Reduced Ca\(^{2+}\)-dependent activation of large-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels from arteries of Type 2 diabetic Zucker diabetic fatty rats

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Burnham, Matthew P., Ian T. Johnson, and Arthur H. Weston. Reduced Ca\(^{2+}\)-dependent activation of large-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels from arteries of Type 2 diabetic Zucker diabetic fatty rats. Am J Physiol Heart Circ Physiol 290: H1520–H1527, 2006. First published November 4, 2005; doi:10.1152/ajpheart.00827.2005.—Although it is well established that diabetes impairs endothelium-dependent vasodilation, including those pathways involving vascular myocyte large-conductance Ca\(^{2+}\)-activated K\(^{+}\) channels (BK\(_{\text{Ca}}\)), little is known about the effects of diabetes on BK\(_{\text{Ca}}\) activation as an intrinsic response to contractile stimulation. We have investigated this mechanism in a model of Type 2 diabetes, the male Zucker diabetic fatty (ZDF) rat. BK\(_{\text{Ca}}\) function in prediabetic (5–7 wk) and diabetic (17–20 wk) ZDF and lean control animals was assessed in whole arteries using myograph and electrophysiology techniques and in freshly dissociated myocytes by patch clamping. Log EC\(_{25}\) values for phenylephrine concentration-tension curves were shifted significantly to the left by blockade of BK\(_{\text{Ca}}\) with iberiotoxin (IBTX) in arteries derived from non- and prediabetic animals but not from diabetic animals. Smooth muscle hyperpolarizations of arteries evoked by the BK\(_{\text{Ca}}\) opener NS-1619 were significantly reduced in the diabetic group. Voltage-clamp recordings indicated that IBTX-sensitive currents were not enhanced to the extent observed in nondiabetic controls by increasing the Ca\(^{2+}\) concentration in the pipette solution or the application of NS-1619 in myocytes from diabetic animals. An alteration in the expression of BK\(_{\text{Ca}}\) \(\beta_1\) subunits was not evident at either the mRNA or protein level in arteries from diabetic animals. Collectively, these results suggest that myocyte BK\(_{\text{Ca}}\) of diabetic animals does not significantly oppose vasoconstriction, unlike that of prediabetic and control animals. This altered function was related to a reduced Ca\(^{2+}\)-dependent activation of the channel not involving \(\beta_1\) subunits.

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Table 1. Basic parameters of animals used

<table>
<thead>
<tr>
<th>Young, Lean</th>
<th>Young, ZDF</th>
<th>Old, Lean</th>
<th>Old, ZDF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight, g</td>
<td>156±7</td>
<td>218±16*</td>
<td>348±8</td>
</tr>
<tr>
<td>Blood pressure, mmHg</td>
<td>154±4</td>
<td>144±5</td>
<td></td>
</tr>
<tr>
<td>Blood glucose, mM</td>
<td>9.7±2.1</td>
<td>17.0±3.1</td>
<td>8.8±0.4</td>
</tr>
<tr>
<td>Insulin, mU/l</td>
<td>33.0±6.1</td>
<td>378.0±103.8*</td>
<td>18.2±5.9</td>
</tr>
</tbody>
</table>

Values are means ± SE. ZDF, Zucker diabetic fatty rats. *P < 0.05, old vs. young (same strain); †P < 0.05, ZDF vs. lean (same age group).

Results of Animals Used

(1). The mesenteric bed was removed and maintained in ice-cold Krebs solution containing (in mM) 118 NaCl, 3.4 KCl, 1.6 CaCl₂, 1.2 KH₂PO₄, 1.2 MgSO₄, 25 NaHCO₃, and 11 glucose while arteries were dissected.

Membrane potential recordings. Segments of third-order mesenteric artery, pinned within a recording chamber, were perfused with Krebs solution containing 100 μM N⁶-nitro-L-arginine (NNA) and 10 μM indomethacin, gassed with 5% CO₂-95% O₂ at 37°C. Myocytes were impaled from the adventitial side using sharp microelectrodes as previously described (4). NS-1619 was added directly to the recording chamber as a bolus calculated to give transiently the indicated concentration.

Myography. Segments of third-order mesenteric arteries were mounted between 40-μm stainless steel wires on a Mulvaney-Halpern-type myograph (model 610, JP Trading). For consistency, Krebs solution, identical to that prepared for microelectrode studies (including t-NNA and indomethacin), was employed. Arteries were normalized to a tension equivalent to 80 mmHg transmural pressure (14). After 30-min equilibration, vessels were stimulated three times with high-K⁺ Krebs solution (an equimolar substitution of 75 mM KCl for NaCl). Cumulative concentration-response relationships for phenylephrine (10⁻⁶ to 10⁻² M) were determined in the absence and presence of 100 nM IBTX in separate vessels.

Patch-clamp experiments. Single myocytes were obtained by enzymatic treatment of mesenteric arteries (20). Only relaxed, spindle-shaped cells were used for experiments. The recording chamber was perfused with external solution containing (in mM) 134 NaCl, 6 KCl, 0.1 CaCl₂, 1 MgCl₂, 10 glucose, and 10 HEPES (pH 7.4 at room temperature), plus 5 μM wortmannin to inhibit contraction (17). Pipettes (3–7 MΩ) were filled with internal solution containing either EGTA [containing (in mM) 10 NaCl, 30 KCl, 110 potassium aspartate, 1 MgCl₂, 0.05 EGTA, and 10 HEPES (pH 7.2)] or 250 mM CaCl₂ [containing (in mM) 10 NaCl, 30 KCl, 110 potassium aspartate, 1 MgCl₂, 1 EGTA, 0.59 CaCl₂, and 10 HEPES (pH 7.2)]. The whole cell, voltage-clamp configuration, without compensation for series resistance, was employed by using an Axopatch 200B amplifier, Digidata 1322A analog-to-digital converter and pClamp 9 software (Axon Instruments). NS-1619 (17 μM) was perfused into the recording chamber, and IBTX (100 nM final) was added directly to the chamber.

Quantitative RT-PCR. Mesenteric artery total RNA was isolated by using RNAeasy Mini kits (Qiagen), treated with DNase I (Invitrogen), and quantified by using Ribogreen RNA kits (Molecular Probes). RNA (1.5μg) was reverse-transcribed by using Superscript II (Invitrogen) and oligo-dT primer. Triplicate samples equivalent to 75 ng RNA were assayed using α subunit (KCNA1)-specific primers 5’-AAA-CAAGTAATTTCTCAAGCTGGTG and 5’-CTGAACTGTCCTGTTGTTTGG or β₁ subunit (KCNC1)-specific primers 5’-CC-ACACGTTGCTCTATTCCTCAAGCAGAG and 5’-ATAGGAGCGGCCCACGTCACG. SYBR Green I Mastermix Plus (Eurorgenetics) reagents and a DNA Engine Opticon 2 (MJ Research) thermal-cycler were employed. Standard curves were prepared from plasmids containing α or β₁ subunit coding regions (a kind gift of G. Richards, Merck UK). 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 and visualization of products on agarose-ethidium bromide gels. Product identity was confirmed by cloning and sequencing.

Immunofluorescence labeling. Immunofluorescence labeling was performed essentially as previously described (4). Briefly, mesenteric arteries were fixed in 4% paraformaldehyde, and cyrostat sections were prepared. Sections were treated with 0.1% (wt/vol) sodium dodecyl sulfate, blocked, and incubated with primary antibodies overnight at 4°C. Anti-BKCaα (APC-107, Alomone) was used at 1:100 dilution (with and without preincubation with immunogen peptide), and anti-BKCa β₁ (No. 444915, Calbiochem) was used at 1:500 dilution (no peptide available). Cy3-conjugated secondary antibodies and 4,6-diamidino-2-phenylindole nuclear label were employed. Images were acquired on a Zeiss Axiosplan 2 microscope equipped with a QICAM cooled, charge-coupled device camera (QImaging). Red fluorescence was quantified with the use of Zeiss KS300 software. For antibody specificity controls, human embryonic kidney (HEK) cells were stably transfected with the α or β₁ subunits, fixed, and immunolabeled as described.

Western blot analysis. Samples of mesenteric artery were dissected from a second group of lean and ZDF animals (12–14 wk old; blood glucose: lean, 7.9 ± 0.2 mM; and ZDF, 40.1 ± 2.7 mM), and Western blot analysis was performed essentially as previously described (4). Briefly, arteries were homogenized, and unfractioned samples were prepared with Laemmli buffer. Samples (10 μg each) were loaded onto 10% acrylamide gels for electrophoresis and blotting. After membranes were blocked with 5% nonfat milk, primary antibodies were applied overnight (4°C). BKCaα subunit was detected by using clone 32 monoclonal antibody (1:100 dilution; No. 611248, BD Transduction Laboratories). Detection was achieved with horseradish peroxidase-conjugated secondary antibodies and chemiluminescent reagents. For antibody specificity controls, lysates of HEK cells expressing BKCaα or β₁ subunits were subjected to Western blot analysis as described.

Drugs. Synthetic IBTX was obtained from Latoxan, France. NS-1619 and all other chemicals were supplied by Sigma.

Data analysis. Phenylephrine responses were calculated as a percentage of the largest 75 mM K⁺ response in that artery. Curve fitting and calculation of log EC₂₅ values (logarithm of molar concentration producing 25% maximal response) were performed with the use of Prism 4 software (GraphPad). Log EC₂₅ data were reported as best-fit values ± SE. All other values are given as means ± SE. Statistical analysis (Student’s t-test and ANOVA) was carried out with Prism 4 software, and a value of P < 0.05 was considered statistically significant unless otherwise stated.

RESULTS

Membrane potential recordings. Differences in endothelial function have been noted in diabetes, and endothelial-derived factors, such as nitric oxide, are known to affect BKCa (2). Therefore, the Krebs solution used for microelectrode studies included t-NNA and indomethacin to reduce these effects. Arterial resting membrane potential was similar between old lean and ZDF animals (−54.3 ± 0.7 mV, n = 7, and −54.6 ± 0.5 mV, n = 6, respectively). Concentrations of 17 and 33 μM were chosen for NS-1619 applications because these evoked approximate EC₃₀ (concentration producing 30% maximal response) and near-maximal responses in these experiments, and these responses are completely inhibited by IBTX in both the normal rat mesenteric artery (data not shown) and in the porcine coronary artery (8). Although both concentrations of NS-1619 evoked hyperpolarizations in arteries from old lean and ZDF animals, the responses recorded in arteries from ZDF animals were significantly smaller than those in lean animals (Fig. 1).
phenylephrine log EC25 values in young lean and ZDF animals were significantly more sensitive to phenylephrine than those from the corresponding young animals (old lean, \(-6.66 \pm 0.06, n = 16\); and old ZDF, \(-6.65 \pm 0.06, n = 14\)). In the absence of IBTX, arteries from old animals of either strain displayed similar phenylephrine log EC25 values and were significantly more sensitive to phenylephrine than those from the corresponding young animals (old lean, \(-6.66 \pm 0.06, n = 16\); and old ZDF, \(-6.65 \pm 0.06, n = 14\)). In the presence of IBTX, the log EC25 value for arteries from old lean animals was reduced significantly (\(-7.14 \pm 0.13, n = 8\)). However, IBTX did not significantly affect the log EC25 value in arteries from old ZDF animals (\(-6.86 \pm 0.14, n = 6\)). Hence, arteries from old ZDF animals maintained an overall vasoconstrictive response to phenylephrine similar to that seen in control animals yet displayed a reduced BKCa-mediated contribution to this response. The sensitivity of arteries to phenylephrine (log EC25) increased with age in both groups.

**Patch-clamp experiments.** After whole cell configuration was established, spontaneous transient outward currents (15) were frequently observed in myocytes and were often superimposed on “bulk” currents evoked by voltage steps. Several minutes were allowed for the pipette solution to fully dialyze the cell and to eliminate this activity from the recorded data. Voltage-clamp protocols were optimized for activation and measurement of BKCa rather than attempting to maintain in vivo membrane potentials in a dissociated and dialyzed cell. Low Ca2+ concentrations and the addition of wortmannin were used to prevent myocyte contraction and, hence, loss of gigaohm seals. Cell capacitance estimates were similar between myocytes from ZDF (19.7 \pm 0.9 pF, \(n = 24\)) and lean animals (18.0 \pm 0.8 pF, \(n = 24\)). Total and IBTX-sensitive currents recorded with the use of EGTA pipette solutions were similar in myocytes from old lean and diabetic ZDF animals (Fig. 3; currents at +140 mV were lean, 111 \pm 11 pA/pF, and ZDF, 101 \pm 12 pA/pF, and in the presence of IBTX, lean, 28 \pm 5 pA/pF, and ZDF, 31 \pm 6 pA/pF). In myocytes from lean animals, currents elicited with Ca2+-containing pipette solutions (160 \pm 20 pA/pF at +140 mV) were significantly larger than those elicited with EGTA pipette solutions and were markedly increased by NS-1619 (348 \pm 25 pA/pF at +140 mV; Fig. 3). However, in myocytes from diabetic ZDF animals, currents evoked with the use of Ca2+-containing pipette solutions (108 \pm 12 pA/pF at +140 mV) were of similar magnitude to those recorded with the use of EGTA pipette solutions, and the increase stimulated by NS-1619 (185 \pm 14 pA/pF at +140 mV) was significantly reduced compared with that observed in myocytes from lean animals (Fig. 3). Application of IBTX reduced NS-1619-stimulated currents to low-level residual currents of similar magnitude in myocytes from both strains (lean, 64 \pm 12 pA/pF, and ZDF, 38 \pm 3 pA/pF, at +140 mV; Fig. 3). Currents recorded with the use of Ca2+-containing pipette solutions in myocytes from young, prediabetic ZDF and lean animals were similar in both the magnitude and the extent to which they were inhibited by IBTX (Fig. 3; young lean, 191 \pm 15 pA/pF, and young ZDF, 177 \pm 20 pA/pF at +140 mV; and young lean + IBTX, 32 \pm 2 pA/pF, and young ZDF + IBTX, 38 \pm 5 pA/pF at +140 mV).

**BKCa subunit expression.** Expression of BKCa \(\alpha\) and \(\beta_1\) subunits in arteries from old lean and diabetic ZDF animals was investigated at the mRNA and protein levels (Fig. 4). Quantitative, real-time RT-PCR analysis under the conditions described was specific for the \(\alpha\) and \(\beta_1\) subunits of BKCa, as

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**Fig. 1.** Effect of NS-1619 (NS) on membrane potential in arteries from old Zucker diabetic fatty (ZDF) and lean animals. Smooth muscle membrane potential of mesenteric arteries was recorded by using sharp microelectrodes during exposure to 17 and 33 \(\mu\)M NS. Representative traces (A) and mean change in membrane potential (B) recorded in arteries from old lean and ZDF animals (\(n = 7\) and 6, respectively) are shown. *\(P < 0.05\), lean vs. ZDF.

**Myograph studies.** Log EC25 values were compared in phenylephrine concentration-response relationships rather than the more traditional log EC50 values. The effects of IBTX were most apparent at lower tensions in arteries from control and prediabetic animals, and, therefore, log EC25 values were a reasonable quantification for the absence of these effects in the diabetic group. Also, arteries mounted on a wire myograph are markedly less sensitive to phenylephrine than those mounted on a pressure myograph, which is closer to the normal physiological situation (5). Therefore, on a wire myograph, responses evoked by lower concentrations of phenylephrine were likely to be the most relevant.

Increases in tension elicited by 75 mM K+ were used for normalizing subsequent phenylephrine responses and were not different between strains (young lean, 0.76 \pm 0.12 mN/mm, \(n = 16\); and ZDF, 0.68 \pm 0.10 mN/mm, \(n = 15\); and old lean 2.34 \pm 0.21 mN/mm, \(n = 24\); and ZDF, 1.89 \pm 0.21 mN/mm, \(n = 20\)). There was no significant difference between the phenylephrine log EC25 values in young lean and ZDF animals (Fig. 2). In the presence of IBTX, log EC25 values were significantly reduced from controls in both strains (young lean, \(-6.22 \pm 0.06 (n = 8)\) vs. young lean + IBTX, \(-6.67 \pm 0.11 (n = 8)\) vs. young ZDF + IBTX, \(-6.86 \pm 0.18 (n = 8)\)). In the absence of IBTX, arteries from old animals of either strain displayed similar phenylephrine log EC25 values and were significantly more sensitive to phenylephrine than those from the corresponding young animals (old lean, \(-6.66 \pm 0.06, n = 16\); and old ZDF, \(-6.65 \pm 0.06, n = 14\)). In the presence of IBTX, the log EC25 value for arteries from old lean animals was reduced significantly (\(-7.14 \pm 0.13, n = 8\)). However, IBTX did not significantly affect the log EC25 value in arteries from old ZDF animals (\(-6.86 \pm 0.14, n = 6\)). Hence, arteries from old ZDF animals maintained an overall vasoconstrictive response to phenylephrine similar to that seen in control animals yet displayed a reduced BKCa-mediated contribution to this response. The sensitivity of arteries to phenylephrine (log EC25) increased with age in both groups.

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demonstrated by the appearance of only a single product on 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 \( \alpha \) subunit (lean, 2,197 \( \pm \) 131, and ZDF, 2,374 \( \pm \) 263 copies/ng total RNA; \( n = 6 \) for both groups) and \( \beta_1 \) subunit (lean 103,800 \( \pm \) 7,094, and ZDF, 100,700 \( \pm \) 9,634 copies/ng total RNA; \( n = 6 \) for both groups) was similar in arteries from lean and ZDF animals. These data indicate that \( \beta_1 \) subunit mRNA was expressed at approximately 40-fold higher copy numbers than \( \alpha \) subunit mRNA in both strains.

Immunolabeling of HEK cells expressing either the BKCa \( \alpha \) or \( \beta_1 \) subunits demonstrated that each antibody only labeled cells expressing the appropriate subunit and did not cross-react with cells expressing the other subunit, confirming the specificity of the antibodies. Furthermore, artery sections were not labeled when primary antibody was preincubated with immunogenic peptide or when secondary antibody was used alone. Images of immunolabeled artery sections were analyzed for labeling intensity in the smooth muscle area (defined as between the inner and outer laminas, visible because of their green autofluorescence). Arteries from ZDF animals displayed significantly less labeling for the \( \alpha \) subunit (129 \( \pm \) 4 units, \( n = 25 \)) compared with arteries from control animals (142 \( \pm \) 4 units, \( n = 29 \)). Immunolabeling for the \( \beta_1 \) subunit was not significantly different between ZDF and control groups (106 \( \pm \) 2 units, \( n = 25 \), and 113 \( \pm \) 5 units, \( n = 25 \), respectively).

Expression of BKCa \( \alpha \) subunit was also quantified by Western blot analysis. Control experiments demonstrated that the anti-BKCa \( \alpha \) subunit antibody recognized the protein when expressed in HEK cells and did not cross-react with other proteins. Artery samples from lean and ZDF animals were subsequently analyzed for \( \alpha \) subunit expression together with \( \beta_1 \)-actin loading controls. Expression of \( \alpha \) subunit (\( n = 5 \)) was not significantly different between samples from lean (27,938 \( \pm \) 6,136 units) and ZDF (16,870 \( \pm \) 1,645 units) animals nor was \( \beta_1 \)-actin loading control (192,888 \( \pm \) 21,762 units and 167,029 \( \pm \) 10,357 units, respectively).

**DISCUSSION**

The current study provides a comprehensive description of myocyte BKCa function in arteries of the ZDF rat in the prediabetic and Type 2 diabetic state. The finding that IBTX did not enhance phenylephrine-induced contraction in arteries from diabetic ZDF animals shows that activation of BKCa during vasoconstriction was impaired in these arteries. Additionally, myocyte hyperpolarizations generated by the BKCa activator NS-1619 were reduced in intact arteries from diabetic ZDF animals compared with controls. In patch-clamp studies using freshly dissociated myocytes, the enhancement of IBTX-sensitive currents by Ca\(^{2+}\) or NS-1619 that was observed in cells isolated from controls was reduced in the diabetic state, suggesting a decreased Ca\(^{2+}\) sensitivity of BKCa. The express-
sion of BK<sub>Ca</sub> β1 subunits, which modulate the Ca<sup>2+</sup> sensitivity of BK<sub>Ca</sub>, was unchanged between groups, and thus alterations in this protein are unlikely to explain the altered BK<sub>Ca</sub> function observed in the diabetic ZDF rat arteries.

Although many studies have examined arterial BK<sub>Ca</sub> function in models of hypertension, few such investigations have been made in models of Type 2 diabetes. In a patch-clamp study of mesenteric myocytes from fructose-fed, insulin-resistant rats, a reduction in both whole cell BK<sub>Ca</sub> currents and the stimulatory effect of NS-1619 was observed, similar to the electrophysiology findings of the present study (7). However, no differences in BK<sub>Ca</sub> subunit expression, single-channel conductance, voltage sensitivity, or Ca<sup>2+</sup> sensitivity were observed, and the mechanism responsible for reducing whole cell BK<sub>Ca</sub> currents was not determined. In a study of endothelium-dependent vasodilator pathways, IBTX-sensitive vasodilations evoked by bradykinin or the prostacyclin analog iloprost were impaired in fructose-fed, insulin-resistant rats, although a direct impairment of the BK<sub>Ca</sub> channel itself was not identified (9, 10). In general terms, therefore, these earlier studies in another rat model are consistent with the findings of the present investigation and suggest that impaired activation and function of pathways involving BK<sub>Ca</sub> are common features of Type 2 diabetes and insulin resistance. We believe that our study is the first to suggest that alterations of vascular function revealed by IBTX in the diabetic ZDF rat are related to a reduced Ca<sup>2+</sup>-dependent activation of the channel itself rather than changes to upstream activators, such as intracellular Ca<sup>2+</sup> concentrations or endothelium-derived factors. The data obtained in young, prediabetic ZDF and older, diabetic ZDF animals do not support a role for hyperinsulinemia per se in the observed alterations to vascular function, because the BK<sub>Ca</sub> activity changed over a period during which serum insulin levels actually declined.

To understand the mechanisms that underlie the observed change in BK<sub>Ca</sub> function in intact arteries observed in the current study, the BK<sub>Ca</sub> currents generated by voltage-clamp protocols in freshly dissociated myocytes were analysed. Voltage-activated BK<sub>Ca</sub> currents recorded with EGTA pipette solutions were similar in myocytes from both lean controls and diabetic ZDF animals, an indication that a similar density of functional channels may be present. Substituting a Ca<sup>2+</sup>-containing pipette solution enhanced BK<sub>Ca</sub> currents in myocytes from lean animals, consistent with the known sensitivity of this channel to Ca<sup>2+</sup>. In contrast, performing the same maneuver in myocytes from diabetic ZDF animals did not significantly increase the BK<sub>Ca</sub>-mediated current, suggesting that the channel from diabetic animals was less sensitive to

![Fig. 3. IBTX-sensitive currents recorded from mesenteric artery myocytes of young and old ZDF and lean animals. Whole cell voltage-clamp currents evoked in freshly dissociated mesenteric artery myocytes by 180 ms, 20 mV steps between -60 and +140 mV from a holding potential of -40 mV. Representative traces obtained by using Ca<sup>2+</sup> pipette solution (Ca) followed by perfusion of 17 μM NS and subsequent addition of 100 nM IBTX to recording chamber (A). Mean data demonstrating reduced effect of NS on IBTX-sensitive currents in myocytes from ZDF compared with lean animals (B). Mean data recorded by using EGTA (E) and Ca<sup>2+</sup>-pipette solutions demonstrate Ca<sup>2+</sup>-dependent activation of current in myocytes from lean but not ZDF animals (C and D, respectively). IBTX-sensitive currents evoked in myocytes from young lean and ZDF animals using Ca<sup>2+</sup> pipette solution were similar (E). *P < 0.05, for the indicated data sets. Number of observations is given in parentheses.](http://ajpheart.physiology.org/).
Fig. 4. Analysis of large-conductance Ca²⁺-activated K⁺ channels (BKCa) α and β₁ subunit expression in arteries from old lean and ZDF animals. A: quantitative real-time RT-PCR analysis using SYBR Green reagent of BKCa α and β₁ subunit mRNA expression. A1: specificity of reaction demonstrated by ethidium bromide-stained agarose gels [gel-resolved products amplified from vector standard (V) and artery cDNA (RT)] and melting-curve analysis [plots of normalized first negative derivative of fluorescence (-dF/dT) vs. temperature for all artery cDNA reactions]. A2: standard curves [threshold cycle (Ct) vs. log copies] showing vector standards (black circles) and artery cDNA samples (shaded circles). A3: mean data obtained from samples analyzed in triplicate (n = 6 animals). No significant differences in subunit mRNA expression were observed between strains. B: immunolabeling of BKCa α and β₁ subunits in lean and ZDF arteries. B1: analysis of anti-α and anti-β₁ subunit antibody specificity by immunolabeling human embryonic kidney (HEK) cells expressing α and β₁ subunits (representative of 3 separate experiments). B2: representative images of immunolabeled artery sections together with negative controls (inset) of primary antibody preincubated with immunogenic peptide (anti-α) and secondary antibody alone (anti-β). B3: mean data from analysis of anti-α (lean, n = 29; and ZDF, n = 25) and anti-β₁ (n = 25, both strains) immunolabeled artery section images. *P < 0.05, lean vs. ZDF. Scale bar = 20 μm. C: quantification of BKCa α subunit expression in lean and ZDF arteries by Western blot analysis. C1: demonstration of anti-α subunit antibody specificity by Western blot analysis of lysates of HEK cells expressing α and β₁ subunits. Molecular mass markers are indicated in kDa. C2: representative images of β-actin (loading control) and BKCa α Western blots of lean and ZDF artery samples. C3: mean data of β-actin and BKCa α expression in lean and ZDF artery samples.
Ca\(^{2+}\). Furthermore, this difference was still apparent in the presence of NS-1619, an agent that may activate BK\(_{\text{Ca}}\) through a mechanism that increases the Ca\(^{2+}\) sensitivity of the \(\alpha\) subunit (12). IBTX was included in the NS-1619 studies to confirm a BK\(_{\text{Ca}}\)-specific effect. Although these differences were observed at positive voltages not normally found in intact arteries, the Ca\(^{2+}\) concentration of the pipette solution (250 nM) was also 1 to 2 orders of magnitude lower than the intracellular Ca\(^{2+}\) levels (4–30 \(\mu\)M) believed to activate BK\(_{\text{Ca}}\) under physiological conditions (16). Given that an increase in Ca\(^{2+}\) concentration from 0.5 to 30 \(\mu\)M shifts the midpoint activation voltage of BK\(_{\text{Ca}}\) almost 160 mV in a hyperpolarizing direction (25), it is likely that a pipette solution containing micromolar Ca\(^{2+}\) concentrations would similarly shift the activation voltage into the typical in vivo range. Furthermore, myocyte hyperpolarizations evoked by NS-1619 in intact arteries were also decreased in arteries from diabetic ZDF animals, suggesting that reduced activation of BK\(_{\text{Ca}}\) by Ca\(^{2+}\) is also a feature of the intact artery.

The auxiliary \(\beta_1\) subunit of BK\(_{\text{Ca}}\) is known to enhance the Ca\(^{2+}\) sensitivity of the pore-forming \(\alpha\) subunit (25). Additionally, single-channel studies in human coronary artery smooth muscle have indicated that the Ca\(^{2+}\) sensitivity of individual channels is consistent with the presence of \(\beta_1\) subunits in the large majority of functional channels (23). Thus changes to the \(\beta_1\) subunit could underlie the observed reduction in Ca\(^{2+}\)-dependent activation of the BK\(_{\text{Ca}}\) in myocytes from diabetic ZDF animals. Such a mechanism has previously been proposed to explain the reduced contribution of BK\(_{\text{Ca}}\) to vascular tone in a study of angiotensin II-induced hypertension in the rat (1). In the present investigation, the expression of \(\alpha\) and \(\beta_1\) subunits was measured both at the mRNA level using quantitative, real-time RT-PCR and at the protein level using immunofluorescence and Western blot analysis. These studies indicated that the mRNA expression of both subunits was unchanged in arteries from diabetic ZDF rats compared with lean controls and that \(\beta_1\) subunit mRNA was expressed at an approximately 40-fold higher copy number than that of the \(\alpha\) subunit. Immunofluorescence studies of subunit protein expression were performed, allowing semiquantitative analysis of expression levels specifically within the smooth muscle of the artery. These studies indicated that, although \(\alpha\) subunit immunolabeling was slightly reduced in arteries from diabetic ZDF animals (~9%), immunolabeling for the \(\beta_1\) subunit was unchanged in the diabetic state. Western blot analysis experiments performed on total artery lysates confirmed that the expression of the \(\alpha\) subunit was not significantly different between groups, a result that supports our suggestion based on patch-clamp data that channel density is unchanged. Collectively, a reduction in the ratio of \(\beta_1\)-to-\(\alpha\) subunit expression within the smooth muscle was not apparent. Therefore, these data suggest that altered expression of \(\beta_1\) subunits is unlikely to be responsible for the changes in BK\(_{\text{Ca}}\) function observed in arteries of diabetic ZDF rats.

Despite the reduced BK\(_{\text{Ca}}\) function in diabetic animals, both the phenylephrine concentration-response curves of arteries (in the absence of IBTX) and the resting blood pressures were similar in diabetic and nondiabetic animals. If the reduced BK\(_{\text{Ca}}\) function observed in the present study were the only change present in arteries from diabetic ZDF animals, a leftward shift in the phenylephrine EC\(_{25}\) would have been expected, but this was not observed. It is thus possible that some other aspect of the vascular contractile response is compensating for the alteration in BK\(_{\text{Ca}}\) function. Alternatively, it is possible that the observed modification to BK\(_{\text{Ca}}\) function is a compensatory mechanism in response to some other effect on vascular function of the underlying diabetic state. Production of both vasodilator and vasoconstrictor prostaglandins is known to be deranged in diabetes (6), although the acute effects of prostaglandin synthase activity were inhibited with indomethacin in the current study. The fact that the KCl-induced tension increases in diabetic and control arteries were similar suggests that the basic relationship between intracellular Ca\(^{2+}\) levels and tension development was unaltered. Thus it seems unlikely that a general inhibition occurs between the coupling of intracellular [Ca\(^{2+}\)] with force generation and BK\(_{\text{Ca}}\) activation in the arteries from diabetic ZDF animals.

The present study has shown that the function and Ca\(^{2+}\)-dependent activation of BK\(_{\text{Ca}}\) channels in vascular myocytes is reduced in ZDF rats, a model of Type 2 diabetes. This effect does not seem to be associated with a reduced expression of the BK\(_{\text{Ca}}\) \(\beta_1\) subunit known to modulate the sensitivity of the channel to Ca\(^{2+}\). Beyond the scope of the present study was a determination of whether the observed change in BK\(_{\text{Ca}}\) was directly associated with the diabetic condition or was a secondary, protective mechanism. Of particular interest is the recent work by Tang et al. (24), who demonstrated that reactive oxidant species inhibit BK\(_{\text{Ca}}\) by reducing the Ca\(^{2+}\) sensitivity of the channel, as observed in the present study. The effect is apparently a direct one, involving modification to a cysteine residue near the proposed Ca\(^{2+}\)-sensing site. Because diabetes can be regarded as a condition associated with an increased level of reactive oxygen species (21), it is possible that such conditions are the cause of the observed change in BK\(_{\text{Ca}}\) function. Indeed, recent studies (10) have shown that IBTX-sensitive vasodilations evoked by iloprost are impaired in fructose-fed, insulin-resistant rats and can be restored by treatment with superoxide dismutase. Future studies are needed to determine whether such oxidant species react with BK\(_{\text{Ca}}\) and whether this is a critical feature of vascular pathologies prevalent in Type 2 diabetes.

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REFERENCES


