunction can simply be defined as a reduced endothelium-dependent vasodilator response to acetylcholine (Ach), and, although the endothelium plays many additional functions beyond the control of vascular tone, this reduced response to Ach serves as an important indicator of vascular dysfunction that implies a decreased bioavailability of nitric oxide (NO). It is well known that a reduction of NO has multiple effects on vascular homeostasis and cardiovascular health (Anderson, 1999; Verma & Anderson, 2002; Ding & Triggle, 2005).

Although the importance of endothelial dysfunction in the pathogenesis of cardiovascular disease has long been recognized (Anderson, 1999; Verma & Anderson, 2002), the cellular processes that lead to dysfunction are debated (De Vriese *et al*., 2000; Triggle *et al*., 2003; Andrews *et al*., 2005). On the

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one hand, diabetes-related hyperglycaemia has been shown to rapidly induce oxidative stress and endothelial cell dysfunction in cell culture (Nishikawa *et al*., 2000), as well as in invasive human studies (Kawano *et al*., 1999). On the other hand, diabetes-related dyslipidaemia has also been shown to be an important contributor to vascular dysfunction, as has been reported in familial hypercholesterolaemic patients before and after cholesterol reduction (Stadler *et al*., 1997), as well as postprandially in relation to remnant lipoprotein levels (Funada *et al*., 2002). In diabetes, triglycerides are typically raised and high-density lipoprotein (HDL) lowered (Grundy *et al*., 1999). Atherosclerosis is, however, associated with macrovascular disease and thus the role of dyslipidaemia *versus* hyperglycaemia in the pathogenesis of microvascular disease remains unclear and continues to be debated (Libby & Plutzky, 2002).

To better understand the link between diabetes and the development of cardiovascular disease, numerous animal models have been developed and transgenic mouse models offer many advantages (Coleman, 1982). Normal mice, however, do not develop atherosclerosis and thus, it may be argued, are unsuitable for comparative studies of the disease process in humans; however, with appropriate pharmacological and/or genetic manipulation, mice can be induced to mimic the human disease. In this regard, the apolipoprotein E (apoE)-deficient mouse has proved a useful model of human atherosclerosis and rapidly develops atherosclerotic lesions even when maintained on a normal chow diet (Meir & Leitersdorf, 2004). Overexpression of the apoE gene results in, dependent upon the promoter, a decrease in diet-induced hypercholesterolaemia and fatty streaks in the arterial wall (Harada *et al*., 1996). Crauwels *et al*. (2003) have reported that endothelial dysfunction was prominent on the plaque-rich regions in the aorta from the apoE-deficient mouse and plaques were predominantly found in the aortic root, the proximal segments and distal aorta segments with the central region of the aorta only minimally affected. Type I diabetes can also be induced in the apoE-deficient mouse with streptozotocin (STZ), and the current investigation was designed to determine whether endothelial dysfunction was exacerbated in the aorta and also whether dysfunction also developed in the microvasculature of the STZ-induced diabetic apoE-deficient mouse.

In the mouse aorta, endothelium-dependent relaxation is entirely mediated by NO (Huang *et al*., 1995) and a reduction in the bioavailability of NO is a common feature of diabetes that, likely, contributes, to the development of atherosclerosis. In the microvasculature there is, as in other species, a variable contribution of endothelium-dependent hyperpolarizing factor, EDHF (Chataigneau *et al*., 1999; Waldron *et al*., 1999; Ding *et al*., 2000). Although the nature of EDHF remains unknown (McGuire *et al*., 2001; Busse *et al*., 2002; Vanhoutte, 2004) and may be purely electrical coupling *via* myo-endothelial gap junctions (Sandow, 2004), the EDHF pathway may serve an important role in the microvasculature and provide the cellular basis for conducted vasodilatation (Takano *et al*., 2004). Of importance for the present study is that myo-endothelial gap junctions have been shown to play an important role in mediating the EDHF response in mouse small mesenteric arteries (SMA) (Dora *et al*., 2003).

Changes in the contribution of EDHF in disease states potentially may have important pathophysiological consequences (Feletou & Vanhoutte, 2004). Decreases in the contribution of EDHF to endothelium-dependent relaxation have been frequently reported in STZ-treated rats (Fukao *et al*., 1997; De Vriese *et al*., 2000; Wigg *et al*., 2001; Matsumoto *et al*., 2003; 2004), but in type II diabetic mice no change in the overall contribution of EDHF is apparent (Pannirselvam *et al*., 2002). In most vascular beds, the EDHF component of endothelium-dependent relaxation is inhibited by the combination of the small-conductance K_{Ca} (SK_{Ca}) blocker, apamin, and the nonspecific intermediate-conductance K_{Ca} (IK_{Ca}) blocker, charybdotoxin (Garland & Plane, 1996; McGuire *et al*., 2001), or the selective IK_{Ca} blocker, TRAM-34 (Wulff *et al*., 2000). In the current study, we thus sought answers to two questions: first, is endothelium-dependent relaxation compromised in the aorta of STZ-treated apoE-deficient mice, and is the endothelial dysfunction associated with the 'plaque-prone' regions of the aorta – as described by Crauwels *et al*. (2003)? Second, is the contribution of EDHF to endothelium-dependent relaxation in SMA from STZ-treated apoE-deficient mice also compromised and does diabetes affect expression of the K_{Ca} channels and connexins (Cx)?

Methods

Experimental procedures

Animals ApoE-deficient mice were purchased from the Heart Research Institute, NSW, Australia. Mice were assigned into age-matched groups. apoE-deficient mice, 6-week old, were injected $55\,mg\,kg^{-1}\,day^{-1}$ STZ or vehicle (citrate buffer) (CIT) intraperitoneal over 5 consecutive days. Mice were monitored for glucosuria 2 weeks after STZ injection to assess the induction of diabetes, and only animals that tested positive were continued in the study. Mice were killed at 12–16 weeks after STZ or vehicle treatment. Animals with blood glucose levels above $20\,mmol\,l^{-1}$ were used. The blood was taken at the time of decapitation, blood was collected in a centrifuged tube with $25\,\mu l$ of heparin and stored in the fridge, the blood samples were analysed the following day for cholesterol and triglycerides and HDL/LDL. All procedures were approved by the RMIT University Animal Ethics Committee.

Plasma levels of total cholesterol and triglycerides HDL and LDL were measured enzymatically (CHOD-PAP and GPO-PAP, respectively, Roche, Basel, Switzerland), performed by Mayne Health Dorevitch Pathology (Victoria, Australia). Blood glucose levels were measured by the *MediSense®2 Blood Glucose Testing System* (MediSense Australia Pty. Ltd, Victoria, Australia) at the time of killing.

Myograph

First-order mesenteric arteries (SMA) were removed and placed in Krebs' solution (composition, mM: NaCl, 120; $NaHCO_3$, 25; KCl, 4.8; NaH_2PO_4, 1.2; $MgSO_4$, 1.2; Glucose, 11.0; $CaCl_2$, 1.8) bubbled with 95% O_2 and 5% CO_2. Arteries were cut into 2 mm rings and mounted on a Mulvany–Halpern myograph as described previously (Mulvany & Halpern, 1977). Tissues were stretched to a set tension of 2 mN. All experiments were performed at 37°C. Tissues were maximally contracted with 120 mM high-potassium salt solution.

Phenylephrine (PE) cumulative concentration–response curves (0.1–30 μM) were performed on all tissues. The concen-

tration of PE required to produce a 70% response was used to contract the vessels for subsequent vasorelaxation curves. Typically, PE produced a contraction that was stable for at least 20 min and only tissues in which the PE contraction was stable were used for the subsequent studies of Ach-mediated relaxation. Concentration–response curves to Ach (0.01–10 μM) were performed on all tissues and then repeated either alone or after a 15-min incubation with N_ω-nitro-L-arginine methyl ester (L-NAME) (100 μM), indomethacin (Indo) (10 μM) and ODQ (10 μM), or after 30-min incubation with: L-NAME, Indo, ODQ plus apamin (10 μM) or L-NAME, Indo, ODQ, TRAM-34 (1 μM), and apamin. Concentration–response curves (0.1–30 μM) were performed up to a maximum of 4 times on each tissue. Time-controls for the maintenance of PE-induced tone and subsequent Ach-mediated relaxations revealed no significant difference between four sequential relaxation–response curves, confirming the reproducibility of Ach response curves.

Aortic ring segments of 3 mm in length (ID 800 μm) were systematically sectioned from the ascending thoracic aorta. Following the description by Crauwels *et al*. (2003), two aortic segments per mouse were dissected 3 mm from the origin of the left subclavian artery towards the diaphragm (the first segment being the 'plaque-prone' and the second the 'plaque-resistant'). Each segment was mounted through the lumen on two parallel 200-μm stainless steel pins in a Mulvany–Halpern myograph for isometric force recording. Each vessel preparation was gradually stretched according to the normalization procedures of Mulvany & Halpern (1977) to determine optimum resting tension (14–18 mN) and was equilibrated for 1 h before commencement of experiments. Preparations were contracted with PE (0.2 μM) and allowed to stabilize before constructing cumulative concentration–response curves to the endothelium-dependent vasodilator (Ach, 1 nM–10 μM) and the endothelium-independent vasodilator sodium nitroprusside (SNP, 1 nM–10 μM). Studies of Ach-mediated relaxation in the presence of L-NAME, Indo, ODQ or K-channel blockers were not performed in the aorta.

Real-time reverse transcription–PCR assay

The entire mesenteric artery arcade was homogenized and total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. The RNA yield was measured by absorbance at 260 nm. The first-strand cDNA was synthesized by Superscript II reverse-transcriptase with oligo(dT)$_{12–18}$ primer from 1 μg of total RNA. To ensure that the primers were specific and efficient, a number of tests were performed. Primers were initially analysed by performing positive control and negative control PCR reactions with SYBR-green (Qiagen) at eight annealing temperatures, ranging from 52 to 62°C. Melt curve analysis was used to visualize primer specificity by revealing the presence or absence of primer dimers. For further verification, 1.0 μl of the PCR reaction was analysed on an Agilent Technologies 2100 Bioanalyzer using a DNA 500 LabChip kit. Sequencing was performed on products from successful reactions. PCR efficiency was determined by performing real-time PCR on serial dilutions of mouse brain cDNA. QRT–PCR was performed in duplicate using the following primer sequences:

eNOS, sense 5′-CAACGCTACCACGAGGACATT-3′
and antisense 5′-CTCCTGCAAAGAAAAGCTCTGG-3′;
SK1, sense 5′-GTGAAGATTGAACAAGGGAAGG-3′
and antisense 5′-TGCCTCCAACTCCTCCTG-3′;
SK2, sense 5′-ACCATCAGACAGCAGCAAAGGG-3′
and antisense 5′-GACCGCCGCCTCCTGGAC-3′;
SK3, sense 5′-GCCAACTCTACCGCCATC-3′
and antisense 5′-GGCTGTGGAACTTGGAGAG-3′;
IK_{Ca}, sense 5′-ATGCTCCTGCGTCTCTAC-3′
and antisense 5′-GAAGCGGACTTGGTTGAG-3′;
β-actin, sense 5′-ACGGCCAGGTCATCACTATTG-3′
and antisense 5′-CCAAGAAGGAAGGCTGGAAAAGA-3′;
Cx37, sense 5′-AGGCAGGCTTCCTCTATGGC-3′
and antisense 5′-AGACATAGCAGTCCACGATGTG-3′;
Cx40, sense 5′-GAGGCCCACGGAGAAGAATG-3′
and antisense 5′-TGGTAGAGTTCAGCCAGGCT-3′;
Cx43, sense 5′-ACAAGGTCCAAGCCTACTCCA-3′
and antisense 5′-CCCCAGGAGCAGGATTCTGA-3′;
Cx45, sense 5′-CGGGCTGTGAGAATGTCTGC-3′
and antisense 5′-CAGGTACATCACAGAGGGAGTTG-3′.

The amplification was carried out in 50 μl of reaction mixture containing 25 μl of iQ SYBR Green Supermix (Bio-Rad), 2 μl of the first-strand cDNA products, 100 nM each of the sense and antisense primers. For each set of primers, a *no template* control was included. The reactions were carried out on a 96-well plate, and a PCR amplification protocol was followed (95°C for 5 min and 40 cycles of amplification at 95°C for 30 s, 62°C (eNOS); 58.2°C (K_{Ca}); 60.1°C (Cx) for 30 s and 72°C for 30 s) using an iCycler iQ machine (Bio-Rad). Post-amplification melting curve analysis was performed to show a single amplification product without contamination. Electrophoresis analysis on a 2% agarose gel was also carried out for quality control. The fold relative to β-actin for the mRNA of interest was calculated using the $2^{-\Delta C_T}$ method (Livak & Schmittgen, 2001).

Drugs

PE, Ach, Indo, apamin, ODQ and L-NAME were purchased from Sigma-Aldrich (Sydney, NSW, Australia). TRAM-34 was a gift from Dr Heike Wulff – Department of Medical Pharmacology and Toxicology, University of California, Davis, CA 95616, U.S.A. PE, Ach and L-NAME were dissolved in distilled water, while TRAM-34 and ODQ were dissolved in 100% DMSO and Indo was dissolved in 1% sodium carbonate.

Data analysis

Data are expressed as pD_2 values and pD_2 is defined as the negative logarithm to base 10 of the EC_{50} values. In all experiments, n equals the number of animals. Relaxation is expressed as percentage of PE-induced tone (plateau phase) $\pm \ominus$ s.e.m. The significance of differences between mean values was calculated by Student's t-test. Statistical significance of differences between the means of data groups was performed using ANOVA for curve analysis. Significance was assumed if $P<0.05$.

Results

Metabolic analysis

Blood glucose levels, total cholesterol, HDL and LDL levels were significantly greater in STZ-treated mice than CIT-

treated mice ($P<0.05$, unpaired Student's t-test, $n=8$). Triglyceride levels for the CIT-treated mice were not significantly different from the STZ-treated group ($P>0.05$, unpaired t-test, $n=8$) (Figure 1).

Myograph – aorta

Relaxation responses to Ach were significantly ($P<0.05$, ANOVA) reduced in the plaque-prone aortic segments from STZ-treated apoE-deficient mice, compared to those in CIT-treated apoE-deficient mice (Figure 2a; STZ group: $pD_2=6.2\pm0.2$; $E_{max}=66.3\pm7.6\%$; $n=4$; CIT group: $pD_2=6.8\pm0.1$; $E_{max}=83.6\pm3.8\%$; $n=6$). In contrast, responses to Ach were not impaired in neighbouring plaque-resistant segments in STZ-treated mice (Figure 2a; STZ group: $pD_2=7.1\pm0.1$; $E_{max}=85.8\pm3.5\%$; $n=4$; CIT group: $pD_2=7.1\pm0.1$; $E_{max}=89.9\pm3.3\%$; $n=7$). SNP responses remained similar in all the segments studied (Figure 2b).

Myograph – SMA

In endothelium-intact first-order SMA from CIT-treated (nondiabetic) apoE-deficient mice contracted with PE (1 μM), Ach induced a concentration-dependent relaxation, as shown in the representative traces (Figure 3a), with a maximum response of $88.6\pm2.7\%$. The representative traces, with a 30-min interval between each trace, illustrate Ach-induced relaxation in the presence of the NOS inhibitor, L-NAME (100 μM), the cyclooxygenase inhibitor, Indo (10 μM), and soluble guanylate cyclase inhibitor, ODQ (10 μM) (Figure 3b), and in the presence of the combination of L-NAME, Indo ODQ, the SK_{Ca} inhibitor apamin, and the combination of the putatively specific IK_{Ca} inhibitor TRAM-34 (Wulff *et al.*, 2000) (Figure 3c). The presence of L-NAME, Indo and ODQ significantly reduced the maximal relaxation to Ach ($69.2\pm4.7\%$) ($P<0.05$) (Figure 3d). In the presence of L-NAME, Indo, ODQ, TRAM-34 and apamin, the maximal

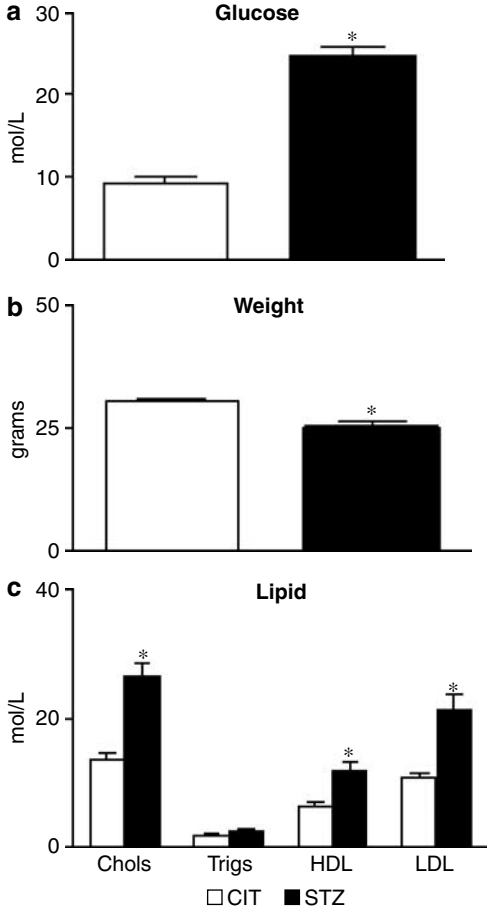

Figure 1 Plasma glucose (a), weights (b) and lipid (c) profile from CIT- and STZ-treated apoE-deficient mice taken at the time of killing.

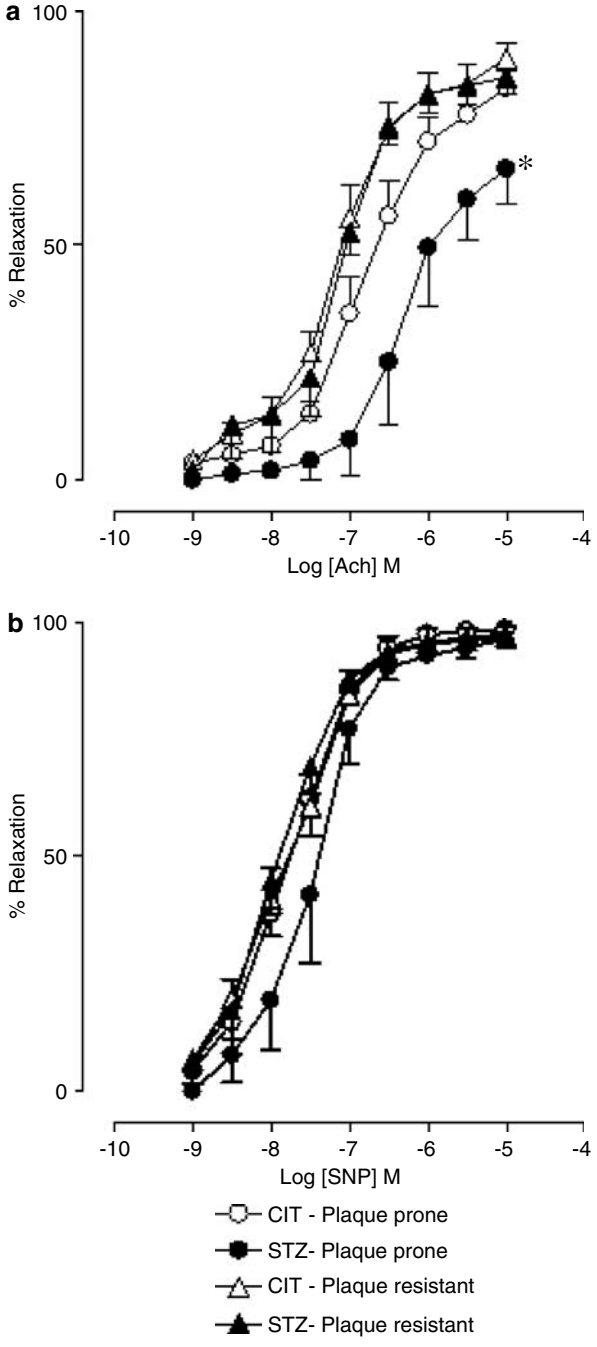

Figure 2 Ach (a) and sodium nitroprusside (SNP, b) concentration–response curves in the absence of inhibitors in the aorta from CIT- and STZ-treated apoE-deficient mice in plaque-prone and -resistant segments.

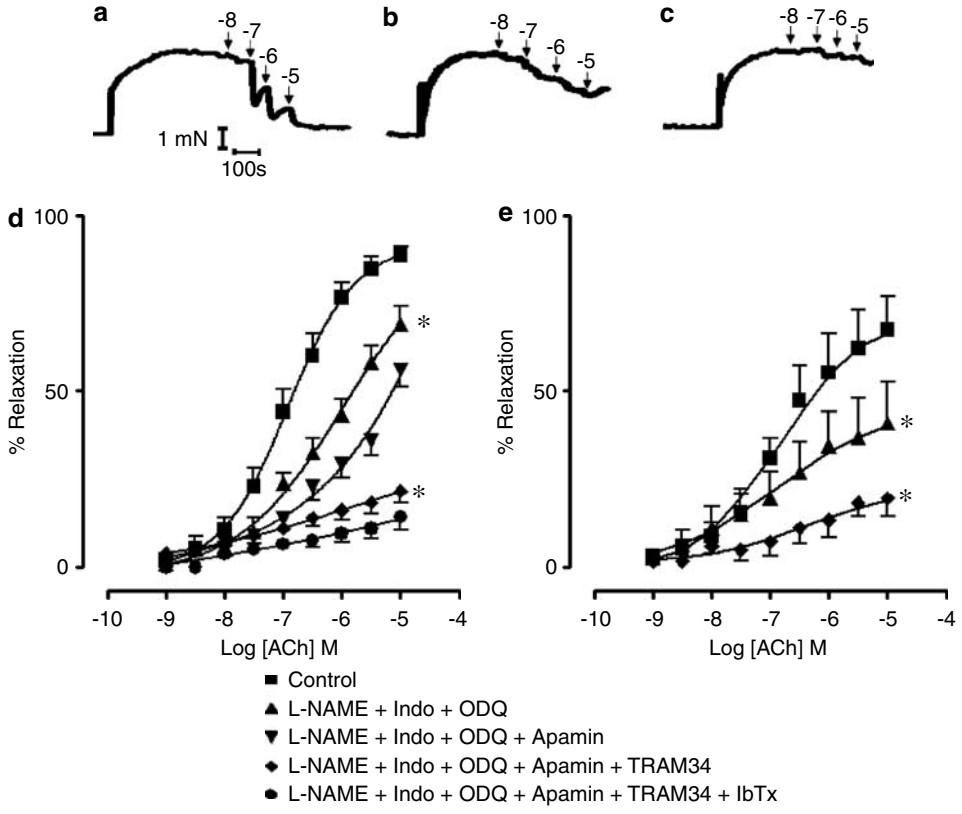

Figure 3 Representative traces of Ach-mediated relaxation in the absence of inhibitors (a), in the presence of L-NAME, Indo and ODQ (b) and in the presence of the combination of L-NAME, Indo, ODQ, apamin and TRAM-34 (c) with a 30-min interval between each trace. Concentration–response curves to Ach in the SMA from CIT- (d) and STZ- (e) treated apoE-deficient mice in the absence or presence of L-NAME, Indo and ODQ (d, e), in the presence of apamin (d) or combination of TRAM-34 and apamin (d, e), as well as L-NAME, Indo, ODQ, apamin and TRAM-34 with iberiotoxin (IbTx) (d). *Significantly different from control responses ($P<0.05$), $n=4$–9.

relaxation to Ach was significantly reduced to $21.6\pm3.5\%$ ($P<0.05$) (Figure 3d). However, the addition of apamin alone, without TRAM-34, produced no additional inhibition of Ach-mediated relaxation to that seen in the presence of L-NAME, Indo and ODQ (Figure 3d). Similarly, the addition of iberotoxin produced no further inhibition to that seen in the presence of TRAM-34 and apamin (Figure 3d). We have demonstrated that there is no time-dependent decrease of responses to Ach by the reproducibility of a series of four Ach-mediated relaxation–response curves (data not shown). We have also demonstrated that a contraction to PE was stable for 20 min in an SMA preparation from a CIT-treated apoE-deficient mouse (data not shown).

In STZ-treated apoE-deficient mouse SMA contracted with PE, Ach also induced a concentration-dependent relaxation with a maximum relaxation of $71.5\pm7.5\%$, which was significantly decreased compared to the Ach relaxation in CIT-treated SMA ($P<0.05$) (Figure 3e). In addition, L-NAME (100 μM), Indo (10 μM) and ODQ (10 μM) significantly inhibited the maximal relaxation to Ach ($P<0.05$; Figure 3e) and the combination of TRAM-34 and apamin (in the presence of L-NAME, Indo and ODQ) also significantly reduced the maximal relaxation ($P<0.05$) (Figure 3e).

Real-time PCR analyses

eNOS mRNA expression was examined by real-time PCR using β-actin as internal control (Figure 4a). There was a 1.9-fold increase of eNOS mRNA expression in STZ-treated apoE-deficient mouse mesenteric arteries compared to expression in CIT-treated apoE-deficient mice ($P<0.05$) (Figure 4b).

As reflected by the RT–PCR data, SK3 is the predominant isoform in apoE-deficient mouse mesenteric arteries, but both SK2 and SK3 are significantly decreased in STZ-treated apoE-deficient mouse mesenteric arteries comparing to that in CIT-treated apoE-deficient mouse mesenteric arteries ($P<0.05$) (Figure 5a and b). We did not detect $SK_{Ca}1$ expression in either CIT- or STZ-treated apoE-deficient mouse mesenteric arteries (Figure 5a). No significant difference was noted in IK_{Ca} expression in mesenteric arteries when CIT-treated was compared to STZ-treated apoE-deficient mice.

Cx 37 mRNA was decreased $23.8\pm3.4\%$ in STZ-treated apoE-deficient mouse mesenteric arteries compared to CIT-treated controls ($P<0.05$). However, Cx 40, 43 and 45 mRNA levels were not significantly changed ($P>0.05$) (Figure 6a and b).

Discussion

The important findings from this study are that the induction of diabetes by STZ in the apoE-deficient mouse results in hyperglycaemia, elevated cholesterol and endothelial dysfunction in the SMA and plaque-prone areas of the aorta. In addition, in the SMA, endothelial dysfunction is associated with a reduced contribution of the EDHF component of the

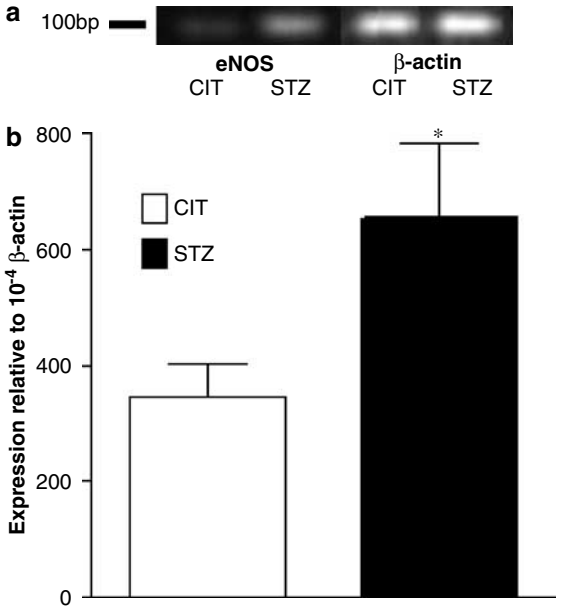

Figure 4 mRNA expression of eNOS in CIT- and STZ-treated apoE-deficient mouse mesenteric arteries by the real-time PCR method. (a) Representative gel. (b) Analytical data of expression ratio. Values are expressed as the relative ratio to β-actin. *Significantly different ($P<0.05$), $n=5$.

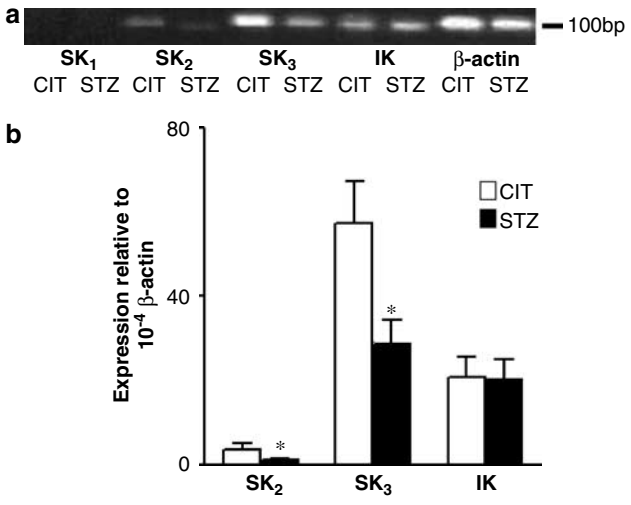

Figure 5 mRNA expression of SK_2, SK_3 and IK in CIT- and STZ-treated apoE-deficient mouse mesenteric arteries by real-time PCR method. (a) Representative gel. (b) Analytical data of expression ratio. Values are expressed as the relative ratio to β-actin. *Significantly different ($P<0.05$), $n=5$.

endothelium-dependent response to Ach, as well as a reduction in the expression of Cx37, SK2 and 3. Although a number of investigators have reported that the induction of diabetes in the apoE-deficient mouse provides a useful animal model of diabetic hyperlipidaemia (for instance, see Yamamoto *et al*., 1995), we believe that the current study is the first to describe endothelial dysfunction in this model.

In a vessel- and species-dependent manner, endothelium-dependent relaxation to Ach is made up of contributions from NO, prostacyclin and the putative EDHF (McGuire *et al*., 2001; Vanhoutte, 2004). In the mouse aorta NO appears to be

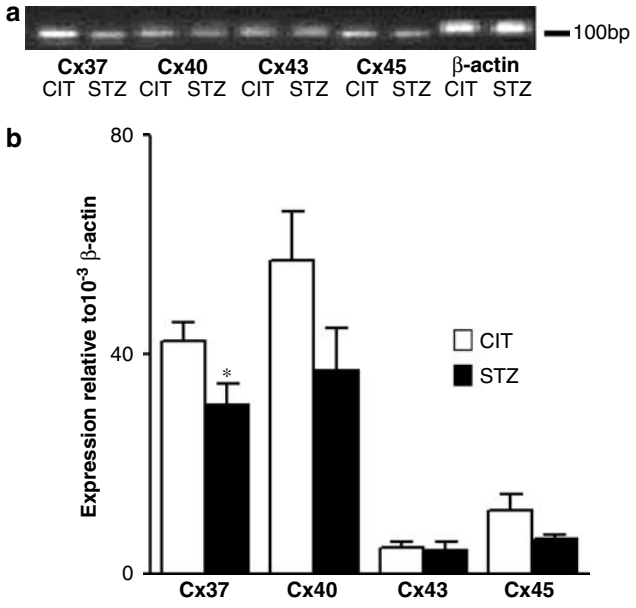

Figure 6 mRNA expression of Cx 37, 40, 43 and 45 in CIT- and STZ-treated apoE-deficient mouse mesenteric arteries by real-time PCR method. (a) Representative gel. (b) Analytical data of expression ratio. Values are expressed as the relative ratio to β-actin. *Significantly different ($P<0.05$), $n=5$.

the sole endothelial-derived mediator, whereas in the SMA both NO and EDHF are important (Huang *et al*., 1995; Waldron *et al*., 1999). In the SMA from the CIT-treated apoE-deficient (control) mouse, the addition of L-NAME, ODQ and Indo significantly reduced the maximal relaxation response to Ach by approximately 20%. In the STZ-treated apoE-deficient mouse, the effects of the L-NAME/Indo/ODQ combination were similar, with the data demonstrating an approximately 30% reduction of the NO component. Thus, despite a 1.9-fold increase in the expression of eNOS in the SMA from the STZ-treated apoE-deficient diabetic mouse, there was a reduction in endothelium-dependent relaxation to Ach. The L-NAME/Indo/ODQ-insensitive component, presumably, reflects the contribution of the putative EDHF. Interestingly, it was the L-NAME/Indo/ODQ-insensitive component that was reduced in the SMA of STZ-treated mice, indicating that the contribution of EDHF is reduced in STZ-induced diabetes – at least in the SMA (Figure 3b).

The relative contribution of EDHF to endothelium-dependent relaxation in a type 1 mouse model of diabetes differs from that which we report for the db/db mouse model of type 2 diabetes; in the SMA, endothelium-dependent relaxation is entirely mediated by EDHF, despite no change in eNOS mRNA expression (Pannirselvam *et al*., 2005). Collectively, these data suggest that the microvascular disease associated with both type I and II diabetes is linked to changes in the contribution of EDHF-mediated pathways in mouse microvasculature; however, in type I diabetes the EDHF-mediated component of endothelium-dependent relaxation is reduced, whereas in type II diabetes the contribution of EDHF is maintained.

As noted in the Introduction, it is the contribution of EDHF to endothelium-dependent relaxation in the microcirculation that is reduced in mesenteric arteries from STZ-treated rats (Fukao *et al*., 1997; Wigg *et al*., 2001; Matsumoto *et al*., 2003; 2004) and the renal vasculature (De Vriese *et al*., 2000). Thus,

our data from mice are consistent with those reported for the STZ-induced diabetic rat and that, in the SMA, STZ-induced diabetes results in endothelial dysfunction that can be linked to a reduced contribution of EDHF. Studies of endothelium-dependent relaxation in larger blood vessels, such as the aorta, have, however, reported a reduction in the NO-mediated component (Kamata *et al*., 1999). Indeed, our study also demonstrated that endothelial function was also reduced in the aorta of the STZ-treated apoE-deficient mice, and this was correlated with the presence of plaque; these data are entirely in agreement with the publication from Crauwels *et al*. (2003) that described endothelial dysfunction only in the plaque-prone areas of the aorta from apoE-deficient mice. It should be stressed, however, that the apoE-deficient mice in the study by Crauwels *et al*. (2003) were 18 months of age, as compared to 12–16 weeks in the present study, and endothelial dysfunction was more dramatic in the older animals and amounted to a 75% reduction of the response to Ach in plaque-prone area, as well as a reduction in relaxation to the endothelium-independent vasodilator, NO. Thus, changes in vascular smooth muscle function are also apparent in older apoE-deficient mice.

The advantages of studying the STZ-treated apoE-deficient mouse as a preferred model to the STZ-induced diabetic rat was first reported by Yamamoto *et al*. (1995) and, in particular, they stressed that hypercholesterolaemia was not stably observed without dietary modification in the rat, but that stable hypercholesterolaemia without dietary modification could be obtained in the STZ-induced diabetic apoE-deficient mouse. Endothelial function has not been previously reported in the STZ-diabetic apoE-deficient mouse model, but has been extensively studied in the STZ-diabetic rat. Endothelial function in the STZ-diabetic rat aorta has been reported to show a triphasic change – enhanced at 1-week post-STZ, unaltered at 1–2 weeks and impaired at 8 weeks (Pieper, 1999). Kobayashi & Kamata (2001) reported a decrease in aortic endothelial function 9 weeks after STZ treatment and this was accompanied by an increase in oxidative stress, but no change in mRNA eNOS expression. In contrast in rats treated for 2 weeks with STZ, aortic eNOS mRNA and NADPH oxidase activation was increased (seven-fold increase in $gp91^{phox}$ mRNA) and endothelial dysfunction was associated with a reduction in the bioavailability of NO as measured by electron spin resonance (Hink *et al*., 2001). An elevated expression of eNOS may not necessarily be beneficial, as Ozaki *et al*. (2002) and Kawashima & Yokoyama (2004) report that the overexpression of eNOS in the apoE-deficient mouse leads to accelerated atherogenesis. Furthermore, an uncoupled eNOS may serve as an NADPH oxidase and result in the overproduction of superoxide anions ($^{\bullet}O_2^-$), and this may further exacerbate endothelial dysfunction. $^{\bullet}O_2^-$ can oxidize tetrahydrobiopterin, an important cofactor in the regulation of NOS, and this will lead to an 'uncoupled eNOS', which will then synthesize $^{\bullet}O_2^-$ rather than NO (Pannirselvam *et al*., 2003; Alp & Channon, 2004). Thus, our finding that eNOS expression is elevated in the STZ-mouse SMA may reflect a pathophysiological event that contributes, *via* enhanced $^{\bullet}O_2^-$ production, to microvascular disease.

Our data indicate that both SK2 and SK3 mRNA for the apamin-sensitive K_{Ca} channels are expressed in the mouse SMA. Western blot data from porcine coronary endothelial cells have also indicated that SK3 protein is abundant in samples of endothelium compared to whole arteries; SK2 protein was only present in whole artery nuclear fractions and immunofluorescent labelling for SK3 was highly expressed at the plasmalemma of endothelial cells, but not in smooth muscle (Burnham *et al*., 2002). The functional significance of the SK2 channel with the nuclear membrane is unknown at this time. The SK3 channel is thought to be essential for mediating the EDHF component of endothelium-dependent relaxation, and this is supported by data from the rat carotid artery (Eichler *et al*., 2003); interestingly we report that the expression of SK3 mRNA was reduced in the SMA of the STZ-diabetic mouse, as was the EDHF component of endothelium-dependent relaxation to Ach. In our study no change in IK_{Ca} expression was noted, and this may reflect the greater significance of the apamin-sensitive SK_{Ca} component in the determination of the EDHF-mediated hyperpolarization event. Two published studies support this conclusion: First, Crane *et al*. (2003) reported that, in rat SMA, TRAM-34 alone did not affect hyperpolarization to Ach, but, in combination with apamin, the hyperpolarization was completely abolished – suggesting that Ach-mediated endothelium-dependent hyperpolarization of vascular smooth muscle can be attributed to SK_{Ca} channels. Second, in the rat SMA the activation of the thromboxane receptor resulted in a differential block of the SK_{Ca}, as determined by a loss of smooth muscle hyperpolarization evoked with the SK_{Ca} activator 100 riluzole, but that due to the IK_{Ca} activator 1-EBIO was unaffected (Crane & Garland, 2004). The role of the apamin-sensitive SK_{Ca} channel in the mouse SMA in determining endothelium-dependent relaxation would appear to be different than in the rat SMA, as, for example, in the mouse SMA the Ach-mediated response cannot be entirely attributed to EDHF (Ding *et al*., 2000). Furthermore, apamin alone does not inhibit either the hyperpolarization or the relaxation mediated by the endothelium-dependent vasodilator peptide, $SLIGRL\text{-}NH_2$ (McGuire *et al*., 2004). Nonetheless, changes in the expression of SK3 channels in the endothelium of a transgenic mouse have been associated with altered arterial tone and blood pressure, and these data reflect, presumably, the importance of SK3 channels in the regulation of resistance artery tone (Taylor *et al*., 2003). Our data that associate a reduction of the EDHF component of endothelium-dependent relaxation to Ach in SMA from STZ-induced diabetic apoE-deficient mice with a reduction in SK2 and SK3 K_{Ca} channels are also supportive of the importance of SK_{Ca} channels in determining the EDHF response.

Myoendothelial gap junctions play an important role in mediating the EDHF response in mouse SMA (Dora *et al*., 2003); in the hypertensive state, both the EDHF response and Cx 37 expression are decreased in the mesenteric artery from the spontaneously hypertensive rat (Rummery & Hill, 2004) and ovariectomy has been shown to reduce Cx 40 and 43 expression, as well as the EDHF component of endothelium-dependent relaxation to Ach in the rat SMA (Nawate *et al*., 2005). We found a decrease in the contribution of EDHF and Cx 37 in the SMA and mesenteric artery arcade, respectively, from the apoE-deficient STZ-diabetic mice and, thus, Cx 37 may also play a critical role in the mesenteric circulation for the mediation of EDHF. We did not detect a change in Cx 40, 43 or 45 mRNA expression; these data, however, must be interpreted carefully as it was not possible, due to the low abundance in the tissue, to determine protein expression, nor

did we determine whether the number or distribution of myoendothelial gap junctions was altered in the STZ-diabetic animals. Additional studies with high-resolution electron microscopy would be valuable. Interestingly, although we did not detect differences in Cx 40 expression in SMA from the apoE *versus* the STZ-apoE, it has been shown that the phenotype of the Cx 40 knockout mouse presents with a profound reduction in Ach-mediated vasodilatation in cremaster arterioles (de Wit *et al*., 2000), and in the rat SMA application of a Cx 40 antibody has been shown to block EDHF-mediated vasodilatation (Mather *et al*., 2005). As stressed by Rummery & Hill (2004), more definitive support for a strong correlation between endothelial dysfunction, blood pressure and Cx expression awaits the use of mice, wherein Cx expression can be rapidly manipulated though an appropriate inducible system.

In conclusion, our studies of the micro- (SMA) vasculature of the STZ-diabetic apoE-deficient mouse reveal that endothelial function is depressed at 12–16 weeks. In the microvasculature, endothelial dysfunction can be linked to a decrease in the contribution of the EDHF component and this was reflected by a decrease in SK2, 3 and Cx 37 expression, but not Cx 40, 43 or 45, and a paradoxical increase in eNOS mRNA. In the macrovasculature (aorta), endothelial dysfunction was only significant in the plaque-prone area. Further studies are required to elucidate the importance of these changes in the contribution of the EDHF pathway, SK_{Ca} and Cx expression to diabetes-related microvascular disease and determine whether the enhanced expression of eNOS mRNA reflects a compensatory protective or pathophysiological process.

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