Impaired function of coronary BK$_{Ca}$ channels in metabolic syndrome

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1Department of Cellular and Integrative Physiology, Indiana University School of Medicine, Indianapolis, Indiana; 2Department of Exercise Physiology, Center for Cardiovascular and Respiratory Sciences, West Virginia University School of Medicine, Morgantown, West Virginia; 3Department of Biomedical Sciences, University of Missouri, Columbia, Missouri; and 4Department of Integrative Medical Sciences, Northeastern Ohio Universities College of Medicine, Rootstown, Ohio

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Borbouse L, Dick GM, Asano S, Bender SB, Dincer UD, Payne GA, Neeb ZP, Bratz IN, Sturek M, Tune JD. Impaired function of coronary BK$_{Ca}$ channels in metabolic syndrome. Am J Physiol Heart Circ Physiol 297: H1629–H1637, 2009. First published September 11, 2009; doi:10.1152/ajpheart.00466.2009.—The role of large-conductance Ca$^{2+}$-activated K$^{+}$ (BK$_{Ca}$) channels in regulation of coronary microvascular function is widely appreciated, but molecular and functional changes underlying the deleterious influence of metabolic syndrome (MetS) have not been determined. Male Ossabaw miniature swine consumed for 3–6 mo a normal diet (11% kcal from fat) or an excess-calorie atherogenic diet that induces MetS (45% kcal from fat, 2% cholesterol, 20% kcal from fructose). MetS significantly impaired coronary vasodilation to the BK$_{Ca}$ opener NS-1619 in vivo (30–100 $\mu$g) and reduced the contribution of these channels to adenosine-induced microvascular vasodilation in vitro (1–100 $\mu$M). MetS reduced whole cell penitrem A (1 $\mu$M)-sensitive K$^+$ current and NS-1619-activated (10 $\mu$M) current in isolated coronary vascular smooth muscle cells. MetS increased the concentration of free intracellular Ca$^{2+}$ and augmented coronary vasoconstriction to the L-type Ca$^{2+}$ channel agonist BAY K 8644 (10 $p$M–10 $n$M). BK$_{Ca}$ channel $\alpha$ and $\beta_1$ protein expression was increased in coronary arteries from MetS swine. Coronary vascular dysfunction in MetS is related to impaired BK$_{Ca}$ channel function and is accompanied by significant increases in L-type Ca$^{2+}$ channel-mediated coronary vasoconstriction.

Obesity is associated with cardiovascular and metabolic risk factors such as insulin resistance, impaired glucose tolerance, hypertension, and dyslipidemia, i.e., metabolic syndrome (MetS) (15). Because each component of MetS is an independent risk factor for cardiovascular disease, it is not surprising that MetS increases morbidity and mortality to many cardiovascular-related diseases, including stroke, coronary artery disease, cardiomyopathies, myocardial infarction, congestive heart failure, and sudden cardiac death (18). However, the mechanisms underlying MetS-induced cardiovascular disease remain poorly understood.

Evidence suggests that coronary microvascular dysfunction contributes to increased cardiovascular events associated with MetS (24, 42). Recent investigations provide evidence for impaired insulin-mediated capillary recruitment, obesity-related endocrine signaling, arterial stiffening, and diminished flow reserve in MetS (40, 42). These vascular defects may play an important role in the end-organ damage associated with MetS. Importantly, MetS is characterized by an imbalance between coronary blood flow and myocardial metabolism that is related to sensitization of angiotensin II, endothelin-1, and $\alpha$-adrenoceptor-mediated vasoconstriction (24). Alterations in these mechanisms could significantly inhibit large-conductance Ca$^{2+}$-activated K$^{+}$ (BK$_{Ca}$) channels (1, 37, 45). BK$_{Ca}$ channels are highly expressed in coronary vascular smooth muscle (5, 33, 43) and have been implicated in exercise- and ischemia-induced coronary vasodilation (5, 33, 38). In addition, BK$_{Ca}$ channel activity was recently shown to be impaired in coronary microvascular smooth muscle of alloxan-diabetic dyslipidemic swine (33), mesenteric microvessels of insulin-resistant rats (10), and coronary arterial myocytes of diabetic fatty rats (7, 28). However, the contribution of BK$_{Ca}$ channel defects to the control of coronary blood flow and vascular dysfunction in MetS has not been examined.

The goal of this investigation was to test the hypothesis that coronary dysfunction in MetS is related to impaired function of vascular smooth muscle BK$_{Ca}$ channels. Experiments were conducted in male Ossabaw miniature swine fed a normal diet or an excess-calorie atherogenic diet that induces many common features of MetS, including obesity, insulin resistance, impaired glucose tolerance, and dyslipidemia (44). Experiments involved in vivo studies of coronary blood flow in open-chest anesthetized swine and in vitro studies in isolated coronary arteries, arterioles, and smooth muscle cells.

**METHODS**

Swine model. An Institutional Animal Care and Use Committee approved our protocols, which were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (NIH Pub. No. 85-23, revised 1996). Male Ossabaw swine were randomized to treatment groups and remained on their respective diets for 3–6 mo from the age of 12 mo (no significant effect of diet duration was detected). Lean swine were fed standard chow (SL80, Purina; ∼2,200 kcal/day) containing (in % kcal) 18 protein, 71 complex carbohydrates, and 11 fat. MetS swine were fed ∼8,000 kcal/day with modified chow (5B4L, Purina) containing (in % kcal) 17 protein, 20 complex carbohydrates, 20 fructose, and 43 fat (lard and hydrogenated soybean and coconut oils). The MetS diet was supplemented with 2% cholesterol and 0.7% sodium cholate by weight.

**Coronary blood flow.** Lean (n = 6) and MetS (n = 5) Ossabaw swine were sedated with telazol (5 mg/kg sc) and xylazine (2.2 mg/kg sc). Anesthesia was maintained with morphine (3 mg/kg im) and α-chloralose (100 mg/kg iv). The left anterior descending coronary artery (LAD) was isolated, and a perivascular Transonic flow transducer (2.5 mm) was implanted. A 24-gauge angiocatheter was inserted into the LAD to infuse the BK$_{Ca}$ channel agonist NS-1619 (3- to 100-$\mu$g bolus) before and after inhibition of BK$_{Ca}$ channels with penitrem A (10 $\mu$g/kg iv, Biomol). The benzimidazolone NS-1619 significantly increases outward K$^+$ current that is fully abolished byiberiotoxin or penitrem A (17). Penitrem A has been shown to be as...
efficacious as iberiotoxin in selectively inhibiting BKCa channels (8, 35). Initial dose responses to NS-1619 were recorded for comparison of microvascular vasodilation in lean versus MetS swine. In lean animals, the NS-1619 dose-response protocol was repeated after administration of the BKCa channel antagonist penitrem A. Additional time control experiments (n = 2) demonstrated similar increases in coronary blood flow following repeated administration of NS-1619 (increase in coronary blood flow ~0.9 ml·min⁻¹·g⁻¹ to 100-μg bolus infusion) in lean swine (i.e., no tachyphylaxis). After in vivo experiments, hearts were fibrillated and excised into 4°C saline for dissection of tissues for in vitro experiments.

Isolated coronary arterioles. Subepicardial coronary arterioles (50- to 150-μm diameter) were isolated from the left ventricular apex, cannulated, and pressurized to 60 cmH2O as described previously (25). Intraluminal diameter was measured continuously with video-microscopes and recorded on a MacLab workstation. Arterioles that were free from leaks were allowed to equilibrate for ~1 h at 37°C with the bath solution changed every 15 min. Arterioles (lean n = 6; MetS n = 6) were preconstricted to 70% tone with endothelin-1 (2 nM), washed, and relaxed with the stable adenosine analog 2-chloroadenosine (2-CA; 0.1 nM–0.1 mM) before and after inhibition of BKCa channels with iberiotoxin (100 nM; 30-min incubation). There is a valid concern about experiments done on vessels with endothelin-induced tone, because endothelin may inhibit BKCa channels. This is unlikely to impact our experiments, because endothelin does not impair hyperpolarization mediated by BKCa channels in human coronary arterioles (31). The arterioles used contracted to vasoconstrictors and relaxed to endothelium-dependent and -independent agonists. Endothelin preconstriction is routinely used to study the dilation of human and pig coronary arterioles (26, 31), suggesting a close similarity in reactivity between human and porcine resistance vessels.

Isolated coronary arteries. Isometric tension studies were performed as previously described (6, 25). Left circumflex coronary arteries from lean (n = 4) and MetS (n = 3) swine were isolated, cleaned of surrounding tissue, and cut into 3-mm ring segments. Arterial rings were then mounted in organ baths and set to optimal length with contractions to 60 mM KCl. Arterial contractile responses to L-type Ca²⁺ channel activators were assessed by the addition of graded concentrations of BAY K 8644 (0.1 pM–10 nM). Maximal contractions were measured by adding U-46619 (1 μM) in the presence and absence of penitrem A (1 μM). L-type Ca²⁺ channel involvement in U-46619 contractions were examined by incubation with nicardipine (100 μM).

Patch-clamp electrophysiology. Coronary smooth muscle cells were isolated from proximal segments of the LAD as previously described (9). Patch-clamp recordings and immunocytochemistry/confocal studies (see below) were performed within 8 h of cell dispersion. Whole cell K⁺ currents were measured at room temperature with the conventional dialyzed configuration of the patch-clamp technique (9). Bath solution contained (in mM) 138 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 glucose, 10 HEPES, and 5 Tris (pH 7.4). Pipettes had tip resistances of 2–4 MΩ when filled with solution containing (in mM) 140 KCl, 3 Mg-ATP, 0.1 Na-GTP, 0.1 EGTA, 10 HEPES, and 5 Tris (pH 7.1). After whole cell access was established, series resistance and membrane capacitance were compensated. Current-voltage relationships were assessed by 400-ms step pulses from ~100 to ~10 mV in 10-mV increments from ~180 mV holding potential. Currents were measured in the absence and presence of the BKCa channel agonist NS-1619 (10 μM) as well as with and without the BKCa channel antagonist penitrem A (1 μM).

Fura-2 microfluorometry. Experiments to assess intracellular Ca²⁺ were performed at room temperature with equipment (Intracellular Imaging, Cincinnati, OH) and techniques described previously (6). Fura-2-loaded cells were placed in a superfusion chamber and observed with an epifluorescence microscopy system. Cells were superfused with physiological saline solution containing (mM) 2 CaCl₂, 143 NaCl, 1 MgCl₂, 5 KCl, 10 HEPES, and 10 glucose, pH 7.4. Excitation light from a 300-W xenon arc lamp was passed through alternating 360- and 380-nm band-pass filters. Fluorescence emission (510 nm) from user-specified regions of interest (selected smooth muscle cells) was synchronized with the appropriate excitation wavelength and reflected with a dichroic mirror to an integrating charge-coupled device (CCD) monochrome video camera. The microscope was equipped with a ×20 objective. Fluorescence images were acquired with InCa dual-wavelength Ca²⁺ imaging software (version 2.1). Background fluorescence was determined before the start of the experiment for online subtraction during data collection, and ratios were calculated on a pixel-by-pixel basis. Intracellular Ca²⁺ concentration was estimated after determining the fluorescence of fura-2 solutions with known Ca²⁺ concentrations (5, 43, 48).

ImmunobLOTS. Western blotting was performed as described previously (6). Arteries from lean (n = 3) and MetS (n = 3) swine were placed in liquid N₂ and stored at ~80°C. Arteries were homogenized, and total membrane protein was collected. Equivalent amounts of protein were loaded onto 7% (for α-subunit, ~125 kDa) and 12% (β-subunit, ~28 kDa) acrylamide gels. Membranes were blocked for 1 h at ambient temperature with 5% nonfat milk and incubated overnight at 4°C with polyclonal antibodies directed against BKCa α or β subunits [both 1:1,000 dilution: Affinity BioReagents PAI-923 (BKCa α) and PAI-924 (BKCa β)]. Blots were washed and incubated with donkey anti-rabbit IgG-horseradish peroxidase (HRP) secondary antibody (1:5,000 dilution; Santa Cruz Biotechnology) for 2 h at ambient temperature. Blots were stripped and reblotted with β-actin antisera (1:3,000; Santa Cruz Biotechnology; ~43 kDa). Immunoreactivity was visualized with an enhanced chemiluminescence detection kit and quantified by scanning densitometry.

Immunocytochemistry. Methods were similar to those described previously (22). Cells were fixed in 4% paraformaldehyde, washed, and permeabilized with 0.2% Triton X-100. Rinsed cells were incubated overnight at 4°C in 2.5% BSA and then incubated for 1 h at 37°C with polyclonal antibodies directed against BKCa α (1:100 in 2.5% BSA in PBS; Affinity BioReagents PAI-923)- and or β1 (1:100 in 2.5% BSA in PBS; Santa Cruz Biotechnology sc-14749)-subunits. Cells were incubated with IgG conjugated to Cy5 or FITC (1:200; Jackson ImmunoResearch) for 45 min. Control cells were prepared in the same manner but without primary antibody; nonspecific staining was not detected. Washed cells were incubated with Fluoromount-G (Southern Biotechnology). For detection of Cy5 or FITC fluorescence, excitation was 488 nm and emission 620–680 nm or 505–570 nm, respectively. Metamorph software was used for analysis. Whole cell immunoreactive fluorescence was calculated for 7–10 cells from each lean (n = 9) and MetS (n = 7) pig.

Heterologous expression of BKCa channels. HEK 293 cells were grown in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin. Plasmids encoding KCNMA1 and KCNMB1 were kindly provided by Dr. Michael J. Davis (University of Missouri). The green fluorescent protein (GFP) plasmid was purchased from Clontech Laboratories.

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<th>Table 1. Phenotypic characteristics of Ossabaw swine</th>
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<td>Body wt, kg</td>
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<td>Heart wt/body wt × 100</td>
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<td>Glucose, mg/dl</td>
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<td>Insulin, μU/ml</td>
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<td>Total cholesterol, mg/dl</td>
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<td>LDL-to-HDL ratio</td>
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Values are means ± SE for n animals. MetS, metabolic syndrome. *P < 0.05 vs. lean.
from AMAXA. Cells (50–70% confluence) in six-well dishes were cotransfected with GFP, KCNMA1, and KCNMB1 with Lipofectamine LTX with PLUS (Invitrogen). For Western blots, cells were harvested 1 day after transfection in RIPA buffer supplemented with 2 mM PMSF, 1 mM Na3VO4, 1 mM DTT, and protease inhibitor cocktail (Santa Cruz). Western blots were performed as described above. For immunostaining, cells were plated on 1% gelatin-coated glass in six-well dishes. One day after transfection, cells were washed with PBS and fixed in 4% formaldehyde in PBS, pH 7.4 for 30 min at room temperature. Cells were washed with PBS and permeabilized with 0.1% Triton X-100 for 2 min. After 1 h in blocking solution (2% goat serum, 3% BSA in PBS), cells were incubated with antibodies for 1 h at room temperature in a humidified chamber. After being washed with PBS, cells were incubated with Alexa Fluor 555 goat anti-rabbit IgG (1:1,000; Invitrogen) for 1 h at room temperature in a humidified environment. Cells were then washed with PBS and visualized with an epifluorescence microscope, and images were obtained with a SPOT RT camera (Diagnostic Instruments).

Statistics. Data are presented as means ± SE; n represents the number of animals. Statistical comparisons were made with unpaired and paired t-tests and one- or two-way repeated-measures analysis of variance (ANOVA; with Student-Newman-Keuls multiple comparison) as appropriate. In all tests, P < 0.05 was considered significant.

RESULTS

Phenotype of Ossabaw swine. Compared with their lean counterparts (Table 1), MetS swine exhibited increased body mass (33%), blood glucose (75%), insulin (210%), total cholesterol (483%), LDL-to-HDL ratio (313%), and triglycerides (105%). Aside from triglyceride levels that are significantly lower in swine than in humans, disease values for MetS swine are consistent with those observed in human patients.

MetS and coronary BKCa channel function in vivo and in vitro. NS-1619, a BKCa channel agonist, dose-dependently increased coronary blood flow in lean swine (Fig. 1). However, the NS-1619-induced increase in coronary flow was markedly attenuated in MetS swine (P < 0.01). Ethanol, the vehicle for NS-1619, had no effect on coronary blood flow (data not shown). The decrease of NS-1619-mediated dilation in MetS swine was similar to that observed in lean swine after blockade of BKCa channels with penitrem A (Fig. 1). Additionally, penitrem A did not alter basal blood flow, suggesting that BKCa channels do not contribute to baseline blood flow. Coronary arteriolar vasodilation in vitro to 2-CADO (0.1 mM–0.1 mM), a stable adenosine analog, was similar between lean and MetS swine (Fig. 2). However, iberiotoxin (100 nM) diminished relaxation to 2-CADO in arterioles from lean swine (1 μM–100 μM; P < 0.01) but had no effect on 2-CADO-induced relaxation in arterioles from MetS swine (Fig. 2). Importantly, adenosine-induced dilation is similar in lean and MetS swine, whether measured in vitro (Fig. 2) or in vivo (2). Furthermore, sodium nitroprusside-induced dilation of coronary arterioles from lean and MetS pigs is not different (2). Thus a general impairment in coronary vasodilation is not responsible for the results shown here. We observed no alterations in baseline tone of isolated coronary arterioles in response to iberiotoxin in lean or MetS swine (Table 2). Iberiotoxin was used for the in vitro experiments because it is the accepted “gold-standard” inhibitor of BKCa channels. Penitrem A is a much less expensive inhibitor of BKCa channels and was used for the in vivo experiments. Penitrem A is as effective as iberiotoxin or charybdotoxin in selectively inhibiting BKCa channels (8, 13, 23).
Whole cell K⁺ current was attenuated ~20% in MetS swine at potentials greater than +50 mV, i.e., currents biophysically consistent with BKCa channels (Fig. 3; P < 0.05). Current elicited by NS-1619 was depressed in smooth muscle cells from MetS swine (Fig. 3A; P < 0.001). The difference between NS-1619-activated current and the current in the presence of penitrem A (i.e., total BKCa current) was 89 ± 2% greater in lean swine (Fig. 3A; P < 0.001). Fura-2 studies demonstrated a significant increase in resting intracellular Ca²⁺ concentration of smooth muscle cells in MetS (144 ± 19 nM) vs. lean (89 ± 5 nM) swine. Vasorelaxation to the L-type Ca²⁺ channel agonist BAY K 8644 was significantly elevated (1 pM–10 nM; P < 0.01) in isolated coronary conduit arteries from MetS relative to lean swine (Fig. 3B). Although baseline tensions were not different, addition of penitrem A (1 μM) significantly increased active tension development to U-46619 (1 μM) in lean swine, whereas no effect was observed in MetS swine (Fig. 3C). These U-46619 contractions were completely abolished by nicardipine (100 μM), indicating a predominant role of L-type Ca²⁺ channels.

**Coronary BKCa protein expression.** Antibody specificity for BKCa α- and β₁-subunits was assessed in HEK 293 cells transfected with cloned BKCa channel subunits (Fig. 4). Western blot showed that the 125-kDa band representing the BKCa α-subunit was detected only in cells expressing KCNMA1, while no band was detected at 125 kDa in cells transfected with the empty vector (Fig. 4A). The 28-kDa band representing the BKCa β₁-subunit was detected only in cells expressing KCNMB1 and not in cells transfected with the empty vector or KCNMA1 alone (Fig. 4B). Additional immunocytochemistry experiments demonstrated that BKCa channel α- and β₁-subunit immunoreactivity (red) was detected only in cells transfected with KCNMA1 and KCNMB1 (Fig. 4, C and D). Nonspecific binding was not detected in cells transfected with the empty GFP vector.

The data thus far indicate diminished function of BKCa channels in MetS in vivo and in vitro (Figs. 1–3), but Western blots revealed a dramatic increase in the expression of coronary BKCa channels in arteries of MetS swine (Fig. 5). Expression of the pore-forming α- and regulatory β₁-subunits was increased 57 ± 13% and 74 ± 15% (P < 0.05), respectively. Immunocytochemistry studies also showed significant increases in immunoreactivity for both BKCa channel α- and β₁-subunits in smooth muscle cells from MetS pigs (Fig. 6). To more quantitatively address the number of BKCa subunits in the

### Table 2. Microvessel diameters

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<th>Passive diameter, μm</th>
<th>Baseline diameter, μm</th>
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<tr>
<td>Lean</td>
<td>80.3 ± 8.4</td>
<td>25.3 ± 5.8</td>
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<tr>
<td>Lean + iberiotoxin</td>
<td>39.2 ± 13.0</td>
<td>35.7 ± 9.7</td>
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<tr>
<td>MetS</td>
<td>108.1 ± 11.5</td>
<td>32.6 ± 10.1</td>
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<tr>
<td>MetS + iberiotoxin</td>
<td>8.4 ± 25.3</td>
<td>13.0 ± 5.8</td>
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Values are means ± SE; n = 6 for lean and 5 for MetS.

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**Fig. 3.** A: reduced BKCa current in coronary artery smooth muscle cells from MetS (n = 7) relative to lean (n = 6) swine. Current was attenuated by penitrem A (1 μM), and the difference between groups was abolished. Activation of BKCa current with NS-1619 (1 μM) was diminished in MetS swine. B: contraction to L-type Ca²⁺ channel agonist BAY K 8644 was increased in coronary arteries from MetS (n = 4) vs. lean (n = 4) swine. *P < 0.01 vs. lean control at same dose. C: penitrem A increased maximal contractile response to U-46619 (1 μM) in lean swine (P < 0.01) but had no effect in MetS swine (P = 0.49). Baseline tensions were not different between lean and MetS swine (P = 0.79). n = 7 pigs for both groups.
sarcolemma, we attempted Western blots on plasma membrane fractions. Unfortunately, our membrane fractionations were unsuccessful and our experimental material was depleted. Thus additional studies will be required to determine whether MetS alters the number of BKCa channels in the sarcolemma. Because multiple immunoreactive bands were detected in the Western blots, we cannot state unequivocally that only BKCa channel proteins were stained in the immunocytochemistry experiments on isolated smooth muscle cells. While our immunocytochemistry experiments in HEK 293 cells demonstrate that the antibodies used are capable of selectively detecting their intended protein targets, multiple immunoreactive bands in coronary artery samples suggest that the antibodies may react with additional proteins.

**DISCUSSION**

We examined the molecular and functional expression of BKCa channels and the mechanisms underlying the deleterious influence of MetS. The primary findings are that 1) MetS significantly impairs BKCa channel-mediated coronary vasodilation in vivo and the contribution of BKCa channels to arteriolar vasodilation in vitro; 2) decreased BKCa channel dilation is not the result of generalized impairment of coronary reactivity because arteriolar relaxation to the adenosine analog 2-CADO is similar in lean and MetS swine; 3) BKCa current in smooth muscle is diminished by MetS; 4) impaired coronary BKCa current in MetS is associated with augmented L-type Ca\(^{2+}\) channel-mediated vasoconstriction; and 5) coronary expression of BKCa channel α- and β-subunits is increased in MetS. These data support the hypothesis that coronary vascular dysfunction in MetS (24) is related to diminished BKCa channel function. The nature of the BKCa channel impairment is not known, but possibilities include 1) reduced Ca\(^{2+}\)/voltage sensitivity, 2) reduced number of channels in the sarcolemma, 3) altered regulation of BKCa channels (e.g., by kinases and phosphates), or 4) a combination of these changes.

BKCa channels are abundantly expressed in coronary smooth muscle cells (5, 33), and we demonstrate that these channels play a functional role in mediating endothelium-independent coronary vasodilation to the adenosine analog 2-CADO (Fig. 2). Although endothelin has been shown to inhibit BKCa channels, preconstriction of isolated coronary arterioles with endothelin is unlikely to impact our findings, because endothelin has no effect on hyperpolarization mediated by BKCa channels in human coronary arterioles (31). In addition, we show that iberiotoxin impairs adenosine-induced dilation in arterioles from lean, but not MetS, swine, which argues against complete inhibition of BKCa channels by endothelin preconstriction. Our findings are consistent with earlier studies documenting that KCa channels contribute to endothelium-dependent (19, 31, 32, 38), metabolic (30), and ischemic (38) vasodilation. Accordingly, we propose that reduced BKCa channel function could be detrimental to the regulation of coronary microvascular...
increased vascular BK$_{Ca}$ channel current in insulin-resistant (10) and diabetic fatty (7, 28) rats. The lack of an effect of iberiotoxin on resting arteriolar tone suggests that the role of BK$_{Ca}$ channels around the resting potential in coronary arteries may not be as prominent as in other vessels (e.g., cerebral circulation). It has been previously demonstrated in rabbit (27) and human (16) coronary smooth muscle that voltage-dependent K$^+$ (K$_V$) channels play the major role in regulating resting membrane potential at physiological levels of intracellular Ca$^{2+}$. Although the contribution of BK$_{Ca}$ channels is minor, elevations of intracellular Ca$^{2+}$ enhance their role. Current magnitudes and activation thresholds documented in the present study agree completely with previous results from our lab (41) and others (46, 47). NS-1619 must indeed activate BK$_{Ca}$ channels near the resting membrane potential, because NS-1619 causes membrane hyperpolarization in pig coronary arteries (12) and elicits penitrem A-sensitive coronary vasodilation (Fig. 1). Importantly, however, patch-clamp studies of NS-1619 in smooth muscle have not shown a significant increase in current in rat cerebral arteries (20) or in rat portal vein or bovine trachea (14) at physiological membrane potentials. An analysis of the data in Fig. 3A indicates that NS-1619 increases whole cell current in lean pigs by 91% at −30 mV. However, current at this potential is very small (∼0.1 pA/pF or around 3 pA of whole cell current). In contrast to lean pigs, NS-1619 did not change current at −30 mV in MetS pigs. The effect of NS-1619 in lean pigs did not reach statistical significance at −30 mV; however, it may be physiologically relevant because of the high input resistance of smooth muscle membranes (calculated from data in Fig. 3 to be 4 GΩ).

Decreases in total K$^+$ current and spontaneous transient outward K$^+$ currents, which are elicited by Ca$^{2+}$ sparks and indicative of BK$_{Ca}$ channel activation (39, 53), have been observed in dyslipidemic (51) and diabetic-dyslipidemic Yucatan swine (33). Importantly, we demonstrate that decreased coronary smooth muscle BK$_{Ca}$ channel function is phenotypically evident in vivo as a reduction in NS-1619-induced vasodilation in MetS swine (Fig. 1). Importantly, however, this reduction is not a generalized impairment in dilation because we observed similar dilation to 2-CADO in lean and MetS swine. These data are consistent with our recent study (2) that documented no significant differences in coronary vasodilation to adenosine (in vitro and in vivo) or sodium nitroprusside (in vitro). Additionally, we found that nicardipine-sensitive contractions to U-46619 were enhanced in the presence of penitrem A in coronary arteries from lean but not MetS swine (Fig. 3C). This finding, along with the augmented coronary vasoconstriction to

Our findings indicate that coronary smooth muscle BK$_{Ca}$ channel function is markedly depressed by MetS. Diminished BK$_{Ca}$ channel function is evidenced by the impaired contribution of BK$_{Ca}$ channels to 2-CADO-mediated coronary arteriolar dilation in coronary arterioles from MetS swine (Fig. 2) and the reduction in BK$_{Ca}$ channel current in isolated coronary vascular smooth muscle cells from MetS swine (Fig. 3). These impairments are consistent with studies demonstrating decreased BK$_{Ca}$ channel function under a variety of physiological/pathophysiological conditions.

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Decreases in total K$^+$ current and spontaneous transient outward K$^+$ currents, which are elicited by Ca$^{2+}$ sparks and indicative of BK$_{Ca}$ channel activation (39, 53), have been observed in dyslipidemic (51) and diabetic-dyslipidemic Yucatan swine (33). Importantly, we demonstrate that decreased coronary smooth muscle BK$_{Ca}$ channel function is phenotypically evident in vivo as a reduction in NS-1619-induced vasodilation in MetS swine (Fig. 1). Importantly, however, this reduction is not a generalized impairment in dilation because we observed similar dilation to 2-CADO in lean and MetS swine. These data are consistent with our recent study (2) that documented no significant differences in coronary vasodilation to adenosine (in vitro and in vivo) or sodium nitroprusside (in vitro). Additionally, we found that nicardipine-sensitive contractions to U-46619 were enhanced in the presence of penitrem A in coronary arteries from lean but not MetS swine (Fig. 3C). This finding, along with the augmented coronary vasoconstriction to

Fig. 5. A–C: Western blot analysis of BK$_{Ca}$ channel α-subunit (A) and β1-subunit (B) in lean and MetS swine. Subunit expression in every sample (each from a different pig) was normalized to β-actin (C) to control for protein loading (average % lean control = 100 ± 1 vs. 102 ± 1 MetS). D: group data (n = 3 different pigs in each group) are expressed as % BK$_{Ca}$ subunit protein observed in lean pigs. MetS increased the expression of both BK$_{Ca}$ channel α (top band in the doublet at 125 kDa) and β1 (28 kDa) subunits. *P < 0.05 vs. lean.

Fig. 6. A: representative immunocytochemistry images of BK$_{Ca}$ channel α- and β1-subunits in coronary smooth muscle cells of lean and MetS pigs. Left: α-subunit reactivity in red. Right: β1-subunit reactivity in green in the same double-labeled cells. B: immunoreactivity for both BK$_{Ca}$ channel α- and β1-subunits was increased in smooth muscle cells from MetS pigs. *P < 0.001 vs. lean. Whole cell immunoreactive fluorescence was calculated for 7–10 cells from each lean (n = 9) and MetS (n = 7) pig.
L-type Ca\(^{2+}\) channel activation (BAY K 8644) in MetS swine (Fig. 3B), supports our previous data documenting increased functional expression of L-type Ca\(^{2+}\) channels in dogs with MetS (24). These changes in coronary electromechanical coupling were accompanied by significant increases in intracellular Ca\(^{2+}\) concentration in coronary smooth muscle cells. We do not yet know the mechanism by which intracellular Ca\(^{2+}\) is increased, but it may be related to impairments in Ca\(^{2+}\) removal (48) or increases in Ca\(^{2+}\) entry. We recently showed (21) that the molecular and functional expression of Ca\(^{2+}\)-permeant transient receptor potential (TRP) channels is increased in adrenal chromaffin cells of MetS pigs. Similarly, preliminary data from our group also show that the function of TRP channels is increased in MetS smooth muscle (36). Increased functional expression of L-type Ca\(^{2+}\) channels in coronary conduit smooth muscle in MetS Ossabaw swine is opposite of that in hyperlipidemia (4) and the combination of gross hyperglycemia and hyperlipidemia (diabetic dyslipidemia) (48). Collectively, these data strongly suggest that the entire MetS milieu is critical in determining ion channel expression, and results cannot be overgeneralized from one disease state to another. Furthermore, sex may have additional effects on adaptations of coronary arterial smooth muscle to metabolic disease (51). We acknowledge that the use of conduit coronary arteries for some in vitro studies is a limitation. However, this concern is tempered by data demonstrating that the functional microvascular response to the BK\(_{Ca}\) channel agonist NS-1619 (Fig. 1) as well as the diminished contribution of BK\(_{Ca}\) channels to 2-CADO-mediated arteriolar dilation (Fig. 2) are entirely consistent with the patch clamp, Western blot, and immunocytochemistry data from conduit artery smooth muscle cells (Figs. 3–5).

Mechanisms underlying impaired function of vascular BK\(_{Ca}\) channels in MetS could possibly be related to modifications of membrane trafficking (29, 37) and/or biophysical properties (52). In addressing these potential mechanisms we found a paradoxical increase in the expression of both the \(\alpha\)- and \(\beta_1\)-subunits in coronary arteries from MetS swine (Figs. 5 and 6). Antibody specificity was confirmed on cloned BK\(_{Ca}\) channels expressed in HEK 293 cells because the 125-kDa band appeared only in cells transfected with a plasmid encoding BK\(_{Ca}\) \(\alpha\)-subunit (Fig. 4A) and the \(\beta_1\) immunoreactivity (28 kDa) appeared only in cells transfected with a plasmid encoding the \(\beta_1\)-subunit (Fig. 4B). Nonspecific binding was not observed in the immunocytochemistry experiments with either \(\alpha\)- or \(\beta_1\)-subunit antibodies (Fig. 4, C and D), suggesting that nonspecific epitopes in the Western blots are recognized only under reducing conditions. While our data are consistent with other investigations that have reported diminished current and channel activity in the presence of cardiovascular risk factors (7, 10, 28, 33, 37), they are at odds with other studies showing downregulation of one or both BK\(_{Ca}\) channel subunits (11, 29). We reiterate that the metabolic milieu of the disease state can modulate channels in multiple ways. It is important to recognize that ours is not the first example of a situation in which BK\(_{Ca}\) channel function and protein expression are inversely related. For example, Benkusky et al. (3) found that functional and molecular expression of BK\(_{Ca}\) channels in myometrial smooth muscle is regulated by gestation such that BK\(_{Ca}\) current decreases while BK\(_{Ca}\) protein increases. Additionally, we previously observed (34) increased BK\(_{Ca}\) current in hyperlipidemic and diabetic dyslipidemic pigs without a change in BK\(_{Ca}\) protein expression. We hypothesize that the increase in BK\(_{Ca}\) channel expression represents compensation in response to diminished function of BK\(_{Ca}\) channels in MetS (Fig. 5). However, we acknowledge that additional studies are needed to more quantitatively address the number of BK\(_{Ca}\) subunits in the sarcolemma. It might be possible that membrane expression of BK\(_{Ca}\) channels could be influenced by alternative mRNA splicing (50) and/or the formation of channels with accessory subunits that inhibit membrane expression (49). These ideas merit further study. Furthermore, alterations in BK\(_{Ca}\) channel open probability, Ca\(^{2+}\)/voltage sensitivity, and unitary conductance in coronary artery smooth muscle cells of MetS swine should also be examined in the future.

In summary, findings from this investigation support the hypothesis that coronary vascular dysfunction in MetS is related to decreased function of BK\(_{Ca}\) channels in vascular smooth muscle cells. Diminished BK\(_{Ca}\) channel function is accompanied by increased L-type Ca\(^{2+}\) channel-mediated coronary vasoconstriction (24). We assert that this impairment of coronary BK\(_{Ca}\) channels could contribute to coronary endothelial dysfunction (31, 32), sensitization of key vasoconstrictor pathways, and diminished functional hyperemia typically observed in obese subjects with MetS (24).

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