Type 2 diabetes: increased expression and contribution of IKCa channels to vasodilation in small mesenteric arteries of ZDF rats

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Schach C, Resch M, Schmid PM, Riegger GA, Endemann DH. Type 2 diabetes: increased expression and contribution of IKCa channels to vasodilation in small mesenteric arteries of ZDF rats. Am J Physiol Heart Circ Physiol 307: H1093–H1102, 2014. First published August 15, 2014; doi:10.1152/ajpheart.00240.2013.—Impaired endothelial function, which is dysregulated in diabetes, also precedes hypertension. We hypothesized that in Type 2 diabetes, the impaired endothelium-dependent relaxation is due to a loss of endothelium-derived hyperpolarization (EDH) that is regulated by impaired ion channel function. Zucker diabetic fatty (ZDF), Zucker heterozygote, and homozygote lean control rats were used as the experimental models in our study. Third-order mesenteric arteries were dissected and mounted on a pressure myograph; mRNA was quantified by RT-PCR and channel proteins by Western blotting. Under nitric oxide (NO) synthesis and cyclooxygenase inhibition, endothelial stimulation with ACh fully relaxes control but not diabetic arteries. In contrast, when small-conductance calcium-activated potassium (KCa) channels and intermediate- and large-conductance KCa (IKCa) are inhibited with apamin and charybdotoxin, NO is able to compensate for ACh-induced relaxation in control but not in diabetic vessels. After replacement of charybdotoxin with 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34; IKCa inhibitor), ACh-induced relaxation in diabetic animals is attenuated. Specific inhibition with TRAM-34 or charybdotoxin attenuates ACh relaxation in diabetes. Stimulation with 1-ethyl-2-benzimidazolinone (IKCa activator) shows a reduced relaxation in diabetes. Activation of BKCa with 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-1H-benzimidazol-2-one NS619 leads to similar relaxations of control and diabetic arteries. RT-PCR and Western blot analysis demonstrate elevated mRNA and protein expression levels of IKCa in diabetes. Our results suggest that the compensatory effect of NO and EDH-associated, endothelium-dependent relaxation is reduced in ZDF rats. Specific blockade of IKCa with TRAM-34 reduces NO and EDH-type relaxation in diabetic rats, indicating an elevated contribution of IKCa in diabetic small mesenteric artery relaxation. This finding correlates with increased IKCa mRNA and protein expression in this vessel.

diabetes; calcium-activated potassium channel; vasodilation; endothelium; nitric oxide; endothelium-derived hyperpolarization; mesenteric artery

CARDIOVASCULAR DISEASE (CVD) is the major cause of mortality in diabetic patients (1). Up to 75% of CVD in diabetes may be attributable to hypertension, with arterial hypertension approximately twice as frequent in individuals with diabetes. Control of blood pressure, therefore, provides an important therapeutic goal (43, 46). Diabetes and hypertension frequently correspond, and endothelial dysfunction is thought to be the initial step in CVD development (7). With the lining of all blood vessels, the endothelium modulates smooth muscle tone and vasorelaxation under healthy conditions. It has been established that endothelial function in diseases, such as diabetes and hypertension, is impaired in animal models and in humans as well (17). Cardiovascular complications develop by deficient function of nitric oxide (NO), following enhanced oxidative stress in diabetes (36). The loss of NO regulation is thought to lead to hypertension by arterial stiffening and chronic activation of the sympathetic nervous system (23, 25). The expected compensatory role for endothelium-derived hyperpolarization (EDH) for a loss of NO regulation is not observed uniformly, and indeed, a reduced function of EDH has been suggested to play an important role in the genesis of endothelial dysfunction in diabetes. Dysregulated, EDH-mediated responses were also observed in Type 1 diabetes. Mesenteric arteries of streptozotocin-induced Type 1 diabetic rats had an enhanced EDH-mediated relaxation (41), whereas other observations show an impaired EDH-dependent response in that vasculature (20, 49). In mice, streptozotocin-induced Type 1 diabetes leads to diminished EDH relaxation with a compensatory increase in the NO response (18, 32). In both studies, isometric force of mesenteric arteries was measured. Type 2 diabetic mice show an attenuated, EDH-mediated vasodilation in coronary arterioles (38), whereas in small mesenteric arteries (SMA), its contribution to endothelium-dependent vasodilation is unaltered or even elevated (37). In humans, NO- and EDH-mediated vasorelaxation is reduced in Types 1 and 2 diabetes patients (3), Otsuka Long-Evans Tokushima fatty (OLETF) Type 2 diabetic rats exhibited an impaired EDH-type relaxation measuring isometric force (30, 31). In an electrophysiological study, using a combination of Zucker diabetic fatty (ZDF) rat SMA and bovine aortic endothelial cells (ECs), an impaired EDH response was observed due to a reduced function of small-conductance calcium-activated potassium channels (SKCa) (8), and diminished EDH relaxation in ZDF rat mesenteric arteries could be restored via pharmacological activation of SKCa and intermediate-conductance KCa (IKCa) (6).

These observations were generated under different conditions and until now, there appears to be an absence of investigations demonstrating a compensatory role for KCa function and their expression levels and role in NO- and EDH-mediated relaxation using pressure myography in Type 2 diabetic rats. Furthermore, it is not known if the commonly used control

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annel for ZDF rats, the Zucker heterozygote lean (ZL) rat, has reduced vasofunction due to its heterogeneity in a leptin receptor defect compared with the Zucker homozygote lean control (Z/+/+) rat, which is negative for a leptin receptor defect. To gain additional insight, the molecular approaches, RT-PCR and Western blot, were used to examine and compare KCa expression levels among these animal models. As SKCa (isoform three of SKCa (SK3), KCa2.3, KCNN3), IKCa (IK1, KC3.1, KCNN4), as well as large-conductance KCa (BKCa; BK, KCa1.1, KCNN1) have been observed in EDH-mediated responses (6, 8, 33), we determined mRNA and protein expression levels of these channels.

METHODS

Animals. Male ZDF rats (homozygote for leptin receptor defect, fa/fa) and their respective heterozygote (ZL, fa/+) and homozygote (Z/+/+, z/W) control rats were purchased from Charles River Laboratories (Brussels, Belgium) at the age of 10 wk. Rats had unlimited access to the diabetogenic diet Purina 5008 (energy rich, containing 26.8% protein, 16.7% fat, and 56.4% carbohydrates). The animals were housed on a 12/12-h light/dark cycle with constant temperature (22–23°C), with access to food and tap water ad libitum. Body weight, blood pressure, and fasting blood glucose concentrations were measured (BPreco No. 8005; W+W electronic AG, Basel, Switzerland; ACCU-CHEK sensor; Roche, Mannheim, Germany). At the age of 18 wk, animals were weighted; systolic blood pressure was measured using an automated tail-cuff, inflator-pulse detection system (CODA2 multichannel, computerized; emka Technologies, Paris, France); and fasting (6 h) blood glucose level was assessed. Afterwards, rats were euthanized by decapitation. Blood samples were collected in tubes, and tibia length (TL) was obtained. All animal care and experimental procedures followed German law as well as the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health, and were approved by the institutional review board at the University of Regensburg.

Drugs and preparation. 4-[(IR)-2-Amino-1-hydroxyethyl]benzene-1,2-diol [noradrenaline (NA)] and sodium pentacyaninotrosylferrate(II) [sodium nitroprusside (SNP)] were purchased from Fluka (Neu-Ulm, Germany); 2-acetoxy-N,N,N-trimethylethanaminium (AcOH), 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619), N0-nitro-l-arginine methyl ester (L-NAME), and 1-[(2-chlorophenyl)diphenylmethyl]-1H-pyrazole (TRAM-34) were purchased from Sigma (Taufkirchen, Germany); 1-propionyl-2-chloroethyl-1,3,4-oxadiazoline (1-EBIO), [2-(2,6-dichlorophenylamino)-1H-pyrazole (SNP), sodium nitroprusside (SNP)] were purchased from Fluka (Neu-Ulm, Germany), and L-NAME, a selective NO synthase (NOS) inhibitor; diclofenac (10 μM), a nonselective cyclooxygenase (COX) inhibitor; apamin (0.1 μM), a blocker of SKCa; charybdotoxin (0.1 μM), a blocker of IKCa and BKCa; as well as voltage-gated K+ channels (Kv) and TRAM-34 (0.1 μM), a selective IKCa inhibitor. These KCa inhibitors block the respective channels selectively at specific concentrations. When vessels were treated with the combination of L-NAME, diclofenac, and apamin plus charybdotoxin, at first, a concentration-response curve to ACh was generated after incubation with given combinations of L-NAME (100 μM), a nonselective NO synthase (NOS) inhibitor; diclofenac (10 μM), a nonselective cyclooxygenase (COX) inhibitor; apamin (0.1 μM), a blocker of SKCa; charybdotoxin (0.1 μM), a blocker of IKCa and BKCa; as well as voltage-gated K+ channels (Kv) and TRAM-34 (0.1 μM), a selective IKCa inhibitor. These KCa inhibitors block the respective channels selectively at specific concentrations. When vessels were treated with the combination of L-NAME, diclofenac, and apamin plus charybdotoxin, a concentration-response curve to ACh was generated after incubation with L-NAME and then again after a 30-min washout period, with the combination of L-NAME plus diclofenac with a 30-min washout afterwards. In experiments with isolated application of TRAM-34, charybdotoxin, and apamin, these inhibitors were applied in respective order with a wash-out period of 30 min afterwards.

For experiments studying the effect of applying individual channel inhibitors (apamin, charybdotoxin, or TRAM-34; see Fig. 3) and the effect of combined channel inhibition (apamin plus charybdotoxin or apamin plus TRAM-34) in the absence and presence of NOS and COX inhibition (by L-NAME and diclofenac), a second set of rats was used (n = 6 for each group).

Quantitative RT-PCR. mRNAs of KCa proteins (SKCa3, IKCa, and BKCa) was measured in the first- through third-order branches of

Table 1. Physiological data

<table>
<thead>
<tr>
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<th>Z/++</th>
<th>ZL</th>
<th>ZDF</th>
<th>P</th>
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<tr>
<td>n</td>
<td>6</td>
<td>6</td>
<td>6</td>
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<tr>
<td>Body weight, g</td>
<td>372 ± 4.2</td>
<td>375.9 ± 8.4</td>
<td>370.7 ± 5.8</td>
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<td>Tibia length, mm</td>
<td>45.0 ± 0.4a</td>
<td>44.7 ± 0.3a</td>
<td>42.8 ± 0.4a</td>
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<tr>
<td>Body weight/tibia</td>
<td>8.3 ± 1.1</td>
<td>8.4 ± 1.9</td>
<td>8.9 ± 1.6</td>
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<td>Blood glucose, mg/dl</td>
<td>90.4 ± 2.2a</td>
<td>86.7 ± 1.9a</td>
<td>73.5 ± 13.1 &lt;0.01</td>
<td></td>
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<tr>
<td>Systolic blood</td>
<td>112.1 ± 4.1</td>
<td>109.9 ± 4.3</td>
<td>103.2 ± 6.3</td>
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<td>pressure, mmHg</td>
<td>585.7 ± 19.7a</td>
<td>622.1 ± 24.9a</td>
<td>493.7 ± 17.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Heart rate, beats/min</td>
<td>265.4 ± 9.1</td>
<td>276.7 ± 8.7</td>
<td>265.4 ± 9.1</td>
<td>0.91</td>
</tr>
</tbody>
</table>

Physiological data and lumen diameter of studied 3rd-order mesenteric resistance arteries from Zucker homozygote lean control (Z/++), Zucker heterozygote lean control (ZL), and Zucker diabetic fatty (ZDF) rats. *P < 0.05 vs. ZDF (1-way ANOVA).
The arteries were homogenized in 300 from the same treatment group were pooled and considered as second-, and third-order branches of superior mesenteric arteries after systems).

RT-PCR was performed on an ABI PRISM 7900 detection system (Applied Biosystems, Darmstadt, Germany) using TaqMan Gene Expression Master Mix (Applied Biosystems, Darmstadt, Germany) containing 1 µl total RNA, 0.5 µg oligo(dT) primer (Sigma-Aldrich, Munich, Germany), 40 U Rnase (Promega, Mannheim, Germany), 0.5 mM dNTP (Amersham, Freiburg, Germany), 4 µl 5× transcription buffer, and 200 U Moloney murine leukemia virus RT, and no template controls were performed. Real-time quantitative RT-PCR was performed on an ABI PRISM 7900 detection system (Applied Biosystems, Darmstadt, Germany) using TaqMan Gene Expression Assays for the genes KCNQ1, KCNQ3, and KCNMA1, together with TaqMan Gene Expression Master Mix (Applied Biosystems). β-Actin was used as a reference gene. All water controls and single-stranded RT controls were negative. Data were analyzed using SDS version 2.2.2 software (Applied Biosystems).

Western blots. Western blots were performed from the first-, second-, and third-order branches of superior mesenteric arteries after carefully dissecting the vessels from surrounding tissue. Two animals from the same treatment group were pooled and considered as n = 1. The arteries were homogenized in 300 µl lysis buffer (100 mM NaCl, 10 mM Tris, 2 mM EDTA, 0.5% wt/vol Na deoxycholate, 1% vol/vol Triton X-100, pH 7.4, plus a protease inhibitor cocktail) on ice. Then, 20% SDS (15 µl) was added and incubated for 10 min. Centrifugation at 800 g for 5 min at 4°C and with 12,000 g for 20 min at 4°C of the supernatant was used to generate aliquots. The total protein concentration was quantified using the two-dimensional Quanti-TI protein assay kit (GE Healthcare, Solingen, Germany), and equal amounts of protein homogenate were subjected to SDS-PAGE (12.5% SDS; Precast; Bio-Rad Laboratories, Hercules, CA) and run for 1.5 h at 35 mA, 100 V (Mini-PROTEAN; Bio-Rad Laboratories), before being transferred (Trans-Blot Turbo; Bio-Rad Laboratories) to a polyvinylidene fluoride membrane. After 10 min at 1.5 A and 25 V, the membrane was blocked with 5% BSA in Tris-buffered saline-Tween 20 (TBS-T) for 1 h. The primary antibodies against SKCa3 AP025 (lot AN-09), IKCa1 AP064 (AN-04), and BKCa2.2 AP021 (AN-12), all 1:500 rabbit polyclonal anti-rat antibodies (Alomone Labs, Jerusalem, Israel), were incubated at 4°C overnight and then washed four times in TBS-T before being incubated for 1 h with the secondary antibody (1:10,000 anti-rabbit horseradish peroxidase; Cell Signaling Technology, Danvers, MA). Afterwards, the membrane was washed four times in TBS-T and once in TBS and then developed using an enhanced chemiluminescence kit (Clarity Western ECL substrate kit; Bio-Rad Laboratories). The relative amount of protein was quantified by densitometry (ChemIDoc; Bio-Rad Laboratories) and expressed as the ratio of loading control (Image Lab 5.0; Bio-Rad Laboratories).

Antibody specificity was assessed by incubation of the antibody with its cognate peptide to block its specific binding site. The peptide was mixed with the antibody (1:1 ratio/wt) and incubated for 1 h at 37°C and afterwards, overnight at 4°C. Further handling of this blocked antibody for Western blotting was as described above. As molecular-weight markers,peqGOLD Protein-Marker IV (M1; Peqlab) and Precision Plus Protein WesternC (M2; Bio-Rad Laboratories) were used.

Statistical analysis. All values are given as means ± SE. Concentration-response curves from isolated rat mesenteric arteries were computer fitted using nonlinear regression (Prism version 6.0; GraphPad Software, San Diego, CA) to calculate the sensitivity of each agonist and presented as pD2 values (pD2 = -log[EC50]). Maximum relaxation (Rmax) to ACh, 1-EBIO, or NS1619 was measured as a percent

Table 2. Role of EDH-type relaxation on Rmax and pD2 values in small mesenteric arteries

<table>
<thead>
<tr>
<th>ACh</th>
<th>Z+/+</th>
<th>ZL</th>
<th>ZDF</th>
<th>Z+/+</th>
<th>ZL</th>
<th>ZDF</th>
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<tr>
<td></td>
<td>Rmax, %, n = 6</td>
<td></td>
<td></td>
<td>pD2, n = 6</td>
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<td>ACh</td>
<td></td>
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<tr>
<td>+L</td>
<td>108.3 ± 7.4*</td>
<td>91.1 ± 16.2*</td>
<td>21.6 ± 7.1*</td>
<td>6.7 ± 0.18*</td>
<td>6.5 ± 0.17</td>
<td>6.2 ± 0.22</td>
</tr>
<tr>
<td>+L + D</td>
<td>103.3 ± 5.4*</td>
<td>70.6 ± 17.4*</td>
<td>16.3 ± 4.4*</td>
<td>6.5 ± 0.14</td>
<td>6.1 ± 0.22</td>
<td>n.d.</td>
</tr>
<tr>
<td>+L + D + A + C</td>
<td>3.7 ± 1.8*</td>
<td>12.9 ± 9.1*</td>
<td>-2.5 ± 2.8*</td>
<td>6.5 ± 0.15</td>
<td>6.2 ± 0.25</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Parameters measured in small mesenteric arteries from 18-wk-old Z+/+, ZL, and ZDF rats. EDH, endothelium-derived hyperpolarization. Sensitivity (pD2) and maximal relaxation (Rmax) to ACh in the absence [control (CTL)] or presence of N-nitro-L-arginine methyl ester (L-NAME (L)); 100 µM; 2-(2,6-dichlorophenylamino)phenyl13C6]acetic acid sodium salt heminonahydrate [diclofenac (D); 10 µM]; apamin (A; 0.1 µM); and charybdotoxin (C; 0.1 µM). n.d., not determined. Values are means ± SE. *P < 0.05 vs. ZDF; **P < 0.05 vs. corresponding CTL; ***P < 0.05 vs. ZL (1-way ANOVA).
pressure was not significantly different in ZDF rats compared with the control groups. Body weight showed no difference among the three groups (Table 1); however, TL was lower in ZDF rats compared with the control groups, resulting in a trend to ($P = 0.08$) elevated relative body weight of ZDF rats (body weight/TL; Table 1).

**NA-induced contraction.** Cumulative doses of NA were applied to the vessel after an equilibration period of 40 min. ZDF and control rats contracted without a significant difference, neither in maximal contraction ($C_{\text{max}}$) nor in NA sensitivity, to ~70% of the initial diameter ($C_{\text{max}}$ ZDF 72.1 ± 1.5% vs. control 70.4 ± 1.2%, $P = $ not significant), indicating neither a loss of contractile ability nor a hyper-responsiveness to catecholamines.

**ACH-induced relaxation in ZDF rats vs. control rats.** The application of increasing ACh concentrations to the arteries of homozygote healthy control rats ($Z+/+$) produced a complete relaxation at high concentrations, whereas vessels from ZDF rats reached only 58% of $R_{\text{max}}$. At nanomolar ACh concentrations, vessels of $Z+/+$ rats are already partly dilated, which is significantly different from the behavior of arteries from ZDF rats.

**Fig. 2.** Cumulative dose-response curves of SMA isolated from (A) $Z+/+$, (B) ZL, and (C) ZDF rats to ACh. Arteries were exposed to cumulative doses of ACh (1 nM–0.1 mM) without incubation [control (CTL)] and after incubation with $N^\omega$-nitro-l-arginine methyl ester (l-NAME; 100 μM), l-NAME plus diclofenac (Diclo; 10 μM), and the combination of apamin (Apa) plus charybdotoxin (Chtx; each 0.1 μM) in the presence of l-NAME (100 μM) and Diclo (10 μM). Arteries were precontracted with NA (10 μM); $n = 6$ experiments. Results are shown as means ± SE. *$P < 0.05$ vs. CTL; **$P < 0.05$ vs. l-NAME; §§$P < 0.05$ vs. l-NAME + Diclo, 2-way repeated-measures ANOVA.

**Fig. 3.** Cumulative dose-response curves to ACh (0.01 nM–0.1 mM) in the absence (CTL) and presence of Apa, Chtx, or 1-[2-chlorophenyl]diphenylmethyl]-1H-pyrazole (TRAM-34), respectively (each 0.1 μM) in $Z+/+$ (A) and ZDF (B) rats. Relaxations to ACh were induced after stable precontraction with NA; $n = 6$ experiments. Results are shown as means ± SE. #$P < 0.05$ vs. TRAM-34; §§$P < 0.05$ vs. Chtx, 2-way repeated-measures ANOVA.
ZL-derived arteries are slightly less sensitive to ACh without statistical significance (Fig. 1 and Table 2).

Endothelium-independent relaxation. Endothelium-independent relaxation was assessed by a dose-response curve to SNP (0.01–100 μM). SMA of ZDF, as well control rats, increased diameter without difference, although complete relaxation was not achieved by high concentrations of SNP (data not shown).

Selective blockade of ACh-induced relaxation. Stimulation with ACh produced a full relaxation in the nondiabetic control groups, Z+/+ and ZL, whereas in diabetic ZDF animals, ACh-induced relaxation was reduced significantly. Inhibition of endothelial NOS (eNOS) with L-NAME (100 μM) significantly reduced relaxation at low doses of ACh in Z+/+ animals (Fig. 2 and Table 2). Further addition of diclofenac (10 μM) did not alter the dose-response curve to ACh significantly. Additional blockade of KCa by apamin plus charybdotoxin (each 0.1 μM) led to a significant suppression of the ACh-induced vasorelaxation in the Z+/+ rat mesenteric artery, indicating a substantial contribution of EDH-type relaxation. In nondiabetic heterozygote (fa+/−) ZL rats, without NOS and COX inhibition, the endothelium-dependent stimulation with ACh led to a complete relaxation of SMA (Fig. 2A and Table 2). In the presence of NOS and COX inhibition, ACh-induced relaxation was not altered significantly, whereas further application of the toxin combination apamin plus charybdotoxin abolished ACh-induced vasorelaxation in ZL animals (Fig. 2B and Table 2). In ZDF rats, ACh-induced relaxation of SMA was decreased significantly, and additive inhibition of NOS further reduced the ACh-induced relaxation in a significant way (Fig. 2C and Table 2). As in the two control groups, COX inhibition did not significantly alter the ACh-induced relaxation. Under NOS and COX blockade and inhibition of SKCa and BKCa by apamin and charybdotoxin, ACh was not able to relax mesenteric arteries from ZDF rats.

Blockade of KCa. In another set of rats, we tested the influence of potassium channels by selective application of apamin, charybdotoxin, and TRAM-34 (block at specific concentrations SKCa, IKCa, BKCa/KV, and IKCa, respectively, each 0.1 μM), comparing ZDF with Z+/+ rats. When applied separately, the channel blockers did not affect ACh-induced relaxation in Z+/+ rats, whereas there was an inhibition in ZDF rats (Fig. 3 and Table 3). In the latter, isolated inhibition of SKCa with apamin did not significantly reduce ACh-induced relaxation. Blockade of IKCa (with TRAM-34) or IKCa, BKCa, and KV (with charybdotoxin) led to a significant reduction in ACh-induced relaxation compared with the control group in diabetic animals (Fig. 3B). Combined application of apamin and charybdotoxin inhibited ACh-induced relaxation in Z+/+ animals in a nonsignificant manner, whereas ACh-induced relaxation of the ZDF SMA was reduced significantly (Fig. 4, A and B, and Table 3). Additional blockade of eNOS and COX in Z+/+ resulted in an abolished ACh-induced relaxation. After substitution of charybdotoxin by TRAM-34, the ACh-induced relaxation was not reduced significantly in Z+/+ rats. However, the substitution of charybdotoxin with TRAM-34 on this combination showed no difference in ZDF animals (Fig. 4, C and D, and Table 3).

Activation of KCa. The stimulation of mesenteric arteries with cumulative concentrations of 1-EBIO, which activates IKCa more specifically than SKCa, showed a significant divergence in relaxations between Z+/+ and ZDF animals at 1, 10, and 100 μM. However, Rmax in ZDF was significantly lower in ZDF than in Z+/+ animals (32.7 ± 6.4% vs. 15.8 ± 5.2%; Fig. 5A), whereas control experiments with denuded arteries showed no difference between the groups (Fig. 5B). The dose-response curve to the BKCa activator NS1619 was significantly different for Z+/+ vs. ZDF at 10 μM vs. both groups treated with iberiotoxin at 5, 10, and 50 μM and vs. the iberiotoxin-treated ZDF group at 100 μM, with no significant difference of Rmax between Z+/+ and ZDF (Rmax Z+/+ 49.2 ± 8.1% vs. ZDF 38.6 ± 9.4%; Fig. 5C). Denudation, however, led to an altered iberiotoxin sensitivity of NS1619-induced relaxation. Relaxation in denuded, iberiotoxin-treated Z+/+ and ZDF vessels was reduced significantly vs. denuded Z+/+ at 10–100 μM NS1619, and denuded ZDF vessels relaxed more than those treated with iberiotoxin at 100 μM (Fig. 5D).

Quantitative RT-PCR studies of KCa. Analyses of mesenteric arteries of ZDF and control rats did not reveal a significant difference in mRNA levels of BKCa (Fig. 6). In ZDF, expression of SK3 was elevated compared with Z+/+, and expression of IKCa (IK1) was significantly augmented compared with Z+/+ and ZL (Fig. 6).

Western blot analysis of KCa. SKCa and BKCa protein expression levels were not altered significantly in ZDF mesenteric arteries (Figs. 7, A and D), whereas expression of IKCa monomeric proteins (appearing at 50 kDa), as well as channel complexes (appearing at high molecular weight, >200 kDa), which were described as the functional homotetrameric channel (11), was upregulated by diabetes compared with Z+/+ mesenteric arteries (Fig. 7, B and F).

DISCUSSION

In SMA, EDH-type relaxation entirely supersedes the loss of NO (Fig. 2, A and B) and vice versa (Fig. 4, A and C). In ZDF, the loss of NO blockade of the EDH pathway with SKCa and IKCa inhibitors could not be overridden by other relaxing mechanisms after stimulation with ACh (Fig. 4, B and D). Inhibition of IKCa with charybdotoxin or with TRAM-34

<table>
<thead>
<tr>
<th>Table 3. ACh-induced Rmax and pD2 under control condition and potassium channel inhibition</th>
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<tr>
<td>ACh</td>
</tr>
<tr>
<td>CTL  91.3 ± 5.9       62.2 ± 5.7*       6.3 ± 0.25       6.0 ± 0.23</td>
</tr>
<tr>
<td>+ A 103.6 ± 6.8       73.4 ± 11.0*       6.4 ± 0.15       n.d.</td>
</tr>
<tr>
<td>+ C 98.9 ± 17.8       17.3 ± 11.4*       6.2 ± 0.14       n.d.</td>
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<tr>
<td>+ T 87.4 ± 8.2       25.2 ± 7.1*        6.2 ± 0.11       n.d.</td>
</tr>
<tr>
<td>+ A + C 64.6 ± 17.4*       10.3 ± 3.4*    5.8 ± 0.29*       n.d.</td>
</tr>
<tr>
<td>+ A + T 72.4 ± 19.6      10.8 ± 2.0*    6.3 ± 0.22       n.d.</td>
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<tr>
<td>+ L + D + A + C 8.7 ± 2.6*       7.4 ± 2.4*    n.d.       n.d.</td>
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<tr>
<td>+ L + D + A + T 55.6 ± 21.4*      12.1 ± 9.6*   6.0 ± 0.28       n.d.</td>
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<td>pD2, n = 6</td>
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<td>Z+/+    11.0 ± 0.23       6.2 ± 0.16       6.0 ± 0.19       n.d.</td>
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<td>ZDF     0.11 n.d.       0.15 n.d.       0.13 n.d.       n.d.</td>
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<tr>
<td>Z+/+    0.28 n.d.       0.01 vs. Z+/+ 0.28 n.d.       0.01 vs. Z+/+ 0.01 vs. Z+/+</td>
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Parameters measured in small mesenteric arteries from 18-wk-old Z+/+, ZL, and ZDF rats. pD2 and Rmax to ACh in the absence (CTL) or presence of apamin (0.1 μM), charybdotoxin (0.1 μM), 1-[2-chlorophenyl]diphenylmethan-1-yl-H-pyrazole [TRAM-34 (T); 0.1 μM], apamin plus charybdotoxin, apamin plus TRAM-34, t-NAME (100 μM) plus diclofenac (10 μM) plus apamin plus charybdotoxin, or t-NAME plus diclofenac plus apamin plus TRAM-34. Values are means ± SE. *P < 0.01 vs. Z+/+; **P < 0.05 vs. Z+/+ (Student’s t-test); *P < 0.05 vs. corresponding CTL; **P < 0.01 vs. corresponding CTL; *P < 0.01 vs. corresponding t-NAME + diclofenac + apamin + charybdotoxin (1-way ANOVA).
resulted in a significant reduction of ACh-induced relaxation in ZDF but not in control SMA (Fig. 3). Given that mRNA and protein expression levels of IKCa are elevated in the animal model (Figs. 6 and 7), the IKCa, therefore, appears to maintain an important role in endothelium-dependent, ACh-induced relaxation of diabetic SMA.

Since it also has been shown in a variety of studies (26, 34, 37) that EDH is able to compensate for decreased availability of the NO pathway, the hypothesis suggesting that EDH-type relaxation is an important compensatory, vasodilatory mechanism is not unanimously accepted, as it has been shown that EDH-type relaxation is severely impaired in diabetes and hypertension (20, 31, 47). Oxidative stress plays an early role leading to endothelial dysfunction and cardiovascular complications in diabetes (36). Hyperglycemia activates reduced NADP oxidases and leads to uncoupling of the homodimer eNOS to form superoxide (12, 24). Elevated formation of reactive oxygen species (ROS) is thought to be a key event in the genesis of endothelial dysfunction (35, 50), and ROS leads to enhanced oxidative stress, especially when antioxidant systems are impaired. One protein involved in this regulation is heme oxygenase, which is responsible for maintaining normal metabolic cellular functions, and when absent, oxidative stress and chronic inflammation markedly increase (2, 21). Metabolites of the heme-containing cytochrome P450 epoxygenases, the epoxyeicosatrienoic (EET) acids, lead to an increased activity of heme oxygenase in rat mesenteric microvessels and to an attenuation of ROS levels, thereby protecting vessels against oxidative stress (27, 28, 39). EET acids can act as an EDH factor (EDHF) via autocrine activation of transient receptor potential channels in ECs with a consecutive opening of SKCa and IKCa to amplify endothelial signals (9), and they also possess a capability to act without involvement of the endothelial S/IKCa system by activating BKCa (33). As BKCa are sensitive to charybdotoxin, and ACh-induced relaxation in ZDF was markedly reduced by this toxin, a contribution of EET acids cannot be ruled out. However, selective inhibition of IKCa with TRAM-34 led to a comparable reduction of ACh-induced relaxation, suggesting no relevant contribution of BKCa for ACh-induced vasorelaxation in this vessel.

A mechanism of ECs to hyperpolarize the cell membrane of smooth muscle cells (SMCs) is mediated by electrical commu-
communication via myoendothelial gap junctions (MEGJs), spreading
the signal from the luminal cell layer into the muscular layer.
In the arterial mesenteric bed, it has been shown that MEGJs
quantitatively increase with the decline of vessel diameter (40).

A prerequisite for this pathway of SMC hyperpolarization is
EC hyperpolarization, which is conducted via activation of
SKCa and IKCa in the membrane of ECs. In the present study,
inhibition of EC hyperpolarization by apamin plus TRAM-34
led to a reduction of ACh-induced relaxation in diabetic vessels
(Fig. 4D). An elongation or amplification of the electrical
signal by gap junctions could be suppressed, so reduced gap
junctional communication in the studied vessel may addition-
ally occur, as observed in other studies (19, 29), and could act
independently of SK/IKCa signaling, as proposed by Chadha et
al. (11).

Our results show a compensation of endothelium-dependent
relaxation when potassium channels are inhibited in control
animals but a loss of compensation in the diabetic model.
Whereas products of COX do not have a role for endothelium-
dependent vasorelaxation in control and diabetic animals in
this study, NO-induced relaxation might be diminished be-
cause of reduced NO bioavailability. In ZDF animals, ACh-
induced relaxation is already severely affected by the combi-
nation of apamin and charybdotoxin, suggesting the NO sys-
tem cannot compensate or alternatively, that EDH-type
relaxation is predominant in the endothelium-dependent relax-
ation in that vessel under diabetic conditions. ROS play an
important role in modulating NO levels, but next to a reduced
bioavailability, the reason for the reduced NO-induced relax-
ation in diabetes can be upstream of NO production. eNOS is

Fig. 5. Concentration-response curves of
SMA precontracted with NA (10 μM) to
increasing concentrations of 1-ethyl-2-benz-
imidazolinone (1-EBIO; 1 nM–100 μM) or
1,3-dihydro-1-[2-hydroxy-5-(trifluorometh-
yl)phenyl]-5-(trifluoromethyl)-2H-benzi-
dazol-2-one (NS1619; 1–100 μM) with en-
dotheleum (A and C) and without endo-
theleum (−E; B and D) in the absence and
presence of TRAM-34 (0.1 μM) or iberi-
toxin (Ibtx; 0.1 μM), respectively, isolated
from Z+/+ and ZDF rats; n = 5–7 experi-
ments. Results are shown as means ± SE.
*P < 0.05 vs. ZDF. A: §P < 0.05 vs.
Z+/+ + TRAM-34 and vs. ZDF + TRAM-
34. C: §P < 0.05 vs. Z+/+ + Ibtx and vs.
ZDF + Ibtx; #P < 0.05 vs. ZDF + Ibtx. D:
§P < 0.05 vs. Z+/+ –E + Ibtx and vs.
ZDF –E + Ibtx; #P < 0.05 vs. ZDF –E +
Ibtx, 2-way repeated-measures ANOVA.

Fig. 6. Comparison of mRNA levels of isoform 3 of small (SKCa3), interme-
diate (IKCa), and large (BKCa)-conductance calcium-activated potassium
channels in mesenteric arteries of Z+/+, ZL, and ZDF rats; n = 6. Results are
shown as means ± SE. *P < 0.05, 1-way ANOVA.
a Ca\textsuperscript{2+}-dependent enzyme (10), so a reduction in endothelial Ca\textsuperscript{2+} concentration could result in reduced enzyme activation and consecutively reduced vasodilation. Furthermore, dampened activation of IK\textsubscript{Ca} or SK\textsubscript{Ca} and attenuated hyperpolarization of EC with decreased driving force for capacitative Ca\textsuperscript{2+} entry through store-operated channels are results of initial attenuation of agonist-induced Ca\textsuperscript{2+} elevation in ECs. It has been shown that Ca\textsuperscript{2+} release from endothelial stores is reduced with no significant alienation in store-operated calcium entry in Type 1 diabetic mice (16). Of course, a reduced NO bioavailability, as well as an altered upstream signaling, can occur coincidentally and lead to reduced vasodilation in diabetes.

In our study, 1-EBIO-induced relaxation is impaired in diabetic arteries, similar to studies using isometric tension in SMA of Type 2 diabetic OLETF and Type 1 diabetic rats (31, 49). IK\textsubscript{Ca} mRNA and protein are quantitatively upregulated in ZDF SMA, suggesting a reduced function of IK\textsubscript{Ca} in diabetes, although besides monomeric channel protein, also channel complexes are upregulated. As SK\textsubscript{Ca} and IK\textsubscript{Ca} have been shown to be expressed exclusively in ECs of rat mesenteric artery (13, 14), our results go in line with other observations, stating an impaired function of the endothelial S/IK\textsubscript{Ca} dilator system in cardiovascular diseases (6, 8). The observed increase in IK\textsubscript{Ca} protein expression in diabetes could be part of a compensatory, regulatory mechanism in mesenteric ECs of ZDF rats. Theoretically, the channel protein could be upregulated in SMCs, as observed in studies of vascular beds other than rat mesenteric artery, and also cultured cells (22, 45). Although the proportion of increased IK\textsubscript{Ca} expression cannot be attributed precisely to EC or SMC, as whole vessel tissue was used in this study, following literature, we suppose an

Fig. 7. Representative Western blots of mesenteric artery revealing expression of SK\textsubscript{Ca}3 (A), IK\textsubscript{Ca} (B), and BK\textsubscript{Ca} (D) expression. Monomeric forms appear at 90 kDa for SK\textsubscript{Ca}3, 50 kDa for IK\textsubscript{Ca}, and 110 kDa for BK\textsubscript{Ca}. Channel protein complexes appear at 250 kDa. Specificity of anti-IK\textsubscript{Ca} was characterized with its antigenic peptide (+p; C). Lanes M1 and M2, molecular-weight markers. Summary of Western blot data with histograms showing normalized values to actin protein levels for SK\textsubscript{Ca}3 (E), IK\textsubscript{Ca} (F), and BK\textsubscript{Ca} (G). Unfilled rectangles represent values for the high molecular-weight region at 250 kDa; pZ+/+ and pZDF, preincubation with their control peptide; n = 3–4 experiments. Results are shown as means ± SE. *P < 0.05 vs. Z+/+ for the same molecular weight, using the Student’s unpaired t-test.
upregulation in EC rather than SMC. As IKCa activation via 1-EBIO stimulation results in reduced vasorelaxation in diabetic SMA, the observed mRNA and protein upregulation of IKCa most likely reflects non- or dysfunctional protein, which leads to a reduced relaxation in ZDF mesenteric arteries when endothelium-dependently stimulated with ACh.

BKCa, which are expressed exclusively in SMCs (15, 48), possibly act as a target of endothelium-derived relaxing factors. In this study EC activation is already reduced, so a lessened downstream activation of BKCa could be expected if EDHF would target this channel. NS1619-induced relaxation showed no difference in ZDF animals, suggesting no impairment of this pathway by diabetes.

Study limitations. Due to technical limitations of our laboratory, we were not able to provide electrophysiological data to demonstrate EDH of SMCs; instead, we use the term EDH-type relaxation. As we used whole mesenteric arteries, third-order branch for myograph experiments, and first- through third-order branches for mRNA and protein quantification, we cannot distinguish between effects on EC or SMC with certainty. However, there are robust data stating that in intact rat mesenteric arteries, BKCa are expressed exclusively in SMC (15, 48) and S1IKCa in EC (13, 14).

The accuracy of the Western blot analysis depends on antibody specificity. The antibodies used in this study to detect SKCa3 and BKCa are well characterized (5, 42, 44). The antibody APC-064 against IKCa is less well characterized; therefore, we determined specificity by preincubation of the antibody with the antigenic peptide, as shown in our data in Fig. 7. We nevertheless note that differences may arise with different antibody batches.

Conclusion. In the present study, we examined the role of endothelium-derived relaxation in Type 2 diabetes with focus on KCa. We introduced a previously uninvestigated control group, the Z+/− rat, which has no allele for the leptin receptor defect. To the best of our knowledge, we present, for the first time, molecular data of the relevant KCa, i.e., SKCa3, IKCa, and BKCa in ZDF, as well as Z+/− SMA, and show functional data of the Z+/− mesenteric vessels in this study.

NO-associated and EDH-type relaxation can compensate for each other in ACh-induced relaxation of mesenteric arteries in control but not in Type 2 diabetic ZDF rats. Particularly, the NOS- and COX-independent relaxation is sensitive to the combination of apamin and TRAM-34 in our diabetes model. Although relaxation after IKCa activation is reduced in diabetess, mRNA and protein levels were elevated in mesenteric arteries of diabetic animals. These observations, when taken together with the shown reduction of ACh-induced relaxation by selective IKCa inhibition, suggest an important role for IKCa in diabetic SMA as a target for innovative treatments of diabetic microvascular complications.

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DISCLOSURES

The authors declare that there is no competing interest.

AUTHOR CONTRIBUTIONS


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