Obesity Up-Regulates Intermediate Conductance Calcium-Activated Potassium Channels and Myoendothelial Gap Junctions to Maintain Endothelial Vasodilator Function

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ABSTRACT

The mechanisms involved in altered endothelial function in obesity-related cardiovascular disease are poorly understood. This study investigates the effect of chronic obesity on endothelium-dependent vasodilation and the relative contribution of nitric oxide (NO), calcium-activated potassium channels (IKCa), and myoendothelial gap junctions (MEGJs) in the rat saphenous artery. Obesity was induced by feeding rats a cafeteria-style diet (~30 kJ as fat) for 16 to 20 weeks, with this model reflecting human dietary obesity etiology. Age- and sex-matched controls received standard chow (~12 kJ as fat). Endothelium-dependent vasodilation was characterized in saphenous arteries by using pressure myography with pharmacological intervention, Western blotting, immunohistochemistry, and ultrastructural techniques. In saphenous artery from control, acetylcholine (ACH)-mediated endothelium-dependent vasodilation was blocked by NO synthase and soluble guanylate cyclase inhibition, whereas in obese rats, the ACh response was less sensitive to such inhibition. Conversely, the intermediate conductance IKCa (IKCa, intermediate conductance KCa) blocker 1-[(2-chlorophenyl)diphenyl-methyl]-1H pyrazole attenuates ACh-mediated dilation in obese, but not control, vessels. In a similar manner, putative gap junction block with carbenoxolone increased the pEC50 for ACh in arteries from obese, but not control, rats. IK1 protein and MEGJ expression was up-regulated in the arteries of obese rats, an observation absent in control. Addition of the small conductance KCa blocker apamin had no effect on ACh-mediated dilation in either control or obese rat vessels, consistent with unaltered SK3 expression. Up-regulation of distinct IKCa− and gap junction-mediated pathways at myoendothelial microdomain sites, key mechanisms for endothelial-derived hyperpolarization-type activity, maintains endothelium-dependent vasodilation in diet-induced obese rat saphenous artery. Plasticity of myoendothelial coupling mechanisms represents a significant potential target for therapeutic intervention.

Introduction

Diet-induced obesity is at epidemic levels, with hypertension, hyperglycemia, and insulin resistance being a common outcome (Haslam and James, 2005) The vascular endothelium modulates vasodilator and vasoconstrictor action and is therefore critical for the control of vascular tone and disease etiology. Endothelium-dependent vasodilation depends on nitric oxide (NO), prostacyclin (PGI2), and the non-NO/PGI2 endothelial-derived hyperpolarization (EDH) mechanism (reviewed in McGuire et al., 2001; Sandow and Tare, 2007; Sandow et al., 2009b).

Key events in the EDH process involve an agonist-induced increase in endothelial cell intracellular calcium and activa-

ABBREVIATIONS: NO, nitric oxide; ACh, acetylcholine; PGL2, prostacyclin; Cx, connexin; CyPPA, cyclohexyl-[2-(3,5-dimethyl-pyrazol-1-yl)-6-methyl-pyrimidin-4-yl]-amine; 1-EBIO, 1-ethyl-2-benzimidazolone; EDH, endothelial-derived hyperpolarization; IEL, internal elastic lamina; KCa, calcium-activated potassium channel; IKCa, intermediate conductance KCa; SKCa, small conductance KCa; L-NAME, N-nitro l-arginine methyl ester; MEGJ, myoendothelial gap junction; ODQ, 1H-[1,2,4] oxadiazolo-[4,3-a]quinoxalin-1-one; PBS, phosphate-buffered saline; TRAM-34, 1-[(2-chlorophenyl)diphenyl-methyl]-1H pyrazole; SMC, smooth muscle cell; MES, 4-morpholinoethanesulfonic acid.
tion of small conductance calcium-activated potassium channels (SKCa; SK3; KCa.3.2/SKCa.3/KCNN3) and intermediate conductance calcium-activated potassium channels (IKCa; IK1; SK4/KCa.3.1/KCNN4). This leads to the subsequent hyperpolarization of the smooth muscle via myoendothelial gap junction (MEGJ)-mediated transfer of current and/or K+ release and activation of inward rectifying potassium channels and/or smooth muscle Na+/K+ ATPase (Hill, 2008; Félétou, 2009; Sandow et al., 2009b). The relationship between MEGJs and EDH activity is supported by studies where gap junction “block” with connexin (Cx)-mimetic “Gap” peptides (Chaytor et al., 2001; Sandow et al., 2004; 43Gap26, 40Gap27, and 37,43Gap27) or licorice derivatives such as carbenoxolone attenuate EDH-mediated vasodilation (Coleman et al., 2001).

In rat mesenteric artery, myoendothelial microdomain IKCa and MEGJs show close spatial association (Sandow et al., 2006, 2009b) that is functionally associated with their activity in EDH (Dora et al., 2008; Félétou, 2009; Sandow et al., 2009b).

Altered endothelial function, like heterogeneity in mechanisms of vascular function in general (Aird, 2007), involves a differential contribution of vasoconstrictor and vasodilator activity to the control of vessel tone (Aird, 2007). The specific action of these stimuli varies within and between beds, disease, development, aging, species, strains, sex, and, unfortunately, experimental methodology (Sandow et al., 2009b). In a similar manner, altered endothelium-dependent vasodilation involves changes in the relative contribution of NO, PGI2, and EDH. Indeed, the contribution of IKCa subtypes and MEGJs to EDH also shows an apparent variation in this manner (McGuire et al., 2001; Sandow et al., 2009b), with alterations in specific such components in pathophysiological states, such as obesity, perhaps contributing to the maintenance of vascular reactivity.

In some forms of vascular disease, altered vessel constriction and/or dilation is associated with reduced NO bioavailability, with EDH acting as a backup pathway to sustain vessel diameter (Taddei et al., 2001). Altered endothelial function is associated with animal models of type I diabetes (Shi et al., 2006), type II diabetes (Park et al., 2008), pulmonary hypertension (Kemp et al., 1995), hypercholesterolemia (Brandes et al., 1997), heart failure (Katz and Krum, 2001), and aging (Gaubert et al., 2007), where NO activity is reduced, and KCa-mediated EDH is preserved or up-regulated to maintain overall vasodilation. The disease-related effects of endothelium-dependent vasodilation in diet-induced obesity are unknown.

In adult rat saphenous artery, endothelium-dependent vasodilation is entirely NO-mediated (Wigg et al., 2001; Sandow et al., 2002, 2004). The inability of an EDH to be transferred to the adjacent smooth muscle is caused by the lack of sufficient MEGJs in this bed to facilitate current/ion transfer between the cell layers (Sandow et al., 2002, 2004), which contrasts with the saphenous artery of juvenile rat, where MEGJ-dependent EDH is down-regulated after development (Sandow et al., 2004).

Using a well characterized rodent model of diet-induced obesity associated with elevated blood pressure, insulin, and leptin that directly reflects the etiology human dietary obesity (Velkoska et al., 2005; Chen et al., 2009), the effect of chronic obesity on endothelium-dependent vasodilation in the small saphenous artery was examined. The hypothesis tested was that the mechanism of endothelium-dependent vasodilation would be altered in obesity-induced vascular disease, with KCa and MEGJ components being up-regulated.

### Materials and Methods

#### Animals

Animal procedures conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes and were approved by the University of New South Wales Animal Care and Ethics Committee.

Eight-week-old male Sprague-Dawley rats were fed normal chow (control; −12% kJ as fat) or a cafeteria-style high-fat diet (obese; chow supplemented with condensed milk and lard, biscuits, cakes, dim sims, meat pies, and pasta; −30% kJ as fat) for 16 to 20 weeks. High-fat and control diets were available ad libitum throughout, with nonfasted glucose, leptin, and insulin being measured immediately upon sacrifice. At 24 to 28 weeks (−6 months) of age, obese rats were significantly heavier than the chow-fed control age-matched animals and were characterized by increased liver, retroperitoneal fat, and testicular fat mass. In addition, obese rats had significantly higher levels of blood glucose, leptin, and insulin (Table 1).

#### Artery Preparation

Rats were anesthetized with sodium pentathol (100 mg·kg−1 i.p.), and vascular beds (saphenous and femoral) from the lower hind limb were removed and placed in cold Krebs' solution containing 112 mM NaCl, 25 mM NaHCO3, 4.7 mM KCl, 1.2 mM MgSO4, 7 mM H2O2, 0.7 mM KH2PO4, 10 mM HEPES, 11.6 mM glucose, 2.5 mM CaCl2, 2 mM H2O2, pH 7.4. The first lateral branch of the saphenous artery that diverged toward the knee (control, 330 ± 7 μm, n = 25; obese, 349 ± 5 μm, n = 43) was isolated and carefully cleaned of connective tissue.

#### Pressure Myography

Saphenous arteries were cannulated in a pressure myograph and continuously superfused with Krebs' solution (37°C) at a rate of 2 ml/min. Arteries were pressurized to 80 mm Hg with incremental increases over 80 min. Vessels were initially preconstricted with superfused phenylephrine (1 μM; to 80% of maximum constriction) before increasing concentrations of acetylcholine (ACh; 1 nM to 30 μM) were added to the bath. Experiments were conducted in the presence and absence of selective blockers of endothelium-dependent vasodilator pathways (see Drugs). In some experiments, SKCa and IKCa blockers, apamin and 1-[(2-chlorophenyl)dimethylsims, meat pies, and pasta; 43) was isolated and carefully cleaned of connective tissue.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control</th>
<th>Obese</th>
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<tr>
<td>Rat Weight (g)</td>
<td>539 ± 10</td>
<td>741 ± 17*</td>
</tr>
<tr>
<td>Length (cm)</td>
<td>25.6 ± 0.2</td>
<td>26.6 ± 0.2</td>
</tr>
<tr>
<td>Retroperitoneal (g)</td>
<td>6.4 ± 0.3</td>
<td>22.2 ± 1.3*</td>
</tr>
<tr>
<td>Kidney (g)</td>
<td>1.6 ± 0.03</td>
<td>1.9 ± 0.04</td>
</tr>
<tr>
<td>Liver (g)</td>
<td>17.8 ± 0.5</td>
<td>25.2 ± 1.0*</td>
</tr>
<tr>
<td>Testicular (g)</td>
<td>9.8 ± 0.5</td>
<td>25.8 ± 1.1*</td>
</tr>
<tr>
<td>Blood glucose (mM)</td>
<td>8.0 ± 0.2</td>
<td>9.8 ± 0.3*</td>
</tr>
<tr>
<td>Leptin (ng/ml)</td>
<td>5.2 ± 0.3</td>
<td>8.6 ± 0.2*</td>
</tr>
<tr>
<td>Insulin (ng/ml)</td>
<td>4.6 ± 0.7</td>
<td>12.1 ± 0.5*</td>
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*P < 0.05, indicates significant difference from control.
percentage of the maximal dilution achieved by replacing the control Krebs’ solution with [Ca\textsuperscript{2+}]-free Krebs’ solution.

**Western Blotting.** Arteries were dissected from both hind legs of age-matched control and obese rats, extraneous tissue was carefully removed, and the arteries were stored in liquid nitrogen. Vessels from four animals per n (i.e., per sample lane; n = 3 and 4, for obese and control groups, respectively) were collected. These arteries were ground in liquid nitrogen with a pestle and mortar, resuspended in phosphate-buffered saline (PBS), pH 7.4 containing complete protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN), and centrifuged (3,000g; 4°C; 5 min). The supernatant was removed and placed on ice, and the pellet was snap-frozen in liquid nitrogen and processed again as described above. After the second spin the supernatants were pooled and centrifuged (25,000g; 4°C; 1 h), and the supernatant, enriched in cytosolic proteins, was aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C. The membrane-enriched pellet was carefully resuspended in PBS containing 0.1% Triton X-100 and protease inhibitor cocktail, aliquoted, snap-frozen in liquid nitrogen, and stored at −80°C. Protein concentration of the samples was determined by using the Bradford protein assay (Bio-Rad Laboratories, Hercules, CA).

Aliquots of protein extracts (5 μg of protein unless otherwise indicated) were dissolved in lithium dodecyl sulfate sample buffer (0.5% lithium dodecyl sulfate, 62.5 mM Tris-HCl, 2.5% glycerol, 0.125 mM EDTA, pH 8.5) for 10 min at 70°C. The samples were separated by electrophoresis in bis-Tris polyacrylamide gels by using MES SDS running buffer and electroblotted onto polyvinylidene difluoride membranes overnight at 4°C, according to the manufacturer’s recommendations (Invitrogen, Carlsbad, CA). After transfer, blots were thoroughly washed, blocked, and probed with primary antibody, and specific binding was visualized by using alkaline phosphatase-conjugated secondary antibody and chemiluminescence according to the manufacturer’s instructions (Invitrogen). The intensity of the band corresponding to each protein was quantified by digital densitometry using ImageJ software (National Institutes of Health, Bethesda, MD). Relative intensity for each protein was determined by comparison with the intensity of actin staining on blots that were stripped and then reprobed with actin primary antibody (Supplemental Fig. S1).

To determine specificity, each antibody was incubated with its cognate peptide to block specific binding. Before use, peptide was added to antibody in a 1:1 ratio (w/w), mixed, and incubated at 37°C for 1 h, then overnight at 4°C. The blocked antibody was then used in Western blotting detection as described above.

**Immunohistochemistry.** The distribution of SKCa and IKCa was examined in the rat saphenous artery by using conventional whole-mount confocal immunohistochemistry (Mather et al., 2005; Hadlock et al., 2006). Rats were perfused via the left ventricle with a clearing solution of 0.1% bovine serum albumin, 0.1% NaNO\textsubscript{3}, and 10 U/ml heparin to fully dilate the vessels and fixed with 2% paraformaldehyde in 0.1 M PBS. Tubular vessel segments were cut along the lateral plane and pinned out as a flat sheet with the intima uppermost.

Whole-mount tissues were incubated in blocking buffer (PBS containing 1% bovine serum albumin, 0.2% Tween 20) for 2 h at room temperature, rinsed in PBS (3 × 5 min), and incubated in primary antibody (SKCa, as SK3, 1:100, Mark Chen, GlaxoSmithKline, Stevenage, United Kingdom, M75; IKCa, as IK1, 1:100, Mark Chen, M20; Supplemental Table S2; compared with those exhibiting less specificity, Supplemental Table S3) in blocking buffer for 18 h at 4°C. Tissue was then rinsed in PBS (3 × 5 min) and incubated in secondary antibody (Alexa Fluor 633; Invitrogen) diluted in 0.01% Tween 20 for 2 h. It was then rinsed in PBS (3 × 5 min), mounted intima uppermost in antifade glycerol, and examined with a confocal microscope (PV1000; Olympus, Tokyo, Japan) using uniform settings. CellR software (Olympus) was used for quantitative measurements, with n being the number of observations, each from a different animal, from four different randomly selected 10\textsuperscript{3} μm\textsuperscript{2} areas per animal (see Figs. 3C and 6E). Controls for antibody specificity involved abolition of staining after preincubation of SK3 (M75) and IK1 (M20) antibodies with their respective antigenic peptides (see Fig. 2B; Supplemental Fig. S2), omission of the primary antibody, and the use of transfected cells, immunoelectron microscopy, and positive controls for SK3 (Boettger et al., 2002; Chen et al., 2004; Mongan et al., 2005; Sandow et al., 2006).

**Electron Microscopy.** Tissue preparation and analysis for serial section electron microscopy were as described previously (Sandow et al., 2002, 2004). In brief, control and obese saphenous artery segments used for functional and Western blot studies were dissected from perfusion fixed rats (1% paraformaldehyde, 3% glutaraldehyde in 0.1 mM sodium cacodylate buffer, with 10 mM betaine, pH 7.4). Serial transverse sections (~100 nm thick) totaling ~5 μm of vessel length were cut, and ME GFJs and their surrounding endothelial and smooth muscle cell (SMC) regions were counted and imaged at ×10,000 to 40,000 on a Philips 7100 transmission electron microscope at 16-megapixel resolution (camera from Scientific Instruments and Applications, Duluth, GA). Quantitative wall properties were made from measurements of vessel cross-sections cut 90° perpendicular to the longitudinal vessel axis from ultrastructural montages taken at ×1500 to 2500 at 16-megapixel resolution. CellR software (Olympus) was used for gross quantitative measurements. In Fig. 6E, wall properties of control and saphenous artery were n = 6, each from a different animal. ME GJ quantification is from n = 4, each from a different animal (Sandow et al., 2004). The number of SMC layers and medial cross-sectional area were determined from the mean of four points 90° apart for each vessel, the former as all ±5-μm-long SMC profiles (Sandow et al., 2002, 2003, 2004).

**Statistical Analysis.** Agonist concentrations causing half-maximal responses (EC\textsubscript{50} value) were calculated by using nonlinear regression analysis and expressed as the negative logarithm of the molar concentration (pEC\textsubscript{50} values). Percentage vasodilation evoked by [agonist] was taken as the maximum (peak) responses. All data are expressed as mean ± S.E.M. from n experiments and were determined by one-way analysis of variance followed by Dunnett’s post test for multiple comparisons or paired t tests for groups of two. P < 0.05 was considered significant.

**Drugs.** Vasodilator blockers/antagonists N-nitro-l-arginine methyl ester (1-NAME), 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ), indomethacin, apamin, and carbenoxolone were purchased from Sigma-Aldrich (Castle Hill, Australia). TRAM-34 was purchased from Toronto Research Chemicals Inc. (North York, ON, Canada). Connexin-mimetic “Gap” peptides, 43Gap26, 40Gap27, and 37,43Gap27 (Australian National University, Canberra, Australia). Scrambled peptide (CNVDTAGKAPTSA) was provided by Caryl Hill (Rad Laboratories, Hercules, CA). 1-EBIO was purchased from Tocris (Cologne, Germany). CyPPA was provided by Palle Christophersen (NeoSearch, Ballerup, Denmark).

**Results**

**KCa Activity Is Present in Obese but Absent in Control Rat Saphenous Artery.** Phenylephrine-induced constriction and ACH-mediated dilatation were unaltered in control compared with obese rat saphenous artery (Fig. 1A; Tables 2 and 3). In saphenous arteries from control and obese rats indomethacin (10 μM) alone had no effect on endothelium-dependent vasodilation to ACH compared with vehicle alone (pEC\textsubscript{50} and E\textsubscript{max} characteristics not being different from vehicle control; Table 3), and it was therefore not included in further protocols. However, the relative contribution of NO and K\textsubscript{Ca} to endothelium-dependent dilatation differed in obese and control arteries. Selective SKCa, and IKCa, block with apamin (50 nM) and TRAM-34 (1 μM), respectively, which, in
combination, caused a 1-log unit rightward shift in the ACh concentration-response curve in saphenous artery of obese rats (P < 0.05; Fig. 1A; Table 3). In contrast, in combination these inhibitors had no effect on ACh-mediated dilation in control (Fig. 1A; Table 3), where dilation was blocked by the NO synthase and soluble guanylate cyclase inhibitors L-NAME (100 μM) and ODQ (10 μM), respectively. In obese rat arteries, L-NAME and ODQ caused a 2-log unit rightward shift in the ACh concentration-response curve (P < 0.05; Table 3). However, in these vessels the sensitivity to L-NAME and ODQ was significantly less than in control (P < 0.05; Fig. 1B; Table 3). In both control and obese vessels, ACh-mediated dilation was blocked in the presence of L-NAME and ODQ plus apamin and TRAM-34 (P < 0.05; Fig. 1C).

Altered IK<sub>Ca</sub> Expression in Obese Rat Saphenous Artery. To determine whether the change in endothelium-dependent dilation in the saphenous artery of diet-induced obese rats was accompanied by a change in SK<sub>Ca</sub> and IK<sub>Ca</sub> (represented by SK3 and IK1, respectively) expression, Western blots of membranes extracted from whole arteries were probed with characterized antibodies (see also Boettger et al., 2002; Chen et al., 2004; Mongan et al., 2005; Sandow et al., 2006) (Fig. 2; Supplemental Figs. S3–S6; Supplemental Table S2). SK3 protein expression was detected as a faint high molecular weight complex in control and obese rat arteries (Supplemental Figs. S3 and S5), whereas IK1 protein expression was ~3-fold higher in arteries from obese rats compared with control rats (P < 0.05; Figs. 2 and 3A; Supplemental Fig. S6; Supplemental Table S4).

IK1 appeared as a series of smeared bands (Fig. 2A), characteristic of glycosylated and/or hydrophobic membrane proteins possessing multiple phosphorylation sites, including a high molecular mass complex (>220 kDa), which represents the functional homotetrameric channel assembled in the membrane and was therefore used to quantify IK1 protein expression (Figs. 2 and 3A; Supplemental Results and Discussion; Supplemental Fig. S6; Supplemental Table S4). Some potassium channels, including SK<sub>Ca</sub> and IK<sub>Ca</sub>, when assembled as homotetrameric complexes, are resistant to dissociation by heat and detergents and migrate in SDS-polyacrylamide gel electrophoresis gels as a smeared complex. Thus, as has been demonstrated (Fig. 1C in Boettger et al., 2002; Fig. 3D in Chen et al., 2004; Fig. 1 in Mongan et al., 2005; Fig. 4C in Absi et al., 2007), IK<sub>Ca</sub> appears as a series of smeared bands between ~50 and >250 kDa, which was also the case in the present study (Fig. 2A). This is also reflected by the specific location and block of bands in SK<sub>Ca</sub>- and IK<sub>Ca</sub>-transfected cells using the same antibodies as the present study (Boettger et al., 2002; Chen et al., 2004; Mongan et al., 2005), with said data mimicking that in the present study, being further confirmation of antibody specificity in the present study. More simply stated, antibody specificity in the present study is reflected by mimic of the specific location and block of bands in SK<sub>Ca</sub>- and IK<sub>Ca</sub>-transfected cells (Boettger et al., 2002; Chen et al., 2004; Mongan et al., 2005). For further details see Supplemental Discussion, including additional references.

The distribution of SK3 and IK1 protein in whole arteries

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TABLE 2: pEC<sub>50</sub> and E<sub>max</sub> characteristics for phenylephrine-mediated constriction

<table>
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<th></th>
<th>Control</th>
<th>Obese</th>
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<tr>
<td>pEC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>E&lt;sub&gt;max&lt;/sub&gt;</td>
<td>n</td>
</tr>
<tr>
<td>Vehicle</td>
<td>6.8 ± 0.2</td>
<td>22.9 ± 7.4</td>
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was examined by conventional confocal immunohistochemistry using characterized antibodies. SK3 was not detected with immunohistochemistry; although as a positive control, with the same protocol, it was present in proximal mesenteric artery endothelium (Supplemental Fig. S2, Inset). In both arteries from control and obese rats, IK1 was expressed in the endothelium, but not the smooth muscle, at a diffuse low level. However, IK1 distribution was altered in arteries from obese compared with control rats (Fig. 3, B ii, iii, v, and vi and C). Bright discrete IK1 densities (IK1 “plaques”) were present in control and obese rat arteries, although the size and distribution of these densities differed in control and obese, control having a larger number of small IK1 plaques compared with obese, where larger IK1 plaques were present (Fig. 3, B ii and iii compare v and vi and C). In addition, in arteries from obese animals, a greater number of IK1 plaques were associated with internal elastic lamina (IEL) holes compared with control (Fig. 3C; \( P < 0.05 \)). For antibody control details, see Fig. 3B, Insets and Supplemental Fig. S2.

**IK\(_{\text{Ca}}\) Mediate Endothelium-Dependent Vasodilation in Obese Rat Saphenous Artery.** The apparent lack of SK3 expression and the increase in IK1 in obese rat artery

| TABLE 3 | pEC\(_{50}\) and E\(_{\text{max}}\) characteristics for Ach-mediated vasodilation |
|------------------------|------------------------|------------------------|------------------------|
|                        | Control                | Obese                  |
|                        | pEC\(_{50}\) | E\(_{\text{max}}\) | % | n | pEC\(_{50}\) | E\(_{\text{max}}\) | % | n |
| Vehicle                | 7.1 ± 0.1 | 99.2 ± 0.6 | 20 | | 7.3 ± 0.03 | 98.1 ± 0.6 | 18 | |
| Indomethacin           | 7.2 ± 0.3 | 98.1 ± 1.0 | 4 | | 7.2 ± 0.2 | 96.6 ± 1.2 | 3 | |
| Apamin + TRAM-34       | 7.3 ± 0.1 | 97.6 ± 1.9 | 6 | | 6.1 ± 0.1*† | 96.7 ± 1.8 | 5 | |
| 1-NAME + ODQ           | n/a       | 22.1 ± 9.1* | 5 | | 5.2 ± 0.1* | 89.4 ± 1.1*† | 5 | |
| 1-NAME + ODQ + Apamin + TRAM-34 | 6.7 ± 0.5 | 9.6 ± 1.7* | 6 | | n/a       | 13.4 ± 7.5 | 4 | |
| Vehicle                | —         | —             | — | | 7.2 ± 0.1 | 99.4 ± 0.3 | 8 | |
| Apamin (50 nM)         | —         | —             | — | | 7.3 ± 0.1 | 98.5 ± 0.4 | 4 | |
| Apamin (100 nM)        | —         | —             | — | | 7.2 ± 0.1 | 98.2 ± 0.4 | 3 | |
| TRAM-34                | —         | —             | — | | 6.4 ± 0.1* | 94.2 ± 3.6 | 5 | |
| Vehicle                | 7.6 ± 0.1 | 99.0 ± 0.4 | 4 | | 7.0 ± 0.03 | 96.4 ± 1.5 | 12 | |
| Carbenoxolone          | 7.4 ± 0.1 | 97.1 ± 0.9 | 4 | | 6.0 ± 0.1* | 95.5 ± 1.8 | 4 | |
| Gap peptides           | —         | —             | — | | 5.3 ± 0.3* | 43.8 ± 17.3* | 5 | |
| Scrambled peptide      | —         | —             | — | | 6.9 ± 0.1 | 98.0 ± 1.0 | 3 | |
| Carbenoxolone + TRAM-34 | —         | —             | — | | 5.1 ± 0.2* | 78.9 ± 8.4* | 4 | |

n/a, not applicable.

* P < 0.05 compared with vehicle treatment.
† P < 0.05 compared with control saphenous artery treatment.

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*Fig. 2.* Western blot of saphenous artery IK\(_{\text{Ca}}\) (IK1/SK4/KCa3.1/KCNN4) expression. Western blot detection of IK\(_{\text{Ca}}\) in membranes extracted from the saphenous arteries of obese and control 26-week-old Sprague-Dawley rats (A) and control (B; peptide block). Lane numbers represent independent samples prepared as described under Materials and Methods. Lane M, molecular mass markers. Upper arrows indicate the position of the high molecular mass complex that is resistant to dissociation by SDS and heat and was used to analyze IK\(_{\text{Ca}}\) expression, and the expected position of 48-kDa monomer, which was not apparent in the samples (see Supplemental Results and Discussion for details).
suggest that KCa-mediated dilation in obese rat saphenous artery is mediated by IKCa alone. Thus, to examine the potential individual role of SKCa and IKCa, obese rat vessels were exposed to apamin and TRAM-34 separately. Apamin (50 and 100 nM) alone had no effect on ACh-mediated dilation (Fig. 4A; Table 3) with no difference in the effect of these two concentrations compared with vehicle alone, in artery from obese rat (Table 3; $P < 0.05$). In contrast, TRAM-34 (1 μM) alone attenuated dilation (0.8 log units; $P < 0.05$; Table 3), compared with vehicle-exposed obese rat vessels (Fig. 4A; Table 3).

In support of the preceding apamin and TRAM-34 data, in both control and obese rat vessels, the concentration-selective IKCa activator, 1-EBIO (Kusama et al., 2005) evoked significant dilation that was not different between arteries from control and obese rats ($P < 0.05$; Fig. 4A; Table 3), compared with vehicle-exposed obese rat vessels (Fig. 4A; Table 3).

Gap Junction Blockers Reduce Endothelium-Dependent Vasodilation in Obese Rat Saphenous Artery. The putative gap junction blockers, carbenoxolone and Cx-mimetic “Gap” peptides, were used to examine MEGJ mediation of endothelium-dependent dilation. Exposure to carbenoxolone (100 μM) for 30 min had no effect on ACh-mediated dilation in control artery (Fig. 5A). In contrast, TRAM-34 (1 μM) attenuated ACh-mediated dilation in obese rat artery (1 log unit; $P < 0.05$; Table 3), compared with vehicle-exposed obese rat vessels (Fig. 5B; Table 2). In a similar manner, 45-min incubation in the triple Gap peptide combination (43Gap26, 40Gap27, and 37,43Gap27; 100 μM each) also inhibited dilation to ACh (1 log unit; $E_{\text{max}}$ reduced 53%; $P < 0.05$; Table 3).

**Fig. 3.** IKCa (IK1) expression and function is altered in obese rat saphenous artery. A, histogram summarizing Western blot IKCa data indicates a significant increase (~3-fold) in IKCa protein expression in obese compared with control rat artery ($*P < 0.05$; for examples of full blots see Fig. 2). B, confocal immunohistochemistry shows IKCa distribution (green) and IEL (blue) characteristics in control (top) and obese (bottom) rat artery. IEL autofluorescence shows sites of IEL holes (dark spots, arrowheads; i and iii) and IKCa predominantly as discrete, bright densities (plaques; arrowheads with asterisk). Predominant IKCa plaque expression occurs within/at IEL holes, with individual plaques being larger in obese (iv and v) compared with control (i and iii). Bar, 20 μm. C, differential IKCa distribution and plaque size in control and obese is confirmed by quantitative analysis. *, $P < 0.05$ relative to control; †, n = number of observations, each from a different animal, from four different randomly selected 10^3-μm^2 areas per animal.
3). Incubation in control scrambled peptide (300 μM) was not significantly different from vehicle (Fig. 5B; Table 3). The maximum inhibitory effect of the Gap peptide combination was 35% greater than that of carbenoxolone (P < 0.05; Fig. 5B; Table 3).

Combined exposure to carbenoxolone (100 μM) and TRAM-34 (1 μM) had a cumulative inhibitory effect on ACh-mediated dilation in obese rat artery (Table 3), causing greater inhibition compared with carbenoxolone or TRAM-34 alone (Fig. 5C).

Myoendothelial gap junctions are frequent in obese rat saphenous artery, but rare in control artery. Serial section electron microscopy was used to examine MEGJ incidence (Fig. 6, A and B) and general vessel characteristics. In control rat arteries, MEGJs were rare, being closely associated with ~3% of IEL holes (P < 0.05; Fig. 6C–E). In arteries from obese rats, MEGJ incidence was ~12-fold higher than control (Fig. 6E), with ~55% of IEL holes having MEGJs. Of interest, the endothelial “head” of MEGJ-related projections in obese rat saphenous artery were multilobed, suggestive of a larger surface area (Fig. 6, A and B), which is consistent with larger IK plaque size (~7-fold) in obese compared with control (control, 34 ± 9; obese, 14 ± 5%; n = 3; P < 0.05). C and D, dose responses to 1-EBIO (C; 1–300 μM) and the SKCa agonist CyPPA (D; 0.01–30 μM, control; 1–30 μM, obese; n = 3–6; see also B, 30 μM, did not differ (P < 0.05).

Discussion

The present study demonstrates that changes in the mechanism of endothelium-dependent vasodilation occur in the small saphenous artery branch of the diet-induced obese rat. As a result of a prolonged high-fat diet, obesity results in an up-regulation of IKCa and MEGJ-mediated activity, thereby maintaining vasodilator function. In contrast, under normal conditions in this vessel, such activity is entirely NO-mediated. Like the rat mesenteric artery (Mather et al., 2005; Dora et al., 2008), EDH-type vasodilator activity in the obese rat saphenous artery depends on specialized myoendothelial microdomain communication sites, where IKCa and MEGJs are localized in close proximity. Such activity is absent in the saphenous artery of normal lean littermate control rats.

In the obese rat saphenous artery, up-regulation of IKCa occurs in conjunction with recruitment and redistribution of the channel, as an increased association with IEL holes, which reflects the functional contribution of IKCa that is blocked by TRAM-34 and activated by 1-EBIO. Although IKCa and MEGJ-dependent activity is absent in control where it is not activated by ACh, dilation to 1-EBIO occurs in both control and obese rat vessels. This suggests that whereas IKCa in control does not contribute to the functional response evoked by ACh, it can be activated by direct stimulation with a concentration-selective agonist (Kusama et al., 2005). The latter observation probably reflects the lower expression and localization of IKCa at MEGJ-associated projections in the site of their primary microdomain-dependent activity.

In arteries from both control and obese rats, functional endothelial SKCa is absent. Apamin had no effect on NO-mediated dilation, and direct activation of SKCa with CyPPA also had no effect, an observation further supported by the lack of functional SK3 detection with Western blotting and immunohistochemistry. The detection of SK3 protein as a monomer, but not as a high molecular weight complex on Western blots, suggests that SK3 is present in the saphenous artery, but not as a functional homotetrameric complex assembled in the membrane. Of interest, in a similar manner to obesity, a dominant role for IKCa as opposed to SKCa in EDH expression and localization of IKCa at MEGJ-associated projections in the site of their primary microdomain-dependent activity.
rat saphenous artery and is down-regulated after development in the adult (Sandow et al., 2004). Although IEL holes are required for MEGJ-related projections in control where IEL holes are prevalent (Sandow et al., 2009a), MEGJ density is 10- to 20-fold less than in obese and juvenile saphenous artery, respectively. Thus, the presence of IEL holes in juvenile and control is consistent with the potential for up-regulation of MEGJs and associated EDH signaling mechanisms in ageing and disease and the up-regulation of myoendothelial microdomain signaling mechanisms in obesity in the present study.

Consistent with previous studies (Wigg et al., 2001; Sandow et al., 2002), endothelium-dependent vasodilation in normal adult rat saphenous artery is NO-mediated, with the response to ACh being blocked by t-NAME and ODQ. The present results demonstrate that the contribution of NO is significantly impaired in obese rat saphenous artery, which is compensated for by the appearance of IKCa and MEGJ-mediated activity, to maintain endothelium-dependent vasodilation. It is noteworthy that the reduction in NO activity in these obese vessels may be caused by increased generation of reactive oxygen species, as shown in diabetes (Guzik et al., 2002), hypercholesterolemia (Ohara et al., 1993), aging (Csiszar et al., 2002), and hypertension (Cifuentes and Pagnano, 2006), where NO bioavailability is reduced. However, further examination of this aspect of altered function is beyond the scope of the present study.

Besides inhibiting cellular coupling, gap junction “blockers” have additional actions unrelated to junction block. In this regard, in addition to gap junction effects, peptides (43Gap26, 40Gap27, and 37,43Gap27; 100 μM each in combination) significantly inhibit ACh-mediated dilation in obese rat artery (*, P < 0.05 indicates difference in pEC50 values relative to vehicle control; n = 4–5; Table 3). Response to scrambled peptide (300 μM) was the same as vehicle. The inhibitory effect of Gap peptides was greater than carbenoxolone (†, P < 0.05 indicates difference in Emax relative to arteries exposed to carbenoxolone; Table 3). C, exposure to carbenoxolone (100 μM) and TRAM-34 (1 μM) had a cumulative effect, causing significantly greater inhibition of ACh-mediated dilation in obese rat vessels compared with exposure with either carbenoxolone or TRAM-34 alone (n = 4–12; *, P < 0.05 indicates difference in pEC50 values relative to either carbenoxolone or TRAM-34 treatment alone; Table 3).

Fig. 5. Gap junctions contribute to endothelium-dependent vasodilation in obese rat saphenous artery. A, the putative gap junction uncoupler carbenoxolone (100 μM) had no effect on ACh-mediated dilation in control (n = 4). B, in contrast, carbenoxolone (100 μM) and Cx-mimetic Gap peptides (43Gap26, 40Gap27, and 37,43Gap27; 100 μM each in combination) significantly inhibit ACh-mediated dilation in obese rat artery (*, P < 0.05 indicates difference in pEC50 values relative to vehicle control; n = 4–5; Table 3). Response to scrambled peptide (300 μM) was the same as vehicle. The inhibitory effect of Gap peptides was greater than carbenoxolone (†, P < 0.05 indicates difference in Emax relative to arteries exposed to carbenoxolone; Table 3). C, exposure to carbenoxolone (100 μM) and TRAM-34 (1 μM) had a cumulative effect, causing significantly greater inhibition of ACh-mediated dilation in obese rat vessels compared with exposure with either carbenoxolone or TRAM-34 alone (n = 4–12; *, P < 0.05 indicates difference in pEC50 values relative to either carbenoxolone or TRAM-34 treatment alone; Table 3).
contributions to EDH-type dilation, we hypothesize that the discrete microdomains at MEJG-associated projections facilitate an integrated EDH-type response. Indeed, in theory such myoendothelial microdomain activity may function in three ways: a MEJG-Cx-dependent signaling site, an IKCa2-dependent signaling site, or a combination of the two, thus conferring considerable potential for functional heterogeneity. Such plasticity of myoendothelial coupling mechanisms represents a significant potential target for therapeutic intervention.

Acknowledgments
We thank Mark Chen (GlaxoSmithKline, Stevenage, United Kingdom) for SK3 and IK1 antibodies, Palle Christophersen (NeuroSearch, Ballerup, Denmark) for CyP2A, and Caryl Hill (Australian National University, Canberra, Australia) for scrambled peptides.

References


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Obesity upregulates $\text{IK}_{\text{Ca}}$ and myoendothelial gap junctions to maintain endothelial vasodilator function

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ONLINE SUPPLEMENTAL DATA

Results & Discussion

Western blotting. Western blotting of SK\textsubscript{Ca} and IK\textsubscript{Ca} in the vasodilator field has the common problem that in general it is assumed that the molecular weight of the native single channels (~90 and ~48 kDa, respectively) reflects that of the functional channel (for example, (Brondum et al., 2010; Burnham et al., 2002; Hilgers et al., 2010). However, this is not the case, as high molecular weight complexes of these channels constitute the functional channel in both isolated cells and intact tissue (Boettger et al., 2002; Chen et al., 2004; Mongan et al., 2005), and thus, the expression of such complexes (and not monomers) is of primary relevance for studies of functional SK\textsubscript{Ca} and IK\textsubscript{Ca} expression; and hence the reason for the focus on such complexes in the Western blotting of the present study.

The SK3 M75 and IK1 M20 antibodies used were characterized by Western blotting and immunolabeling of transfected cells, and have been demonstrated to be highly selective in several independent studies (Online Supplemental Table S2 (Boettger et al., 2002; Chen et al., 2004; Mongan et al., 2005). Furthermore, it has been demonstrated that some potassium channels, including SK\textsubscript{Ca} (as SK3 / K\textsubscript{Ca2.3} / SK\textsubscript{Ca3} / KCNN3) and IK\textsubscript{Ca} (as IK1 / SK4 / K\textsubscript{Ca3.1} / KCNN4), when assembled as homotetrameric complexes are resistant to dissociation by heat and detergents and migrate in SDS-PAGE gels as smeared complexes (Arkin et al., 1998; Boettger et al., 2002; Chen et al., 2004; Corey et al., 1998; Mongan et al., 2005). Exhaustive and independent studies by Boettger et al., (2002) Chen et al., (2004) and Mongan et al., (2005) characterize a series of antibodies recognising each K\textsubscript{Ca} subtype, and show that IK\textsubscript{Ca} appears as a series of smeared bands migrating between ~50kDa and >250 kDa (see also Figure 4C in Absi et al. (Absi et al., 2007). This is also the case in the present study (Figure 2), which was carried out under mild conditions for the extraction and electrophoresis of
membrane proteins from whole arteries. The high molecular weight complex >220 kDa represents the functional homotetramer assembled in the plasmalemma and we therefore used this to quantify IKCa protein expression. Lower molecular weight bands represent a combination of degradation products or trimers, dimers, and monomers, and which therefore do not represent the functional channel assembled in the cell membrane (Boettger et al., 2002; Chen et al., 2004). Peptide block of the primary antibody abolished staining that corresponded to specific IKCa protein (Figure 2B), with bands and block reflecting that present in cultured IKCa-transfected cells (Boettger et al., 2002; Chen et al., 2004; Mongan et al., 2005). Thus, simply stated, antibody specificity in the present study is reflected by mimic of the specific location and block of bands in SKCa and IKCa transfected cell (Boettger et al., 2002; Chen et al., 2004; Mongan et al., 2005). Additional IKCa targeted antibodies were also used to probe Western blots, but were found to not optimally identify bands at predicted molecular weights, and/or had significant background (Online Supplemental Table S3). The apparent faint appearance of the specific IKCa bands (Figure 2A) is likely a reflection of the titre and affinity of these antibodies for the IKCa membrane proteins in the saphenous artery and an indication that the proteins are not highly abundant in the samples which included all membrane material extracted from whole arteries consisting of endothelium, smooth muscle and connective tissue.

Of interest, SK3 (as SKCa) was only faintly visible as a high molecular weight complex, mainly appearing as a smeared band, characteristic of hydrophobic membrane proteins possessing multiple phosphorylation sites (Kohler et al., 1996; Neylon et al., 1999), located ~90 kDa; the size of the monomeric form of the protein in the saphenous artery (Online Supplemental Figure S3). Peptide block of the primary antibody abolished staining of the band corresponding to the expected size of full length SKCa (Online Supplemental Figure S4). Hence, SK3 expression (Online Supplemental Figures S3-5) is calculated from the ~90 kDa monomeric form of the protein and not the high molecular weight complexes that represent
the functional homotetramer assembled in the plasmalemma and which were absent on Western blots of membranes from the saphenous artery. Furthermore, the paucity of SK3 complexes in these membrane extracts suggests that although SK3 protein is present in the saphenous artery, only a small proportion may be assembled in the membrane as functional homotetramers (Arkin et al., 1998; Boettger et al., 2002; Chen et al., 2004; Corey et al., 1998). Such a situation may arise as a consequence of the interaction of SK3 with another protein that may act to regulate trafficking to the cell surface and/or assembly as a functional channel complex in the membrane, such as was demonstrated to occur in interactions between SK3 and SK1 (Monaghan et al., 2004).
### TABLE S1

Sympathetic innervations density in control and diet-induced obese rat saphenous artery.

<table>
<thead>
<tr>
<th></th>
<th>External SMC layer circ (µm)</th>
<th>Number of bundles</th>
<th>Number of nerves</th>
<th>Mean no. nerves / 100 µm vessel wall</th>
<th>No. bundles / 100 µm vessel wall</th>
<th>No. nerves / 100 µm vessel wall</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>790 ± 121</td>
<td>29 ± 4</td>
<td>157 ± 10</td>
<td>5.3 ± 0.7</td>
<td>3.8 ± 0.4</td>
<td>20 ± 5</td>
</tr>
<tr>
<td>Obese</td>
<td>868 ± 49</td>
<td>41 ± 9</td>
<td>210 ± 66</td>
<td>4.9 ± 0.8</td>
<td>4.9 ± 1.3</td>
<td>24 ± 9</td>
</tr>
</tbody>
</table>

For analysis method, see (Sandow et al., 1998). n=3, each from a different rat. SMC, smooth muscle cell.
TABLE S2

Western blot and immunohistochemistry primary antibody characteristics.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Antibody Description</th>
<th>Source &amp; Identifier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKCa, * hu N’ aa 2-17</td>
<td>Rabbit</td>
<td>Affinity purified polyclonal</td>
<td>Chen et al., 2004; M75</td>
<td>1:250</td>
</tr>
<tr>
<td>IKCa, # hu N’ aa 2-17</td>
<td>Rabbit</td>
<td>Affinity purified polyclonal</td>
<td>Chen et al., 2004; M20</td>
<td>1:250</td>
</tr>
<tr>
<td>Actin</td>
<td>Rabbit</td>
<td>Affinity purified polyclonal</td>
<td>Sigma, A2066</td>
<td>1:1000</td>
</tr>
<tr>
<td>Goat IgG (Fc portion)</td>
<td>Rabbit</td>
<td>Affinity purified polyclonal AP conjugate</td>
<td>Invitrogen, WB7108</td>
<td>n/a</td>
</tr>
</tbody>
</table>

*, as SK3 / KCa2.3 / SKCa3 / KCNN3. #, as IK1 / SK4 / KCa3.1 / KCNN1. aa, amino acid; AP, alkaline phosphatase; hu, human; n/a, not indicated by manufacturer.

IKCa and SKCa antibodies, batches M75 and M20, respectively, were gifts from Mark Chen (GSK, Stevenage, UK).
**TABLE S3**

Additional Western blot primary antibody characteristics.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Antibody</th>
<th>Source &amp; Identifier</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>SKCa,* hu N’ aa 2-21</td>
<td>Rabbit</td>
<td>Affinity purified polyclonal</td>
<td>Sigma P0608 (10K0932)</td>
<td>1:500</td>
</tr>
<tr>
<td>SKCa,* hu N’ aa 2-21</td>
<td>Rabbit</td>
<td>Affinity purified polyclonal</td>
<td>Alomone APC-025 (AN-04), ‡</td>
<td>1:500</td>
</tr>
<tr>
<td>IKCa,# hu N’ aa 2-17</td>
<td>Rabbit</td>
<td>Affinity purified polyclonal</td>
<td>Chen,(Chen et al., 2004) M4</td>
<td>1:500</td>
</tr>
<tr>
<td>IKCa,# rat C’ aa 350-363</td>
<td>Rabbit</td>
<td>Affinity purified polyclonal</td>
<td>Alomone APC-064 (AN-02)</td>
<td>1:500</td>
</tr>
<tr>
<td>IKCa,# rat C’ aa 350-363</td>
<td>Rabbit</td>
<td>Affinity purified polyclonal</td>
<td>Sigma P4997 (03K1673)</td>
<td>1:500</td>
</tr>
</tbody>
</table>


‡, shows cross-reactivity and overlap with Alomone APC-064 1K1 antibody.

IKCa antibody, batch M4, gift from Mark Chen (GSK, Stevenage, UK).
TABLE S4

Expression of total membrane and functional IK$_{Ca}$ protein in the saphenous artery of 24-28 week control and obese rat. *, indicated significant difference compared to control ($P < 0.05$).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Obese</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total membrane IK$_{Ca}$/actin protein</td>
<td>4.59 ± 0.46</td>
<td>4.97 ± 0.78</td>
</tr>
<tr>
<td>% functional: total IK$_{Ca}$/acting protein</td>
<td>2.39 ± 0.75</td>
<td>6.06 ± 0.42*</td>
</tr>
</tbody>
</table>

References


Supplemental Figure S1. Western blot of saphenous artery actin expression for comparative SK3 and IK1 quantification. Western blot detection of actin in membranes extracted from the saphenous arteries of control and obese 26 week Sprague Dawley rats. Blots were first probed with SK3 or IK primary antibodies, then stripped and reprobed with actin primary antibody. Lane numbers represent independent samples prepared as described in Online Supplementary Methods. Lane M, molecular weight markers. Arrow indicates the position of the full length protein.

Supplemental Figure S2. Confocal immunohistochemistry of SKCa expression in saphenous artery whole mounts. Images show autofluorescence of the internal elastic lamina (IEL; blue; A,D), sites of IEL holes (dark spots, examples with arrowheads; A,D) and absence of SKCa (SK3; KCa2.3; SKCa3; KCNN3) in endothelium (B,E) and smooth muscle (C,F) in control (upper panels) and obese (lower panels). n=4, as number of observations, each from a different animal. Endothelial patency is confirmed by positive endothelial IK1 labelling in adjacent tissue segments (Figure 3B), whilst positive endothelial SK3 in proximal (8 wk male) mesenteric artery acts as positive control (inset). Further controls are detailed in Materials and Methods. Bar, 20 μm.
Supplemental Figure S3. Western blot of saphenous artery SKCa (SK3; KCa2.3; SKCa3; KCNN3) expression. Western blot detection of SKCa in membranes extracted from the saphenous arteries of obese and control 26 week Sprague Dawley rats. Lane numbers represent independent samples prepared as described in Materials and Methods. Lane M, molecular weight markers. The position of the ~90 kDa monomeric protein which was used to analyse SKCa expression is indicated, as is the site of the high molecular weight SDS and heat resistant complex which has been demonstrated for functional, homotetrameric SKCa, which was not apparent in these samples (see text for details). As with immunohistochemistry (Online Supplemental Figure S2), positive Western blot SK3 expression in mesenteric artery (ma) using the same protocol acts as positive control.

Supplemental Figure S4. Western blot SKCa expression control (ie. + peptide block). Peptide block of antibody recognition of SKCa in membranes extracted from saphenous arteries of obese and control 26 week Sprague Dawley rats. Lanes and arrow are as described in Online Supplemental Figure S3.
Supplemental Figure S5. Histograms summarizing Western blot data (Online Supplemental Figure S3) of saphenous artery $SK_{Ca}$ expression. $SK_{Ca}$ expression is the same in control and obese saphenous arteries ($P>0.05$).

Supplemental Figure S6. Relative % of functional to total $IK_{Ca}$ protein (A) and total $IK_{Ca}$ membrane protein expression (B). As per Figures 2-4 and Table S4, functional $IK_{Ca}$ expression is ~3-fold higher in obese compared to control rat saphenous artery; although total IK expression in maintained (B).
Supplemental Figure S7  Myoendothelial microdomain upregulation of gap junction (1) and IK_{Ca} (2) endothelium-dependent vasodilation mechanisms in diet-induced obese rat saphenous artery.

Mechanism 1 involves transfer of EC-derived hyperpolarization via myoendothelial gap junction (MEGJ) connexins (Cxs), whilst mechanism 2 involves localized potassium release from IK_{Ca}, activation of smooth muscle Na+/K+ATPase, with EC inward rectifying potassium channels (Kir; Hill et al., 2008; Smith et al., 2008) modulating such localized K⁺ activity. Modified from Sandow et al., (2009), with permission.