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Impaired small-conductance Ca^{2+}-activated K^+ channel-dependent EDHF responses in Type II diabetic ZDF rats

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1 We have examined the relative contributions of small- and intermediate-conductance Ca^{2+}-activated K^+ channels (SK_{Ca} and IK_{Ca}) to the endothelium-derived hyperpolarizing factor (EDHF) pathway response in small mesenteric arteries of Zucker Diabetic Fatty (ZDF) rats, before and after the development of Type II diabetes, together with Lean controls.

2 Smooth muscle membrane potential was recorded using sharp microelectrodes in the presence of 10 μM indomethacin plus 100 μM N^{ω}-nitro-L-arginine. SK_{Ca} was selectively inhibited with 100 nM apamin, whereas IK_{Ca} was blocked with 10 μM TRAM-39 (2-(2-chlorophenyl)-2,2-diphenylacetonitrile).

3 Resting membrane potentials were similar in arteries from 17- to 20-week-old control and diabetic rats (approximately -54 mV). Responses elicited by 1 and 10 μM acetylcholine (ACh) were significantly smaller in the diabetic group (e.g. hyperpolarizations to -69.5 ± 0.8 mV (ZDF; $n=12$) and -73.2 ± 0.6 mV (Lean; $n=12$; $P<0.05$) evoked by 10 μM ACh).

4 The IK_{Ca}-mediated components of the ACh responses were comparable between groups (hyperpolarizations to approximately -65 mV on exposure to 10 μM ACh). However, SK_{Ca}-mediated responses were significantly reduced in the diabetic group (hyperpolarizations to -63.1 ± 1.0 mV (ZDF; $n=6$) and -71.5 ± 1.2 mV (Lean; $n=6$; $P<0.05$) on exposure to 10 μM ACh.

5 Impaired ACh responses were not observed in arteries from 5- to 6-week-old (pre-diabetic) animals. SK_{Ca} subunit mRNA expression was increased in the diabetic group.

6 The EDHF pathway, especially the SK_{Ca}-mediated response, is impaired in Type II diabetic ZDF rats without a reduction in channel gene expression. These results may be particularly relevant to the microvascular complications of diabetes. The functional separation of SK_{Ca} and IK_{Ca} pathways is discussed.

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Abbreviations: ACh, acetylcholine; BAE, bovine aortic endothelial cell; ChTX, charybdotoxin; DMSO, dimethyl sulphoxide; 1-EBIO, 1-ethyl-2-benzimidazolinone; EDHF, endothelium-derived hyperpolarizing factor; IbTX, iberiotoxin; IK_{Ca}, intermediate-conductance Ca^{2+}-activated K^+ channel; SK_{Ca}, small-conductance Ca^{2+}-activated K^+ channel; ZDF, Zucker Diabetic Fatty (rat)

Introduction

Patients with overt Type II diabetes mellitus and obese patients with insulin resistance display reduced responsiveness to endothelium-dependent vasodilators such as acetylcholine or methacholine (Saenz de Tejada *et al*., 1989; McVeigh *et al*., 1992; Steinberg *et al*., 1996). Impaired endothelium-dependent vasodilation, 'endothelial dysfunction', may also be induced in healthy subjects by acute elevation of blood glucose or free fatty acids (Steinberg *et al*., 1997; Williams *et al*., 1998). Endothelial dysfunction is primarily interpreted as a deficiency of nitric oxide-mediated signalling related to reduced production combined with increased destruction of nitric oxide (reviewed by Cai & Harrison, 2000; Calles-Escandon & Cipolla, 2001). However, endothelium-dependent vasodilation may also occur *via* the endothelium-derived hyperpolarizing factor (EDHF) pathway, particularly in smaller resistance vessels, which may be important in the microvascular complications of diabetes (Hwa *et al*., 1994).

Non-nitric oxide, non-prostacyclin-mediated endothelium-dependent vasodilation can be attributed to the EDHF pathway. EDHF responses are blocked by inhibition of small- and intermediate-conductance Ca^{2+}-activated K^+ channels (SK_{Ca} and IK_{Ca}) located on the vascular endothelium, classically with the combination of apamin plus charybdotoxin (ChTX) but not apamin plus iberiotoxin (IbTX) (reviewed by Busse *et al*., 2002). Recently, a series of clotrimazole derivatives (TRAM-39 and TRAM-34) that selectively block IK_{Ca} but do not inhibit other Ca^{2+}-activated K^+ channels or cytochrome *P*450 enzymes have been developed (Wulff *et al*., 2000). Studies with these compounds have strengthened the idea that 'classical' EDHF responses are indeed due to the activation of endothelial SK_{Ca} and IK_{Ca} (Crane *et al*., 2003; Eichler *et al*., 2003), whereas additional hyperpolarizing

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factors involve smooth muscle large-conductance Ca^{2+}-activated K^+ channels and products of *P*450 enzymes (Weston *et al*., 2005). Studies suggest that the endothelial IK_{Ca} is composed of IK1 subunits (Bychkov *et al*., 2002; Eichler *et al*., 2003), whereas SK3 is apparently the relevant SK_{Ca} isoform (Burnham *et al*., 2002; Eichler *et al*., 2003; Taylor *et al*., 2003).

The inbred Zucker Diabetic Fatty (ZDF) rat is a model of Type II, adult-onset diabetes derived from the insulin-resistant but non-diabetic Obese or Fatty Zucker rat (Peterson *et al*., 1990). Male ZDF rats consistently develop hyperglycaemia over a period between 7 and 12 weeks of age and aged diabetic ZDF rats exhibit microvascular retinopathy and nephropathy (Yang *et al*., 2000; Hoshi *et al*., 2002). Little is known about endothelium-dependent hyperpolarization involving the EDHF pathway in arteries from this model of Type II diabetes. We have examined non-nitric oxide, non-prostacyclin-mediated responses to ACh using sharp microelectrodes to record smooth muscle membrane potential in arteries from ZDF rats before and after the development of diabetes. The dependence of these responses on SK_{Ca} and IK_{Ca} was determined by selective inhibition of these channels using either apamin or TRAM-39, respectively. Expression of channel mRNA in artery samples from control and diabetic groups was quantified by real-time RT–PCR.

Methods

Animals

Procedures were performed in accordance with the United Kingdom Animals (Scientific Procedures) Act 1986. Zucker Diabetic Fatty (ZDF/Gmi-fa/fa) rats and Lean Zucker controls were obtained from Charles River (six male animals per group). All ZDF rats tested positive for glucosuria (Uristix, Bayer) at 10 weeks of age. Systolic blood pressures in conscious animals were measured by tail cuff plethysmography (Pressure Computer LE 5007, Letica Scientific Instruments) at 16 weeks of age and animals were killed at 17–20 weeks (henceforth referred to as 'old' Lean or ZDF animals). Separately, 4- to 5-week-old pre-diabetic male ZDF and Lean animals (six animals per group) were obtained and killed within 2 weeks (referred to as the 'young' groups). Rats were killed by decapitation following CO_2 asphyxiation. Serum for blood chemistry analysis was collected post mortem and tests performed by the Manchester Royal Infirmary Biochemistry laboratory (Table 1). The mesenteric bed was removed into ice-cold Krebs solution (mM, 118 NaCl, 3.4 KCl, 1.6 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 25 $NaHCO_3$, 11 glucose) for artery dissection.

Membrane potential recordings

Third-order mesenteric arteries were pinned within a recording chamber and superfused with Krebs solution containing 100 μM N^{ω}-nitro-L-arginine and 10 μM indomethacin gassed with 5% CO_2 in O_2 at 37°C. Myocytes were impaled from the adventitial side using sharp microelectrodes filled with 3 M KCl (resistance 40–80 MΩ). Recordings were obtained using an Intra 767 amplifier (WPI Instruments) together with a Humbug active processing circuit (Digitimer) and digitized using a MacLab system (AD Instruments). Only recordings displaying a stable baseline for at least 5 min before application of drugs were included in the study. Acetylcholine and 1-EBIO were added directly to the recording chamber as a bolus calculated to give transiently the indicated concentration. Apamin and TRAM-39 were added to the superfusing solution.

Patch-clamp recordings

Bovine aortic endothelial (BAE) cells were maintained in a 5% CO_2 atmosphere in a humidified incubator at 37°C in Dulbecco's modified Eagle's medium (Gibco 41965-070) containing 10% fetal bovine serum, 2 mM glutamine and 1% penicillin/streptomycin (Gibco 15070-063). Cells were transferred to a recording chamber superfused with external solution (mM, 135 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 KH_2PO_4, 1.2 $MgCl_2$, 10 HEPES pH 7.30, 11 glucose) gassed with O_2 at room temperature. Pipettes (5–7 MΩ) were filled with internal solution (mM, 5 NaCl, 120 KCl, 1.2 K_2HPO_4, 1.2 $MgCl_2$, 10 HEPES pH 7.30, 11 glucose, 5 oxalacetate, 2 pyruvic acid, 5 succinic acid). The whole-cell, voltage-clamp configuration was employed using an Axopatch-1C amplifier and pClamp 5.5 software (Axon Instruments). Drugs (1-EBIO, TRAM-39 and DMSO vehicle) were perfused into the recording chamber at the desired concentration while toxins were added directly to the chamber after halting the superfusion system.

Quantitative RT–PCR

Mesenteric artery total RNA was isolated using RNeasy Mini kits (Qiagen, Crawley, U.K.), treated with DNase I (Invitrogen, Paisley, U.K.) and quantified using Ribogreen RNA quantification kits (Molecular Probes, Paisley, U.K.). RNA (1.5 μg) was reverse-transcribed using Superscript II (Invitrogen) and oligo-dT primer. Triplicate samples equivalent to 75 ng RNA were assayed using SK3 (KCNN3)-specific primers 5′-CTATACCTGATCGCCCGAGTCAT and 5′-TGATCCA CAGAGAGATGCTGAACA or IK1 (KCNN4)-specific primers 5′-GCCATGCTGCTACGTCTCTACCT and 5′-TCATG TATAGTTTGGCCACGAACC. SYBR Green I Mastermix

Table 1 Basic parameters of animals used

		Body weight (g)	*Blood pressure* (mmHg)	*Serum glucose* (mM)	*Serum insulin* ($mU\,l^{-1}$)	*Serum lipid index*
Young	Lean	156±7		9.7±1.2	33.0±6.1	14±2
	ZDF	218±16[a]		17.0±3.1	378±103[a]	104±31[a]
Old	Lean	348±8[b]	154±4	8.8±0.4	18.2±5.9	35±6[b]
	ZDF	372±8[b]	144±5	38.0±2.7[a,b]	35.3±6.6[b]	211±63[a]

[a]$P<0.05$ ZDF *versus* Lean (same-age group).
[b]$P<0.05$ Old *versus* Young (same strain).

Plus (Eurogentec, Southampton, U.K.) reagents and a DNA Engine Opticon 2 (MJ Research, Hemel Hempstead, U.K.) thermal-cycler were employed. Standard curves were prepared from plasmids containing full-length SK3 or IK1 coding regions (a kind gift of Dr G. Richards, Merck, U.K.). The threshold for determining threshold-cycle values was chosen to maximize precision between sample replicates. Specific product amplification was confirmed by analysis of melting curves (calculated amplicon melting temperatures were 82°C for SK3 and 84°C for IK1) and visualization of products on agarose–ethidium bromide gels (calculated amplicon lengths were 175 bp for SK3 and 139 bp for IK1). Product identity was confirmed by cloning and sequencing.

Drugs

TRAM-39 was a kind gift of Dr Heike Wulff (University of California, U.S.A.). Apamin and synthetic ChTX and IbTX were obtained from Latoxan, France. All other chemicals (including 1-EBIO) were supplied by Sigma Aldrich (Gillingham, U.K.).

Data analysis

All values are given as mean ± s.e.m. Statistical analysis (Student's *t*-test) was carried out using Microsoft Excel and a value of $P<0.05$ was considered statistically significant.

Results

Patch-clamp recordings

The first series of experiments (Figure 1a and b) were designed to validate our patch-clamp protocols for measuring IK_{Ca} currents in BAE cells. Basal currents recorded from BAE cells (102 ± 12 pA at +50 mV; $n=3$) were markedly increased by 600 μM 1-EBIO, an activator of IK_{Ca} (1017 ± 145 pA at +50 mV; $n=3$). Further application of 100 nM IbTX was without effect (1006 ± 290 pA at +50 mV; $n=3$). Subsequent addition of 100 nM ChTX significantly inhibited 1-EBIO-evoked currents (168 ± 43 pA at +50 mV; $n=3$). Thus, this protocol using 1-EBIO activated IK_{Ca} in BAE cells without contribution from large-conductance Ca^{2+}-activated K^+ channels also expressed in cultured endothelial cells.

In the second series of experiments (Figure 1c), we tested the effects of a range of TRAM-39 concentrations and DMSO vehicle on IK_{Ca} currents evoked in BAE cells. Basal currents (77 ± 25 pA at +50 mV; $n=3$) were unaffected by DMSO vehicle (112 ± 51 pA at +50 mV; $n=3$). Currents stimulated by 600 μM 1-EBIO (1077 ± 192 pA at +50 mV; $n=3$) were not significantly changed by 10 nM (1032 ± 314 pA at +50 mV; $n=3$) or 100 nM (862 ± 297 pA at +50 mV; $n=3$) TRAM-39. However, 1 μM (500 ± 123 pA at +50 mV; $n=3$) and 10 μM (183 ± 52 pA at +50 mV; $n=3$) TRAM-39 significantly inhibited 1-EBIO-stimulated currents. Thus, 10 μM TRAM-39 effectively abolished the native IK_{Ca} current.

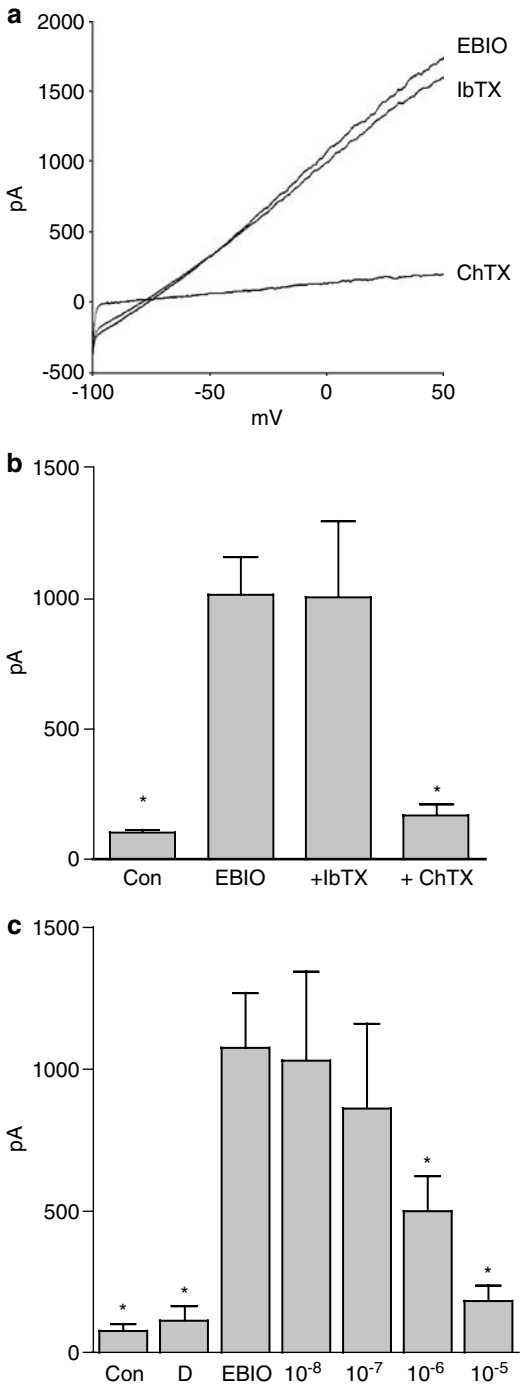

Figure 1 Effects of IbTX, ChTX and TRAM-39 on 1-EBIO-stimulated outward currents in BAE cells. Representative traces (a) and mean data measured at +50 mV (b) showing effects of cumulative addition of 100 nM IbTX and then 100 nM ChTX on 600 μM 1-EBIO (EBIO)-stimulated whole-cell currents in BAE cells ($n=3$). Mean data (c) showing effects of TRAM-39 (molar concentrations indicated) on EBIO-evoked outward currents measured at +50 mV ($n=3$). Outward currents under control (Con) conditions and in the presence of DMSO vehicle (D) before addition of EBIO are also given. *$P<0.05$ compared to EBIO.

Microelectrode studies: 17- to 20-week (Old) animals

In the present study, ZDF rats aged 17–20 weeks (Old group) were frankly diabetic (Table 1). Smooth muscle membrane potentials in arteries from Lean and ZDF rats were recorded using sharp microelectrodes. Responses were evoked by activation of the EDHF pathway with 1 and 10 μM ACh. Responses to 600 μM 1-EBIO were also recorded. To assess

responses due to activation of either SK_{Ca} or IK_{Ca} alone, ACh and 1-EBIO were each applied in the presence of either 10 μM TRAM-39 or 100 nM apamin, respectively.

Under basal conditions, resting membrane potentials were similar in arteries from old Lean and ZDF animals (-54.2 ± 0.5 mV, $n=12$ and -54.7 ± 0.5 mV, $n=12$, respectively). Application of 1-EBIO hyperpolarized the membrane to -71.0 ± 0.8 mV ($n=12$) in Lean animals and -71.6 ± 0.6 mV ($n=12$) in ZDF animals (Figure 2). Hyperpolarizations to -64.4 ± 0.6 mV ($n=8$) and -73.2 ± 0.6 mV ($n=12$) were evoked by 1 and 10 μM ACh, respectively, in Lean animals. In ZDF animals, hyperpolarizations to both concentrations of ACh were significantly reduced (1 μM, -61.4 ± 0.8 mV, $n=8$ and 10 μM, -69.5 ± 0.8 mV, $n=12$).

In the presence of 100 nM apamin, the resting membrane potential was similar in arteries from Lean (-51.1 ± 1.3 mV, $n=6$) and ZDF (-52.7 ± 0.8 mV, $n=6$) animals. Responses evoked by 1-EBIO in arteries from Lean (hyperpolarization to -69.2 ± 1.0 mV, $n=6$) and ZDF (hyperpolarization to -69.3 ± 1.0 mV, $n=6$) animals were similar and were not significantly different to 1-EBIO responses recorded in the absence of apamin (Figure 3a). Responses evoked by 1 μM ACh were similar in arteries from Lean (hyperpolarization to -56.4 ± 1.2 mV, $n=4$) and ZDF (hyperpolarization to -59.6 ± 1.8 mV, $n=4$) animals, as were responses elicited by 10 μM ACh (Lean, hyperpolarization to -64.7 ± 2.3 mV, $n=6$ and ZDF, hyperpolarization to -64.6 ± 0.6 mV, $n=6$). Relative changes in membrane potential were also similar between Lean and ZDF groups for 1 μM (-5.1 ± 0.6 and -7.3 ± 1.8 mV, respectively) and 10 μM ACh (-14.1 ± 1.7 and -12.5 ± 1.0 mV, respectively).

In the presence of 10 μM TRAM-39, resting membrane potential was similar in arteries from Lean (-53.9 ± 0.6 mV, $n=6$) and ZDF (-53.9 ± 0.9 mV, $n=6$) animals, and these values were similar to those obtained in the absence of TRAM-39. Under these conditions, responses to 1-EBIO were abolished (Lean, -53.9 ± 0.6 mV, $n=6$ and ZDF, -54.4 ± 0.6 mV, $n=6$; Figure 3b). Responses evoked by 1 μM ACh were not significantly different in arteries from Lean (hyperpolarization to -60.7 ± 0.6 mV, $n=4$) and ZDF (hyperpolarization to -57.8 ± 1.7 mV, $n=4$) animals, although the relative membrane potential changes were (Lean, -7.3 ± 0.8 mV and ZDF, -3.1 ± 1.0 mV, $P<0.05$). Responses elicited by 10 μM ACh were significantly different in arteries from Lean (hyperpolarization to -71.5 ± 1.2 mV, $n=6$) and ZDF (hyperpolarization to -63.1 ± 1.0 mV, $n=6$) animals, as were the relative responses (Lean, -17.9 ± 1.2 mV and ZDF, -9.6 ± 1.2 mV, $P<0.05$).

In the combined presence of 100 nM apamin and 10 μM TRAM-39, resting membrane potentials (Lean, -51.1 ± 1.7 mV, $n=4$ and ZDF, -50.8 ± 0.9 mV, $n=4$) were unaffected by 1-EBIO (Lean, -51.1 ± 1.7 mV, $n=4$ and ZDF, -50.8 ± 0.9 mV, $n=4$) or 10 μM ACh (Lean, -51.1 ± 1.6 mV, $n=4$ and ZDF, -50.6 ± 0.9 mV, $n=4$).

Microelectrode studies: 6- to 7-week (Young) animals

Resting membrane potentials in arteries from young Lean and ZDF animals were similar (Lean, -59.5 ± 0.3 mV, $n=4$ and ZDF, -58.7 ± 1.1 mV, $n=4$) and were significantly more negative than the corresponding values from old groups. Responses evoked by 1-EBIO were similar (Lean, hyperpolarization to -73.6 ± 0.7 mV, $n=4$ and ZDF, hyperpolarization to -74.3 ± 1.8 mV, $n=4$), as were responses elicited by 1 μM (Lean, hyperpolarization to -65.2 ± 0.8 mV, $n=4$ and ZDF, hyperpolarization to -65.8 ± 0.3 mV, $n=4$) and 10 μM ACh (Lean, hyperpolarization to -76.1 ± 1.1 mV, $n=4$ and ZDF, hyperpolarization to -76.1 ± 1.8 mV, $n=4$).

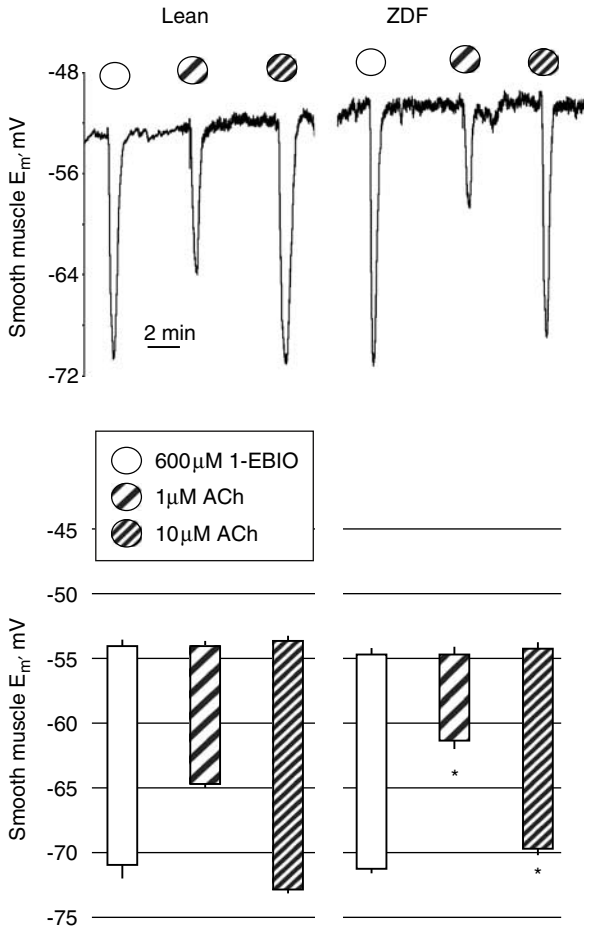

Figure 2 ACh- and 1-EBIO-evoked responses in arteries from Old Lean and ZDF rats. Membrane potential (E_m) recordings obtained from smooth muscle impalements using sharp microelectrodes are shown. Representative traces (top) and mean data (bottom; bar top represents mean membrane potential before response, whereas bar bottom represents the mean maximum change in membrane potential during response) showing responses elicited by 600 μM 1-EBIO, 1 μM ACh and 10 μM ACh. *$P<0.05$ Lean compared to ZDF.

In the presence of 10 μM TRAM-39, resting membrane potentials (Lean, -57.3 ± 0.5 mV, $n=4$ and ZDF, -58.2 ± 1.4 mV, $n=4$) were unaffected by 1-EBIO (Lean, -57.3 ± 0.5 mV, $n=4$ and ZDF, -58.2 ± 1.4 mV, $n=4$). Hyperpolarizations evoked by 1 μM (Lean, hyperpolarization to -64.1 ± 0.7 mV, $n=4$ and ZDF, hyperpolarization to -64.7 ± 1.1 mV, $n=4$) and 10 μM ACh (Lean, hyperpolarization to -73.8 ± 1.0 mV, $n=4$ and ZDF, hyperpolarization to -75.1 ± 0.9 mV, $n=4$) were similar between Lean and ZDF groups.

Quantitative RT–PCR studies

Expression of SK3 and IK1, the pore-forming subunits of SK_{Ca} and IK_{Ca}, respectively, was quantified in mRNA obtained from arteries of old Lean and ZDF animals

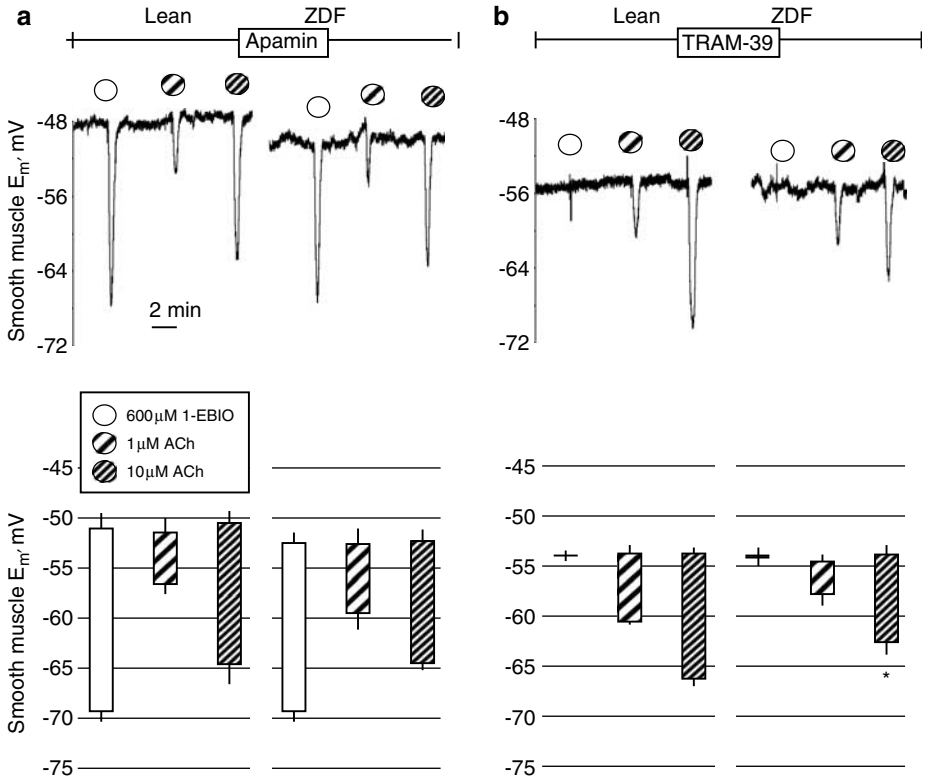

Figure 3 SK_{Ca}- and IK_{Ca}-mediated components of ACh- and 1-EBIO-elicited responses in arteries of Old Lean and ZDF rats. Membrane potential (E_m) recordings obtained from smooth muscle impalements using sharp microelectrodes in the presence of 100 nM apamin (a) or 10 μM TRAM-39 (b). Representative traces (top) and mean data (bottom; bar top represents mean membrane potential before response, whereas bar bottom represents mean maximum change in membrane potential during response) showing responses evoked by 600 μM 1-EBIO, 1 μM ACh and 10 μM ACh. *$P<0.05$ Lean compared to ZDF.

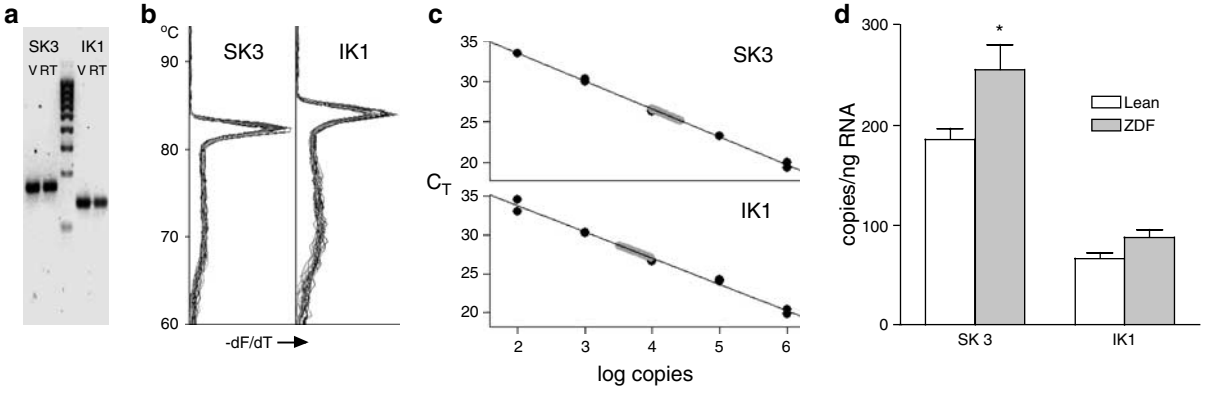

Figure 4 Quantification of SK3 and IK1 subunit mRNA expression by real-time RT–PCR in the arteries of Old Lean and ZDF rats. Specificity of reactions demonstrated by (a) ethidium bromide-stained agarose gels (products amplified from vector standard (V) and artery cDNA (RT)) and (b) melting curve analysis (plots of first negative derivative of fluorescence ($-dF/dT$) *versus* temperature for all artery cDNA reactions). (c) Standard curves (threshold cycle (C_T) *versus* log copies) for SK3 and IK1 quantification showing vector standards (black circles) and artery cDNA samples (grey circles). (d) Mean data obtained from artery cDNA samples. *$P<0.05$ Lean *versus* ZDF.

(Figure 4). Quantitative, real-time RT–PCR analysis under the conditions described was specific for these gene products, as demonstrated by the appearance of only a single product upon resolving reactions by either agarose–ethidium bromide gels or melting-curve analyses. Product identity was confirmed by sequencing (data not shown). Expression of mRNA encoding the SK3 subunit (Lean 186 ± 11 copies ng^{-1} total RNA, $n=6$ and ZDF 256 ± 25 copies ng^{-1} total RNA, $n=6$) was significantly increased in the diabetic group. Expression of IK1 mRNA was not significantly different in the diabetic group (Lean 67 ± 6 copies ng^{-1} total RNA, $n=6$ and ZDF 88 ± 8 copies ng^{-1} total RNA, $n=6$).

Discussion

General findings

The objective of this study was to characterize the EDHF pathway in arteries from the Type II diabetic ZDF rat and to examine the effects of diabetes on the Ca^{2+}-activated K^+ channels which underpin this pathway. TRAM-39 abolished activity of the native IK_{Ca} of BAE cells under whole-cell voltage-clamp conditions and, together with apamin, was used in whole artery studies to dissect the SK_{Ca}- and IK_{Ca}-mediated components, respectively. Overall, ACh-evoked hyperpolarizations recorded from smooth muscle impalements of mesenteric arteries were reduced in the diabetic state. This deficit was also apparent in the TRAM-39-resistant, SK_{Ca}-mediated responses but not in IK_{Ca}-mediated responses recorded in the presence of apamin or in IK_{Ca} responses to 1-EBIO. Responses to ACh in arteries from young, pre-diabetic ZDF rats were not different from controls, nor was the SK_{Ca}-mediated component of these responses. The combination of apamin plus TRAM-39 abolished membrane hyperpolarizations in all arteries, suggesting that SK_{Ca} and IK_{Ca} are the only K^+ channels involved in ACh-evoked EDHF responses in mesenteric arteries of ZDF rats. Expression of mRNA encoding SK3 subunits was increased in the diabetic state, whereas IK1 mRNA was unaltered, as quantified by real-time RT–PCR.

Type II diabetes and the EDHF pathway

Type II diabetes incurs endothelial dysfunction related to reduced nitric oxide signalling (Cai & Harrison, 2000; Calles-Escandon & Cipolla, 2001). However, only a small number of observational studies have reported impairment of the EDHF pathway in animal models of this condition. One laboratory has demonstrated that overall relaxation to ACh of sciatic nerve epineurial arterioles was impaired in diabetic ZDF rats (4 weeks hyperglycaemia) and, in a subsequent study, that ACh-evoked relaxation mediated by the EDHF pathway was absent from these arteries (Coppey *et al*., 2002; 2003). Mesenteric artery relaxation elicited by activation of the EDHF pathway with ACh was also impaired in fructose-fed, insulin-resistant rats (Katakam *et al*., 1999). As experimental observations confirm that hyperpolarization is directly linked to vasorelaxation in the EDHF pathway (Chen & Cheung, 1997; Crane *et al*., 2003), these myograph experiments are in agreement with our microelectrode studies and demonstrate that the EDHF pathway is impaired in several models of Type II diabetes and in several vascular beds. We have begun to dissect the mechanism(s) responsible, as discussed below, and it is noteworthy that other vascular K^+ channels are also affected by Type II diabetes. We have previously reported a reduced function of large-conductance Ca^{2+}-activated K^+ channels from diabetic ZDF rat mesenteric artery myocytes that may be related to direct effects on activation of the channels by Ca^{2+} (Burnham *et al*., 2006).

Studies in healthy humans have demonstrated that acute elevation of either blood glucose or free fatty acids is sufficient to impair endothelium-dependent vasodilation in these subjects (Steinberg *et al*., 1997; Williams *et al*., 1998). Studies of vessels isolated from patients with Type II diabetes have associated dyslipidaemia with impaired ACh-evoked vasodilation (Schofield *et al*., 2002). In the present study, impaired EDHF responses were apparent in arteries from ZDF animals with hyperglycaemia and hyperlipidaemia (raised serum lipid index, a clinical measure derived from sample turbidity). However, in pre-diabetic ZDF animals, hyperinsulinaemia and hyperlipidaemia were not associated with a reduction in EDHF responses, suggesting that this deficit is not an immediate consequence of these conditions. It may be the case that endothelium-dependent vasodilation utilizing alternative pathways (e.g. nitric oxide or EDHF) is susceptible to distinct metabolic disturbances.

Separating the SK_{Ca} and IK_{Ca} pathways

Whether the impairment to the EDHF pathway in diabetes is specific to endothelial cells and the activation of channels present there, or whether the transfer to or induction of smooth muscle hyperpolarization is also impaired, is currently unknown. We have begun to address these questions by examining the component hyperpolarizations apparent upon pathway activation in the presence of selective inhibitors of either SK_{Ca} or IK_{Ca}. A deficit was apparent in the TRAM-39-resistant, SK_{Ca}-mediated responses but not in IK_{Ca}-mediated responses to ACh or 1-EBIO recorded in the presence of apamin. SK3 mRNA expression was increased in these arteries, possibly reflecting a compensatory effect, although a study of streptozotocin-treated ApoE-deficient mice found that SK3 mRNA was decreased (Ding *et al*., 2005). Levels of SK3 protein were not determined owing to insufficient material for mRNA, protein and functional studies. Functional assessment of SK_{Ca} expression in sharp microelectrode recordings awaits the development of SK_{Ca}-selective channel openers. The fact that 1-EBIO responses were similar in diabetic and control groups suggests that levels of functional IK_{Ca} were unaltered, a result consistent with mRNA expression analysis. Additionally, the similarity of IK_{Ca}-mediated responses argues that at least one route by which endothelial hyperpolarization is transferred to the smooth muscle remains intact. A remaining consideration is the mechanism by which the channels are activated.

Activation of endothelial SK_{Ca} and IK_{Ca} is thought to occur *via* a common mechanism, namely an increase in intracellular Ca^{2+}. However, studies of the Ca^{2+} sensitivity of these channels have reported a range of values and, in those studies utilizing mammalian expression rather than *Xenopus* oocytes, EC_{50} values of 95 and 270 nM have been reported for human IK1 (hSK4) (Joiner *et al*., 1997; Logsdon *et al*., 1997). Similarly, an isoform of SK3 expressed in rat liver and differing from the isoform expressed in rat brain by five N-terminal amino acids displayed an EC_{50} of 630 nM Ca^{2+} (Barfod *et al*., 2001), whereas a native SK_{Ca} from human TE671 cells apparently composed of SK3 subunits exhibited a lower EC_{50} of 104 nM (Carignani *et al*., 2002). Perhaps the data most relevant to the present study are those from the investigation by Marchenko & Sage (1996), in which native, 6.7 pS ChTX-sensitive channels on inside-out patches from rat aortic endothelium were activated by Ca^{2+} concentrations greater than 100 nM and displayed an EC_{50} of 340 nM. Furthermore, 2.8 pS apamin-sensitive channels were activated by Ca^{2+} concentrations greater than 500 nM and activation was not complete at 1 μM. Thus, the IK_{Ca} of rat aortic endothelium would appear to be more sensitive to Ca^{2+} than the SK_{Ca}. Assuming that the same is true in mesenteric artery endo-

thelium in the present study, an impaired ACh-evoked elevation of intracellular Ca^{2+} in endothelial cells of diabetic ZDF rats could result in a maximal activation of the IK_{Ca} while only partially activating the SK_{Ca} in the same cells. However, direct effects of the diabetic state on SK_{Ca} function could also underlie these observations.

Role of SK_{Ca} in resting and stimulated arteries

Two previous studies have examined the effects of rat mesenteric artery pre-contraction on the individual contributions of IK_{Ca} and SK_{Ca} to EDHF-dependent hyperpolarizations (Chen & Cheung, 1997; Crane *et al*., 2003). In the resting state, it was found that hyperpolarizations to ACh were entirely SK_{Ca} dependent and that blockade of both IK_{Ca} and SK_{Ca} was only required in pre-contracted arteries. In the present study, ACh elicited hyperpolarizations from the resting membrane potential that involved both SK_{Ca} and IK_{Ca}. In other membrane potential studies of the EDHF pathway under resting conditions, apamin alone did not abolish responses in the porcine coronary artery (Bychkov *et al*., 2002) and had no effect or only partially inhibited responses in the guinea-pig carotid artery (Corriu *et al*., 1996; Chataigneau *et al*., 1998). These differences may be related to the use of a wire myograph, where the artery is tensioned in its resting state, compared to other studies, where the resting state is untensioned. Parkington *et al*. (1993) demonstrated that the degree of stretch to which an artery is subjected to affects the hyperpolarizing responses elicited by activation of the EDHF, nitric oxide and prostacyclin pathways. It remains to be determined how SK_{Ca} and IK_{Ca} may be differentially activated in endothelial cells.

Use of 1-EBIO

In the present study, responses evoked by 1-EBIO were unaffected by apamin and abolished by TRAM-39, consistent with the activation of IK_{Ca} only and not SK_{Ca} by the compound. A similar conclusion was reached in studies of the porcine coronary artery, guinea-pig submucosal arteriole and Wistar rat mesenteric artery (Coleman *et al*., 2001; Walker *et al*., 2001; Burnham *et al*., 2002). However, 1-EBIO in concentrations comparable to the present study has been shown to activate SK_{Ca} composed of the rat SK3 subunit when expressed in mammalian HEK293 cells (Grunnet *et al*., 2001). Although channel activation by 1-EBIO was Ca^{2+} dependent, a concentration of 100 nM was sufficient to observe activation. Likewise, although SK3 subunits may co-assemble with SK1 or SK2 subunits (Monaghan *et al*., 2004), both of these subunits are also activated by 1-EBIO (Pedarzani *et al*., 2001). Thus, the absence of SK_{Ca} activation by 1-EBIO in rat mesenteric artery endothelium in the present study may reflect an association of the native SK_{Ca} with additional regulatory proteins or pathways.

Conclusions

The results of the present study demonstrate that EDHF-mediated hyperpolarizations, particularly responses mediated by SK_{Ca}, are impaired by diabetes in the ZDF rat without reduction in SK_{Ca} or IK_{Ca} gene expression. Differential activation of these channels could be explained to some extent by distinct Ca^{2+} sensitivities, although direct effects on the channels cannot be excluded. The data are consistent with a role for hyperglycaemia, but not acute hyperinsulinaemia or hyperlipidaemia, in the deficit of the EDHF pathway. Additional studies are currently underway to determine whether SK_{Ca} and IK_{Ca} can be differentially activated in endothelial cells and whether these channels are regulated by glucose-sensitive pathways.

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