Impaired capsaicin-induced relaxation of coronary arteries in a porcine model of the metabolic syndrome

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Bratz IN, Dick GM, Tune JD, Edwards JM, Neeb ZP, Dincer UD, Sturek M. Impaired capsaicin-induced relaxation of coronary arteries in a porcine model of the metabolic syndrome. Am J Physiol Heart Circ Physiol 294: H2489–H2496, 2008. First published April 4, 2008; doi:10.1152/ajpheart.01191.2007.—Recent studies implicate channels of the transient receptor potential vanilloid family (e.g., TRPV1) in regulating vascular tone; however, little is known about these channels in the coronary circulation. Furthermore, it is unclear whether metabolic syndrome alters the function and/or expression of TRPV1. We tested the hypothesis that TRPV1 mediates coronary vasodilation through endothelium-dependent mechanisms that are impaired by the metabolic syndrome. Studies were conducted on coronary arteries from lean and obese male Ossabaw miniature swine. In lean pigs, capsaicin, a TRPV1 agonist, relaxed arteries in a dose-dependent manner (EC50 = 116 ± 41 nM). Capsaicin-induced relaxation was blocked by the TRPV1 antagonist capsazepine, endothelial denudation, inhibition of nitric oxide synthase, and K+ channel antagonists. Capsaicin-induced relaxation was impaired in rings from pigs with metabolic syndrome (91 ± 4% vs. 51 ± 10% relaxation at 100 μM). TRPV1 immunoreactivity was prominent in coronary endothelial cells. TRPV1 protein expression was decreased 40 ± 11% in obese pigs. Capsaicin (100 μM) elicited divalent cation influx that was abolished in endothelial cells from obese pigs. These data indicate that TRPV1 channels are functionally expressed in the coronary circulation and mediate endothelium-dependent vasodilation through TRPV1-mediated vasodilation in the metabolic syndrome is associated with decreased expression of TRPV1 and cation influx. Ossabaw miniature swine; transient receptor potential vanilloid 1; potassium channels; nitric oxide; endothelial cells

THE METABOLIC SYNDROME COMBINES multiple cardiovascular risk factors including hypertension, insulin resistance, and obesity and is associated with a higher incidence of cardiovascular diseases, including peripheral vascular and coronary artery disease (18–20, 26) and Type 2 diabetes (12, 31). Mechanisms underlying the elevated incidence of cardiovascular disease in patients with the metabolic syndrome are unresolved, but arterial dysfunction has been suggested as a contributing factor (24, 40). For instance, vascular disorders associated with the metabolic syndrome involve alterations in local blood flow supply (21), endothelial dysfunction (14, 54), vascular remodeling of arterial diameter (22), reduced nitric oxide (NO) concentration (3, 14), and increased vascular oxidative stress (14, 54).

A potential mechanism of coronary vascular dysfunction in metabolic syndrome is transient receptor potential (TRP) channels, cation-permeable channels receiving their name from the Drosophila mutant. Most TRP channels function as Ca2+-entry pathways, thus contributing to numerous Ca2+-dependent functions (34), but many of their vascular physiological functions remain unclear. Recent studies have revealed TRP channel expression in vascular smooth muscle and endothelium and suggested they may regulate vascular tone (23, 34, 47, 49). The TRP vanilloid (TRPV) channels represent one of the known subfamilies and are characteristically gated by chemical and physical stimuli including acid, heat, ethanol, and vanilloid compounds, such as capsaicin, a component from red chili peppers.

Several studies have implicated various TRPV channels in the regulation of vascular tone. For example, Yang et al. (51) demonstrated that TRPV1, TRPV3, and TRPV4 mRNA expression in both pulmonary and aortic smooth muscle cells. In addition, Earley et al. (10) reported that TRPV4 channels form a novel Ca2+-signaling complex with ryanodine receptors and large-conductance K+ channels that mediate vasodilation in cerebral artery smooth muscle cells. In contrast, Lizanecz et al. (29) demonstrated that capsaicin constricted rat skeletal gracilis resistance arteries. Thus, depending on the type and region of circulation, TRPV channels may exert a positive or negative regulation on blood flow and pressure.

The ability of TRP channels to function as Ca2+-entry pathways has led to the emergence of their connection to vascular diseases. TRP channel dysfunction could prove to be a mechanism underlying altered vascular reactivity and thus be involved in the development of cardiovascular diseases. Liu et al. (27), Wang and Wang (45), and Zhang et al. (55) reported that TRPV1 activation prevents adipogenesis and obesity. Despite the expression of TRPV1 channels in many different cell types and their role in vascular health and disease, their function in regulation of coronary vascular tone in normal or obese subjects has not been previously examined.

Accordingly, this study was designed to test the hypothesis that TRPV1 channels mediate coronary vasodilation through endothelium-dependent mechanisms and that this pathway is disrupted in the metabolic syndrome. This hypothesis was tested in lean and obese Ossabaw swine. The effects of selective endothelial and smooth muscle pathways on TRPV1-mediated vasodilation were also studied. Our data provide direct evidence that TRPV1 channels function as Ca2+-entry pathways and indicate the possible mechanisms, which may have important clinical implications.
METHODS

Ossabaw miniature swine tissue collection. Protocols were approved by an Institutional Animal Care and Use Committee and complied with the Guide for the Care and Use of Laboratory Animals (National Academy Press, Washington, DC, 1996). Male Ossabaw swine were used for the study. Control swine were fed standard chow for 24 wk, which contained 22% kcal from protein, 70% kcal from carbohydrates, and 8% kcal from fat (Purina Test Diet, Richmond, IN). Obese swine were fed a high-fat/high-cholesterol diet for 24 wk, which was composed of standard chow supplemented with 2.0% cholesterol, 46% kcal from fat, and 20% kcal from fructose. Control group pigs ate ~725 g/day, whereas obese group pigs ate ~800–1,200 g/day (See Table 1 for metabolic data). Animals were housed in individual pens and provided a 12-h:12-h light-dark cycle. On the day of tissue collection, preanesthesia consisted of (in mg/kg im) 0.05 atropine, 2.2 xylazine, and 5.5 telazol. A surgical plane of anesthesia was maintained with isoflurane (1–4%) supplemented with oxygen. A sternotomy was performed, and the heart was excised into cold saline. The right coronary artery was harvested. For molecular and biochemical experiments, artery segments were frozen in liquid N2 and stored at −80°C. For immunohistochemistry, artery segments were fixed in Zn-formalin. Arteries isolated for contractile studies were placed in cold physiological saline solution (PSS) containing (in mM) 138 NaCl, 5 KCl, 10 HEPES, 1 MgCl2, 10 glucose, and 2 CaCl2 at pH 7.4.

Intravenous glucose tolerance test. Swine were acclimatized to a restraint in a specialized sling for 5–7 days before the intravenous glucose tolerance test (IVGTT) was conducted. Swine were then fasted overnight and anesthetized with isoflurane (maintained at 2% by mask with supplemental O2). The right jugular vein was catheterized percutaneously. Following catheterization, the swine were allowed to recover for 3 h before the IVGTT to avoid any effect of isoflurane on insulin signaling (35). For IVGTT, conscious swine were restrained by sling, and baseline blood samples were obtained. Glucose (1 g/kg body wt iv) was administered, and timed blood samples were collected (35). Blood glucose was measured using a YSI 2300 STAT Plus Glucose analyzer. Plasma insulin assays were performed by Linco Research (St. Charles, MO).

Plasma lipid assays. Venous blood samples were obtained following overnight fasting. Fasting samples were analyzed for triglyceride and total cholesterol [fractionated into high-density lipoprotein (HDL) and low-density lipoprotein (LDL) components]. Cholesterol in lipoprotein fractions was determined after the precipitation of HDL using minor modifications of standard methods (9). Specifically, apolipoprotein-B-containing lipoproteins were precipitated with heparin-MnCl2, and the supernatant was assayed. LDL was calculated from the Friedewald equation: LDL = total cholesterol – HDL – (triglyceride × 5).

Isometric tension studies. Right coronary arteries were cleaned of connective tissue and surrounding fat and cut into 3-mm segments. In some experiments, endothelial cells were removed by gently rubbing the lumen with fine-tipped forceps. Tissue rings were suspended in a bath containing warmed PSS containing (in mM) 131.5 NaCl, 5 KCl, 1.2 NaH2PO4, 1.2 MgCl2, 25 NaHCO3, 10.1 glucose, and 2.5 CaCl2 at pH 7.4, maintained at 37°C and bubbled with 95% O2:5% CO2 using metal hooks attached to force transducers. The rings were stretched with 4 g of passive tension and equilibrated for 60 min. After equilibration, the functional integrity of the arterial rings was confirmed by contraction to KCl (60 mM). Endothelial integrity was assessed by relaxation to bradykinin (1 µM) in rings contracted with the thromboxane mimetic U-46619 (1 µM). Segments relaxing >80% were used as endothelium intact, whereas those with relaxing <5% were used as endothelium denuded. Tissues were washed to remove KCl and bradykinin, exposed to U-46619 for 1 h, and followed by bradykinin. These segments were then subjected to increasing concentrations of the TRPV1 agonist capsaicin. Capsaicin-induced relaxations were evaluated in endothelium-intact and -denuded rings from each animal. In addition, capsaicin concentration–response conducted were in the presence of the NO synthase inhibitor Nω-nitro-L-arginine methyl ester (L-NAME, 300 µM, n = 7 rings and 7 pigs), the cyclooxygenase (COX) inhibitor indomethacin (1 µM, n = 5 rings and 5 pigs), the TRPV1 channel antagonists capsazepine (30 µM, n = 7 rings and 7 pigs), the K+ channel inhibitor tetraethylammonium (TEA, 10 mM, n = 5 rings and 5 pigs), and the Ca2+-dependent K+ channel inhibitor ibetoxin (IBTX, 100 nM, n = 5 rings and 5 pigs). Rings were subjected to only one capsaicin concentration-response curve to avoid the effects of tachyphylaxis. Inhibitors were preincubated with rings 5–10 min before the addition of U-46619.

Inmunohistochemistry. The fixed artery segments were embedded in paraffin and cross-sectioned. The samples were washed in 0.02 M PBS (pH 7.4), permeabilized with 0.5% Triton X-100 (Sigma) for 1 h, and immunoblocked in 10% goat serum and 1% bovine albumin for 1 h. The tissues were incubated for 1 h at 25°C with rabbit monoclonal anti-TRPV1 (Alomone). After being washed with PBS, the sections were covered for 3 h with biotinylated secondary goat anti-rabbit IgG and then reacted with an avidin-biotin complex (Vectastain Elite; Vector). Signal detection was performed using 3,3′-diaminobenzidine.

Isolation and quantification of total RNA. Right coronary arteries from control pigs were isolated, placed in liquid N2, and stored at −80°C. Total RNA was extracted according to manufacturer’s instructions [SV Total RNA Isolation System (Promega)]. The total RNA quantity and quality were determined using the Experion semi-automated electrophoresis system (Bio-Rad).

Preparation of first-strand cDNA via reverse transcriptase reactions. RNA samples were used as templates for synthesis of first-strand cDNAs as described previously (6, 7). Briefly, 1 µl of oligo(dT)15 primer (Promega) was added to equivalent amounts of total RNA obtained from coronary arteries. The mixtures were then placed into a thermocycler (My Cycler, Bio-Rad) and held at 70°C for 5 min. The samples were then transferred into an ice bath for 3 min. For the poly(A) tail of the mRNA utilized, the oligo(dT)15 primer (Promega) was added to equivalent amounts of total RNA samples were used as templates for synthesis of the first-strand cDNA reaction. The samples were then subjected to a 15-min incubation at 42°C.

Amplification detection of cDNA. The first-strand cDNA was synthesized with an InvitroScript reverse transcriptase kit (Promega). Primers were designed using the Genomatix panel based on published sequences for different species in the GenBank. Different TRPV1 gene-specific primers were designed for semiquantitative [sense 5′-AGG TAC TTT GAC TGA CTT GA-3′ and antisense 5′-CAG GAG TCC AGC TCA CCT TC-3′ (986 bp)] and real-time

Table 1. Phenotypic characteristics of lean and obese Ossabaw swine

<table>
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<tr>
<th>Phenotypic characteristic</th>
<th>Lean</th>
<th>Obese</th>
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<tr>
<td>Start body wt, kg</td>
<td>42±2</td>
<td>48±2</td>
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<tr>
<td>End body wt, kg</td>
<td>60±3</td>
<td>81±5*</td>
</tr>
<tr>
<td>Heart wt, g</td>
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<td>Heart w/body wt × 100</td>
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<td>0.44±0.05*</td>
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<td>Heart rate, beats/min</td>
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<td>95±5*</td>
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<tr>
<td>Fasting glucose, mg/dl</td>
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<td>101±6*</td>
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<tr>
<td>Fasting insulin, µU/ml</td>
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<td>13.4±1.8</td>
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<tr>
<td>Total cholesterol, mg/dl</td>
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<tr>
<td>LDL, mg/dl</td>
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<td>HDL, mg/dl</td>
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<td>HDL-to-LDL ratio</td>
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<tr>
<td>Triglycerides, mg/dl</td>
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<td>Diastolic blood pressure, mmHg</td>
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<tr>
<td>Mean arterial pressure, mmHg</td>
<td>95±7</td>
<td>104±3</td>
</tr>
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Values are means ± SE. Pigs fed a diet high in fat and cholesterol were obese and demonstrated symptoms associated with the metabolic syndrome.

*p < 0.05 by unpaired t-test.

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PCR [sense 5'-GCG TGG AGC TGT CCA AGT TC-3' and antisense 5'-TCT CCT GTG CGA TCT TG-3' (177 bp)] reactions. Amplified cDNA sequences were sequenced, and partial cDNA sequences were submitted for sus scrofa TRPV1 to the GenBank data base (accession number, EF100781). β-Actin primers were also designed based on published sequences in the GenBank database (sense 5'-AGG TGG ACA TCA GGA AGG AC-3' and antisense 5'-ACA TCT GCT GGA TGG AC-3'; accession number, U-07786).

Real-time PCR reactions were performed for TRPV1 and β-actin in triplicate with SYBR Green (Molecular Probes) and fluorescein calibration dye (Bio-Rad) in 50 μl of total reaction using Taq DNA polymerase (Promega) (7). The amplification was carried out with iCycler iQ multicolor real-time PCR detection system (Bio-Rad). β-Actin was amplified in each reactions to serve as an internal reference during quantitation to correct for operator and/or experimental variations. The mean threshold cycle (Ct) values for both the target (TRPV1) and internal control (β-actin) genes were determined in each sample.

Western immunoblot analysis of TRPV1 proteins. Isolated endothelial and vascular smooth muscle cells were lysed, and arteries were homogenized in buffer containing 10 mM Tris-HCl (pH 7.6) and 0.5 mM MgCl2 with protease inhibitors (10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, and 1.8 mg/ml iodoacetamide). Homogenates were centrifuged at 20,000 g for 20 min at 4°C. Membrane pellets were resuspended in lysis buffer containing 300 mM NaCl and 50 mM Tris-HCl (pH 7.6) and 0.5% Triton X-100 with protease inhibitors, incubated on ice, and spun down at 20,000 g for 20 min at 4°C. Supernatant was collected and used for analysis. Protein quantity and quality were determined (Experion). Equivalent amounts of protein were separated by gel electrophoresis (10 – 20% polyacrylamide gel) and transferred onto nitrocellulose membranes by electroblotting overnight at 4°C and 70 V.

Membranes were soaked in 10 mM Tris-HCl containing 5% nonfat dry milk and 0.7% Tween 20 (pH 7.2) for 4 h at room temperature to block nonspecific sites. Membranes were incubated overnight at 4°C with TRPV1 primary antibody (1:500 in TBS with 5% nonfat dry milk, 0.1% Tween 20; Alamone). β-Actin antisemur (Santa Cruz) was used for internal control (1:3,000 in TBS with 5% nonfat dry milk and 0.1% Tween 20 for 2 h at room temperature). Blots were washed and incubated with donkey anti-rabbit IgG-horseradish peroxidase secondary antibody (1:3,000 dilution; Santa Cruz) for 2 h at room temperature. Immunoreactivity was visualized with an ECL Western blotting detection kit (Amersham Biosciences). Quantitative assessment of band densities was performed by scanning densitometry.

Fura-2 experiments. Porcine coronary endothelial and smooth muscle cells were prepared similar to that previously described (16, 28, 44, 48). Briefly, arterial segments were incubated at 37°C for 1 h in a low-Ca2+ solution containing 294 U/ml collagenase and (in mg/ml) 2 bovine serum albumin, 1 soybean trypsin inhibitor, and 0.4 DNAse. Endothelial and smooth muscle cells were differentiated by distinct morphology (15, 16, 44). All experiments were performed on freshly dispersed cells within 24 h of death and within 6 h of isolation from the artery.

Fura-2 experiments were performed at room temperature using an InCyt Basic IM Calcium Imaging System (Intracellular Imaging, Cincinnati, OH) as previously described by our laboratory (48). Briefly, freshly dispersed cells were incubated with 2.5 μM fura-2 AM (Molecular Probes) in a shaking water bath at 37°C for 20 min. Cells were spun down and washed. An aliquot of fura-2-loaded cells was placed on a coverslip in a superfusion chamber mounted on an inverted epifluorescence microscope. Light from a 300-W xenon arc lamp was passed through 360 ± 10- and 380 ± 10-nm band-pass filters. Emitted light (510 nM) was collected using a monochrome charge-coupled device camera (COHU) attached to a 100-MHz Pentium data acquisition computer. Initial studies of cytosolic Ca2+ showed virtually no capsaicin-induced Ca2+ response in Ca2+-free solution (data not shown), thus indicating selective Ca2+ influx rather than Ca2+ release from intracellular stores. Accordingly, TRPV1-mediated Ca2+ influx pathways were monitored with greater sensitivity using extracellular Mn2+ as a surrogate ion for Ca2+ since we have used previously in endothelial and smooth muscle studies (16, 28). The Mn2+ quench technique evaluates divalent cation (Ca2+/Mn2+) influx with greater sensitivity than the net measures of bulk cytosolic Ca2+ (fura-2 ratio) because 1) fura-2 has a 40-fold greater affinity for Mn2+ than Ca2+; 2) Mn2+ is not transported by other Ca2+ transporters; and 3) since there are no Mn2+ stores, no intracellular release is measured. The extracellular medium contained 2 mM MnCl2 instead of CaCl2. The entry of Mn2+ entry was measured as quenching of fura-2 at the excitation wavelength of 360 nm, the Ca2+-insensitive isosbestic wavelength (11, 16, 28).

Data analysis and statistics. Data are reported as means ± SE; n represents the number of animals. Data were analyzed by ANOVA or t-test as appropriate. Post hoc tests were performed using Student-Newman-Keuls tests. Differences were considered significant for P < 0.05.

RESULTS

Characteristics of Ossabaw swine. In Ossabaw swine, a diet high in fat and cholesterol produced many phenotypic characteristics associated with metabolic syndrome in humans (Table 1). Body weight was significantly higher in animals fed an excess high-fat and high-cholesterol diet. Mean arterial blood pressure and heart rate were higher in obese Ossabaw swine. In addition, total cholesterol and triglycerides were significantly higher in obese swine. Obese swine displayed a significantly higher fasting blood glucose concentration, with no change in insulin levels.

Effect of capsaicin on coronary vascular tone. The addition of capsaicin, a TRPV1 channel agonist, did not change baseline tension in either group. Importantly, however, capsaicin relaxed U-46619-precontracted coronary artery rings in a concentration-dependent manner (Fig. 1). The apparent EC50 was 116 ± 41 nM. Capsazepine (30 μM), a TRPV1 channel antagonist, significantly attenuated capsaicin-induced relax-
respectively; maximum relaxation (Fig. 1), indicating that the response to capsaicin is receptor mediated. Ethanol, the vehicle for capsaicin, did not elicit relaxation at the highest concentration used. Capsaicin-induced relaxation is endothelium dependent, since endothelial denudation attenuated the response (Fig. 1; endothelium-denuded rings relaxed <5% in response to 1 μM bradykinin).

**Role of NO and K+ channels in capsaicin-induced relaxation.** NO is the primary endothelium-derived relaxing factor produced with capsaicin stimulation, since L-NAME (300 μM; an inhibitor of NO synthase) inhibited relaxation to the same extent as endothelial denudation (Fig. 2A). Interestingly, the combination of L-NAME and indomethacin, a COX inhibitor, did not further attenuate the response to capsaicin (Fig. 2A). Furthermore, in endothelium-denuded coronary segments, the inhibition of endothelial NO synthase (eNOS) or COX did not affect the relaxation response to capsaicin (data not shown). Iberiotoxin (100 nM) attenuated capsaicin-induced relaxation, suggesting that large conductance Ca2+-activated K+ channels mediate a portion of the response (Fig. 2B). TEA (10 mM; a nonselective inhibitor of K+ channels) attenuated relaxations to capsaicin, suggesting that multiple types of K+ channels contribute to measures of bulk cytosolic Ca2+ release, rather than Ca2+ influx in isolated endothelial cells.

**Impaired capsaicin-induced vasodilation in obese swine.** Capsaicin-mediated relaxation was significantly attenuated in coronary artery rings from obese swine (EC50 = 36 ± 16 μM, Fig. 3). Neither L-NAME (300 μM) nor endothelial denudation further inhibited capsaicin-induced relaxations in artery rings from obese pigs (Fig. 3). Similarly, neither indomethacin (10 μM) nor K+ channel inhibitors (iberiotoxin or TEA) had any further inhibitory effect on capsaicin-induced relaxation of coronary artery rings from obese pigs (Fig. 3, and data not shown). Importantly, bradykinin-mediated relaxations were attenuated in obese animals compared with controls (n = 5 in each group; EC50 = 9 ± 4 vs. 78 ± 39 nM in lean and obese, respectively; maximum relaxation = 97.1 ± 0.3% lean vs. 86.3 ± 5.2% obese). Furthermore, TRPV1 channel inhibition, with capsaazepine, did not affect relaxation to bradykinin in either group (EC50 = 19 ± 12 vs. 104 ± 5 nM in lean and obese, respectively; maximum relaxation = 97.6 ± 1.5% lean vs. 86.3 ± 13.2% obese). However, no change in relaxations to sodium nitroprusside was seen between groups (EC50 = 1.8 ± 1.6 vs. 0.4 ± 0.1 μM in lean and obese, respectively, n = 5 in each group; maximum relaxation 81.9 ± 2.2% lean vs. 87.7 ± 3.8% obese).

**Reduced TRPV1 expression in obese swine.** Localization of TRPV1 channels was evaluated by immunohistochemistry (Fig. 4). TRPV1 immunoreactivity was observed in coronary arteries from lean (Fig. 4A) and obese (Fig. 4B) swine, including prominent staining in coronary endothelium. The vascular smooth muscle cell (VSMC) staining appears largely nonspecific, because of the uniform cytosolic rather than focal plasma lamellar staining. Note that the counterstained nuclei clearly show the endothelial cell layer is decreased in membrane staining of TRPV1 in obese swine (Fig. 4B) compared with lean (Fig. 4A). Real-time PCR showed a significant increase in TRPV1 channel mRNA expression in coronary arteries from obese Ossabaw swine (Fig. 5A); however, Western blot analysis demonstrated a 40 ± 11% decrease in TRPV1 protein expression in the coronary arteries of obese swine (Fig. 5B).

**Reduced capsaicin-induced cation (Ca2+/Mn2+) influx in obese swine.** Fura-2 imaging was performed to determine the ability of the TRPV1 channels to influence intracellular Ca2+ concentration. Capsaicin increased intracellular Ca2+ concentrations in Ca2+-containing buffer, which was inhibited in the presence of capsazepine (data not shown). Importantly, there was virtually no capsaicin-induced Ca2+ response in Ca2+-free solution (data not shown), thus indicating selective Ca2+ influx, rather than Ca2+ release, from intracellular stores. Accordingly, we used the Mn2+-quench method, which is superior to measures of bulk cytosolic Ca2+ in studying Ca2+ influx pathways. In freshly isolated cells, the TRPV1 channel agonist, capsaicin, elicited Mn2+ influx in isolated endothelial cells (Fig. 6), which was blocked by the TRPV1 channel antagonist.

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**Fig. 2.** Nitric oxide (NO) and K+ channels mediate capsaicin-induced coronary artery relaxations: group data from isometric tension experiments performed on coronary arteries from lean pigs. Rings were contracted with 1 μM U-46619 and relaxed with increasing concentrations of capsaicin. A: control data are from Fig. 1 (n = 9 animals). The effect of capsaicin is mediated by NO, since 300 μM Nω-nitro-arginine methyl ester (L-NAME), an inhibitor of NO production, attenuates relaxation (n = 7 animals). Indomethacin (Indo, 10 μM), a cyclooxygenase blocker, had no inhibitory effect in addition to L-NAME (n = 5 animals). *P < 0.05; L-NAME or L-NAME + Indo vs. control. B: capsaicin-induced relaxation involves K+ channels, since it attenuated by iberiotoxin (100 nM; n = 5 animals) and tetrodoylamin (TEA, 10 mM; n = 5 animals). Iberiotoxin is a selective inhibitor of Ca2+-activated K+ channels, whereas TEA is a nonselective inhibitor of K+ channels. *P < 0.05, iberiotoxin vs. control; †P < 0.05, TEA vs. control. C: K+ channel inhibition did not further attenuate capsaicin-induced relaxation in endothelium-denuded rings (TEA, 10 mM; n = 4 animals).
capsazepine (data not shown). In contrast, there was minimal Mn$^{2+}$ influx in vascular smooth muscle cells (Fig. 6B). Freshly isolated endothelial cells were identified by their typical cobblestone or cluster of grapes morphology (16, 44). Furthermore, unlike smooth muscle cells, which have voltage-dependent Ca$^{2+}$ channels, the identity of endothelial cells was confirmed through the absence of a response to membrane depolarization (80 mM K$^{+}$; Fig. 6A). Capsaicin-induced cation influx was abolished in endothelial cells from obese swine (Fig. 6B) compared with lean controls. The results of these physiological experiments clearly demonstrate that TRPV1 channels are functional Ca$^{2+}$ entry pathways in coronary artery endothelium.

**DISCUSSION**

This study was designed to delineate the specific mechanisms by which TRPV1 channels affect coronary vascular tone in lean and obese (metabolic syndrome) Ossabaw swine. Major findings include the following: 1) TRPV1 channels are functionally expressed in the porcine coronary circulation, 2) capsaicin-induced coronary artery relaxation is endothelium dependent and involves NO and K$^{+}$ channels, 3) capsaicin-induced vasodilation is markedly attenuated in obese Ossabaw swine with the metabolic syndrome, and 4) reduced capsaicin-induced responses correlate with diminished TRPV1 protein expression and impaired cation influx into endothelial cells. Taken together, these data indicate that TRPV1 channels are functionally expressed in the coronary circulation and mediate endothelium-dependent vasodilation through a mechanism involving NO and K$^{+}$ channels.

TRPV channels have been described in numerous tissues (33, 34, 52), including vascular endothelial cells (2, 17, 43). Recently, a role for TRPV channels in the control of vascular tone and cardiovascular system has been established. For instance, TRPV1 agonist infusion increased blood pressure, which was largely reversed by selective TRPV1 antagonists (56). Similarly, TRPV1 channel activation by protons following an ischemic event, elicited a sympathoexcitatory reflex which was abolished by antagonist treatment (53). The discrepancies in the role of TRPV1 channels in vascular tone may be due to a positive or negative regulation on blood flow and pressure depending on the type and region of circulation.

**Mechanisms of TRPV1 channel-mediated coronary vasodilation.** Previous results from our laboratory suggest that TRP channels play a role in porcine coronary circulation. Based on these previous observations, we hypothesized that the impair-
ment of endothelial TRPV1 channel-mediated Ca\textsuperscript{2+} signaling contributes to the impairment of porcine coronary artery vasodilation. Our current findings are consistent with this hypothesis. In the present study, we found the TRPV1 agonist capsaicin (41) dose-dependently relaxed coronary conduit arteries, which was attenuated by the TRPV1 antagonist capsazepine (Fig. 1), thus confirming a role of TRPV1 channels in coronary circulation. TRPV1 channel involvement in vascular relaxation has been previously documented. Domenicali et al. (8) reported that anandamide-mediated mesenteric artery relaxation was inhibited by capsazepine. Similarly, Scotland et al. (37) reported that a large part of the “myogenic response” in small mesenteric resistance arteries was susceptible to capsazepine or desensitization with capsaicin, implicating TRPV1 channels.

To further characterize the signaling cascade behind the capsaicin-mediated relaxation, endothelial cell components were examined. Specifically, the role of COX metabolites and eNOS were investigated since both components have been shown to be involved in TRP channel involvement of vascular tone. For instance, Marrelli et al. (30) demonstrated a connection between BK channels and TRPV4 in cerebral vasodilation, whereas Breyne and Vanheel (4) demonstrated that TRPV1 stimulation led to VSMC hyperpolarization via activation of

Fig. 5. Metabolic syndrome alters TRPV1 channel mRNA and protein expression in obese Ossabaw swine. A: quantitative RT-PCR data demonstrating increased TRPV1 channel mRNA expression in obese Ossabaw swine compared with lean controls. Inset: representative gel of TRPV1 transcript products for lean and obese animals. B: summary data from Western blots indicate decreased TRPV1 protein expression in obese Ossabaw swine. Inset: representative blot for TRPV1 protein expression in 4 animals from lean and obese groups. *P < 0.05, lean vs. obese pigs.

channels may be essential in the signal transduction mechanism of endothelial mechanotransduction. Poblete et al. (36) reported that anandamide led to endothelial NO production through the TRPV1 receptor. We found that capsaicin-induced coronary vasodilation is endothelial NO dependent (Figs. 1 and 2A). In the present study, endothelial denuding and eNOS inhibition attenuated capsaicin relaxation in lean pigs, with no additive effect of COX inhibition. However, in obese pigs, inhibition of eNOS and COX did not appear to affect the relaxation to capsaicin. Importantly, COX inhibition may lead to increased AA metabolism via lipoxygenase (LOX) and cytochrome P-450 (CYP) pathways. Various products of the LOX and CYP pathway have been shown to directly activate TRPV1 channels (5, 37). Thus the inhibition of COX could lead to an enhanced production of LOX and CYP metabolites that lead to TRPV1 activation and thus vascular relaxation seen in our study. Future studies are needed to investigate a role for both pathways in capsaicin-induced relaxation.

Previous studies have demonstrated a link between K\textsuperscript{+} and TRPV channels in vascular tone regulation, thus the role of various K\textsuperscript{+} channels in the vasorelaxant response to capsaicin was examined. Specifically, Earley et al. (10) found a connection between BK channels and TRPV4 in cerebral vasodilation, whereas Bryne and Vanheel (4) demonstrated that TRPV1 stimulation led to VSMC hyperpolarization via activation of

Fig. 6. Metabolic syndrome impairs capsaicin-induced divalent cation (Ca\textsuperscript{2+}/Mn\textsuperscript{2+}) influx in endothelial cells. A: data from a fura-2 imaging experiment in a representative endothelial cell. Solutions were applied to the cell for durations indicated by horizontal lines. Capsaicin (100 μM) activated a Mn\textsuperscript{2+}-permeable influx pathway and thus quenched fura-2 signals. B: group data for capsaicin-induced Mn\textsuperscript{2+} influx (rate of fura-2 fluorescence quench) in endothelial cells (ECs) and coronary VSMCs. F380 and F360, fluorescence at 380 and 360 nm, respectively. *P < 0.05, between lean (n = 4 animals) and obese (n = 6 animals).
Mn\(^{2+}\) (K\(_{ATP}\)) channel. Similarly, Fujimoto et al. (13) reported that capsaicin-mediated relaxation of guinea pig ileum via voltage-dependent K\(^+\) channels. Presently, we found that TEA and IBTX (BK channels) administration attenuated capsaicin relaxation responses (Fig. 2B), thus suggesting that capsaicin is working via K\(^+\) channels, as an end result of a second messenger, primarily NO, yet others such as EDHF, PGI\(_2\), or even CGRP and substance P cannot be ruled out. Clearly, our data in coronary artery of the Ossabaw miniature swine show that TRPV1-mediated actions are specific to the endothelium.

Impaired TRPV1 channel-mediated coronary vasodilatation in metabolic syndrome. In the present study, we found that capsaicin-induced vasodilatation is virtually abolished in obese Ossabaw swine with the metabolic syndrome (Fig. 3). Importantly, in obese pigs, the inhibition of eNOS and COX did not appear to affect relaxation to capsaicin (Fig. 3), suggesting a loss of endothelial-mediated relaxation.

Molecular studies were next performed to determine whether decreased TRPV1 channel expression could account for the attenuated capsaicin-mediated relaxation. This study for the first time detected TRPV1 transcript (Fig. 5) and protein in isolated endothelial and VSMCs and right coronary arteries from Ossabaw miniature swine (Figs. 4 and 5). Importantly, TRPV1 protein expression was decreased in obese Ossabaw swine. The altered expression patterns could be due to a number of possibilities involving TRPV1 degradation in the obese pigs. Elevated intracellular free Ca\(^{2+}\), as seen in numerous cardiovascular diseases, could regulate transcription factors in smooth muscle including cAMP response element binding protein and nuclear factor of activated T cells (1). Importantly, this could induce changes in transcriptional or posttranslational modifications of TRPV1 channels. Protein degradation pathways, such as proteases in programmed cell death (interleukin-1β-converting enzyme family), Ca\(^{2+}\)-activated proteases (calpains), and cell-cycle control and stress response elements such as ubiquitin and proteasomes, have been shown to directly or indirectly be involved in surface expression of ion channels at the plasma membrane. For instance, ubiquitin has been shown to regulate the expression of ENaC (38, 39) and K\(_{ATP}\) channels (42, 50). Recently, the HECT ubiquitin ligase AIP4 has been shown to regulate the expression of various members of the TRP channel family, including TRPV4 (46). Similarly, TRPV1 and TRPC3 have been shown to interact and colocalize with proteins of the exocytic machinery (32). Since the presence of channels at the cell membrane is tightly controlled, adjusting the rate of endocytosis or exocytosis of functional plasma membrane proteins (i.e., TRPV1 channels) could exert this control. Future studies are needed explore many of these processes.

Finally, studies were performed to determine whether the attenuated endothelial-mediated relaxations observed in obese arteries was related to decreased cation influx into endothelial cells. Endothelial NO release triggered by TRPV1-mediated Ca\(^{2+}\) influx has been described (36). We found that capsaicin caused Mn\(^{2+}\) quenching of fura-2 fluorescence in both endothelial and VSMCs, although the Mn\(^{2+}\) quench, i.e., divalent cation influx, was almost 10-fold greater in endothelial cells compared with VSMCs (Fig. 6B). Additionally, Mn\(^{2+}\) influx was almost completely abolished by metabolic syndrome (Fig. 6B). Importantly, the reduced capsaicin-induced responses were associated with diminished TRPV1 protein expression and impaired cation (Mn\(^{2+}\)/Ca\(^{2+}\)) influx into endothelial cells. Thus capsaicin-induced Mn\(^{2+}\) quench (Ca\(^{2+}\) influx) signaling mechanism could at least partially explain the impaired endothelium-dependent relaxation.

In summary, the present study provides strong evidence for a physiological and/or pathophysiological role of TRPV1 channels in endothelial cells. Collectively, our results suggest that TRPV1 channels play an important role in the regulation of vascular tone, largely through Ca\(^{2+}\) entry via endothelial TRPV1 channels, which triggers NO-dependent vasodilatation in the endothelium of conduit coronary arteries from Ossabaw swine. TRPV1 channel signaling is virtually abolished in the metabolic syndrome and thus could be a mechanism contributing to the endothelial dysfunction and the development of vascular dysfunction and coronary disease. Furthermore, since activation of TRPV1 channels resulted in robust vasodilatation, endothelial TRPV1 channels may represent a novel pharmacological target for the treatment of coronary disease.

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