Lean and Obese Coronary Perivascular Adipose Tissue Impairs Vasodilation via Differential Inhibition of Vascular Smooth Muscle K⁺ Channels

Jillian N. Noblet, Meredith K. Owen, Adam G. Goodwill, Daniel J. Sassoon, Johnathan D. Tune

Objective—The effects of coronary perivascular adipose tissue (PVAT) on vasomotor tone are influenced by an obese phenotype and are distinct from other adipose tissue depots. The purpose of this investigation was to examine the effects of lean and obese coronary PVAT on end-effector mechanisms of coronary vasodilation and to identify potential factors involved.

Approach and Results—Hematoxylin and eosin staining revealed similarities in coronary perivascular adipocyte size between lean and obese Ossabaw swine. Isometric tension studies of isolated coronary arteries from Ossabaw swine revealed that factors derived from lean and obese coronary PVAT attenuated vasodilation to adenosine. Lean coronary PVAT inhibited K<sub>Ca</sub> and K<sub>V7</sub>, but not K<sub>ATP</sub> channel-mediated dilation in lean arteries. In the absence of PVAT, vasodilation to K<sub>Ca</sub> and K<sub>V7</sub> channel activation was impaired in obese arteries relative to lean arteries. Obese PVAT had no effect on K<sub>Ca</sub> or K<sub>V7</sub> channel-mediated dilation in obese arteries. In contrast, obese PVAT inhibited K<sub>ATP</sub> channel-mediated dilation in both lean and obese arteries. The differential effects of obese versus lean PVAT were not associated with changes in either coronary K<sub>V7</sub> or K<sub>ATP</sub> channel expression. Incubation with calpastatin attenuated coronary vasodilation to adenosine in lean but not in obese arteries.

Conclusions—These findings indicate that lean and obese coronary PVAT attenuates vasodilation via inhibitory effects on vascular smooth muscle K⁺ channels and that alterations in specific factors such as calpastatin are capable of contributing to the initiation or progression of smooth muscle dysfunction in obesity. (Arterioscler Thromb Vasc Biol. 2015;35:1393-1400. DOI: 10.1161/ATVBAHA.115.305500.)

Key Words: adipose tissue • coronary • obesity • vasodilation
PVAT. Obesity has also been found to diminish the contribution of end-effector K+ channels to coronary vasodilator responses. These channels include voltage-dependent (Kᵥ), Ca²⁺-activated (KᵥCa), and ATP-sensitive (KᵥATP) channels, which regulate smooth muscle membrane potential and participate in the regulation of coronary vascular resistance. However, the extent to which coronary PVAT-derived factors modulate the role of these channels has not been investigated.

Accordingly, the purpose of this investigation was to delineate the effects of lean and obese coronary PVAT on end-effector mechanisms of coronary vasodilation and to identify potential PVAT-derived factors involved. Studies were specifically designed to test the hypothesis that lean and obese PVAT differentially attenuate KᵥCa, Kᵥ, and KᵥATP channel-mediated vasodilation in the coronary circulation and that calpastatin contributes to these effects. Findings from this investigation add to growing evidence supporting a role for PVAT in the pathogenesis of vascular dysfunction in obesity-induced coronary disease.

Materials and Methods
Materials and methods are available in the online-only Data Supplement.

Results
Phenotype of Lean and Obese Ossabaw Swine
Compared with their lean counterparts, obese swine exhibited significant increases in body weight, fasting glucose, total cholesterol, and triglycerides (Table 1). Histopathologic analyses to examine the morphology of perivascular adipocytes were performed on sections of coronary arteries with the adjacent PVAT intact. Hematoxylin and eosin staining revealed apparent similarities in perivascular adipocyte size between lean (Figure 1A) and obese (Figure 1B) swine. Specifically, adipocyte diameter averaged 70±1 μm in lean and 67±2 μm in obese swine (P=0.24). These values are consistent with measures of coronary perivascular adipocyte diameter (Figure 1C; average, 66±2 μm; n=2) from human subjects with evidence of coronary artery disease (Figure 1F). Verhoeff–van Gieson elastin stain demonstrated the presence of atheroma formation in obese (Figure 1E) compared with lean (Figure 1D) swine. These data are consistent with findings from other studies from our investigative team, which documented ≈15% to 20% stenosis of major coronary arteries (using intravascular ultrasound) in obese Ossabaw swine. Immunostaining for CD163, a marker for cells of the monocyte/macrophage lineage, revealed prominent staining in the medial layer of obese arteries (Figure 1I) with only modest staining evident in lean arteries (Figure 1H) relative to isotype control (Figure 1G). These findings are consistent with previous reports of inflammation in coronary arteries from obese swine.

Lean and Obese PVAT Attenuate Coronary Vasodilation
To initially examine the effects of PVAT on vasodilation, coronary arteries cleaned of surrounding PVAT from lean and obese swine were incubated with or without a known quantity of coronary PVAT (0.3 g) from the same animal for 30 minutes (Figure 2 in the online-only supplement). Arteries were then preconstricted with the thromboxane A₂ mimetic U46619 (1 μmol/L). Active tension development of control arteries to U46619 (1 μmol/L) in the absence of PVAT averaged 9.01±0.41 g in lean and 10.20±0.61 g in obese arteries (P=0.09). In arteries treated with PVAT, active tension development averaged 9.61±0.42 g in lean and 9.88±0.53 g in obese arteries (P=0.68). Vasodilation to adenosine (30 μmol/L) was reduced ≈25% in obese (Figure 2B) compared with lean (Figure 2A) arteries in the absence of PVAT (P<0.001). The presence of PVAT significantly attenuated adenosine relaxation at concentrations >3 μmol/L in arteries from both lean and obese swine. Although maximal responses to adenosine were lower in obese arteries, the overall degree of PVAT inhibition on maximal adenosine-induced dilation (30 μmol/L) was similar in lean (≈31%) and obese (≈32%) arteries (Figure 2A versus Figure 2B). Preconstriction with KCl (60 mmol/L), which averaged 7.19±0.22 g in lean and 8.27±0.90 g in obese arteries (P=0.36), essentially abolished dilation to adenosine in both lean and obese arteries. Additional experiments in endothelium-denuded coronary arteries demonstrated that adenosine-induced dilation was unaffected by removal of the endothelium in both control (P=0.94) and PVAT-treated (P=0.99) arteries. Denudation was confirmed in these studies by <15% relaxation to bradykinin (1 mmol/L).

Lean Coronary PVAT Inhibits KᵥCa and Kᵥ7 Channels
The contribution of KᵥCa channels to coronary vasodilation in lean and obese hearts was examined by comparing responses to the KᵥCa channel agonist NS-1619 (1–30 μmol/L). Overall responses to NS-1619 (30 μmol/L) were reduced ≈30% in obese compared with lean control arteries in the absence of PVAT (P=0.01; Figure 3A versus Figure 3B). Compared with control responses, the addition of PVAT attenuated dilator responses to the NS-1619 (30 μmol/L) by ≈30% in lean arteries (P<0.001; Figure 3A). In contrast, NS-1619-mediated dilation was unaffected by the addition of PVAT in obese arteries (P=0.90; Figure 3B).

Table 1. Phenotypic Characteristics of Lean and Obese Ossabaw Swine

<table>
<thead>
<tr>
<th></th>
<th>Lean</th>
<th>Obese</th>
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<tbody>
<tr>
<td>Body weight, kg</td>
<td>62±6</td>
<td>100±5*</td>
</tr>
<tr>
<td>Heart weight, g</td>
<td>182±16</td>
<td>222±15</td>
</tr>
<tr>
<td>Mean arterial pressure, mm Hg</td>
<td>102±9</td>
<td>107±5</td>
</tr>
<tr>
<td>Glucose, mg/dL</td>
<td>154±14</td>
<td>232±21*</td>
</tr>
<tr>
<td>Insulin, μIU/mL</td>
<td>12±1</td>
<td>14±3</td>
</tr>
<tr>
<td>Total cholesterol, mg/dL</td>
<td>74±4</td>
<td>340±61*</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>46±5</td>
<td>78±14*</td>
</tr>
</tbody>
</table>

Values are mean±SE for a subset of lean (n=10) and obese (n=10) swine. *P<0.05 vs lean.

Nonstandard Abbreviation and Acronym
PVAT  perivascular adipose tissue
To investigate the role of Kv7 channels in coronary vasodilation, responses to the Kv7 channel agonist L-364,373 (10 nmol/L–10 μmol/L) were examined in lean and obese arteries. Dilation to L-364,373 was significantly attenuated at doses >3 μmol/L in obese compared with lean control arteries in the absence of PV AT (P<0.05; Figure 3C versus Figure 3D). The presence of PV AT attenuated L-364,373-mediated dilation (10 μmol/L) by ≈20% in lean arteries (P=0.02; Figure 3C) but had no effect in obese arteries (P=0.98; Figure 3D). Western blot analyses of Kv7 channel (KCNQ1) protein indicated that the abundance of KCNQ1 was not significantly different in lean versus obese coronary arteries (P=0.11; Figure 4A and 4C). Abundance of β actin was not significantly different in lean versus obese arteries (P=0.91), indicating equal protein loading between samples.

Figure 1. Representative images of immunohistochemical analyses of coronary arteries and associated perivascular adipose tissue (PVAT) obtained from human (n=2), and lean and obese swine (n=4, each group). Hematoxylin and eosin-stained sections (×10) illustrated similarities in perivascular adipocyte morphology between humans and swine (A–C). Verhoeff–van Gieson–stained sections (×4) showed evidence of atheroma formation in human (F) and obese (E) compared with lean (D) arteries. CD163 staining (×10) indicated a marked increase in macrophages in obese (I, arrows) compared with lean (H) arteries relative to isotype control (G).

Figure 2. Coronary perivascular adipose tissue (PVAT) attenuates adenosine-induced vasodilation. In control arteries cleaned of PVAT (n=6 each group), maximal vasodilation to adenosine was reduced ≈25% in lean (A) compared with obese (B) arteries. The presence of PVAT from the same animal (n=6 each group) impaired dilation to a similar extent and constriction with KCl (n=3 each group) abolished adenosine dilation in lean and obese arteries. *P<0.05, PVAT vs control. †P<0.001, lean vs obese control.
Obese Coronary PVAT Inhibits $K_{\text{ATP}}$ Channels

Studies to investigate the effect of coronary PVAT on $K_{\text{ATP}}$ channel-mediated dilation were performed by comparing responses to the $K_{\text{ATP}}$ channel agonist cromakalim (30 nmol/L–1 μmol/L) in lean and obese arteries. Control responses to cromakalim (1 μmol/L) were not significantly different in lean versus obese arteries ($P=0.90$; Figure 3E and 3F). The presence of coronary PVAT from the same animal had no effect on dilation to cromakalim (1 μmol/L) in lean arteries ($P=0.57$; Figure 3E). In contrast, PVAT significantly attenuated dilation to cromakalim in obese arteries ($P=0.02$; Figure 3F). Western blot analyses show that abundance of $K_{\text{ATP}}$ channel pore-forming unit ($K_{\text{ir}}$ 6.1) protein was not different in obese compared with lean arteries ($P=0.40$; Figure 4B and 4D). Abundance of β actin was not significantly different in lean versus obese arteries ($P=0.34$), indicating equal protein loading between samples.

Differential Effects of Lean Versus Obese Coronary PVAT

To evaluate the specific effects of lean versus obese PVAT on $K^+$ channel function, independent of differences in coronary artery responsiveness, control coronary arteries (cleaned of PVAT) from lean swine were incubated with known quantities of PVAT (0.3 g) from either lean or obese swine euthanized on the same day. In contrast to the inhibitory effects of lean PVAT, obese PVAT had no effect on relaxation to NS-1619 (30 μmol/L) or L-364,373 (10 μmol/L) in lean arteries ($P=0.40$; Figure 5A and $P=0.10$; Figure 5B). Alternatively, obese PVAT significantly attenuated relaxation to cromakalim (1 μmol/L; $P=0.001$; Figure 5C), whereas lean PVAT had no effect ($P=0.57$).
On the basis of previous findings, additional proof-of-principle studies were performed to investigate the effects of calpastatin (10 μmol/L) on coronary vasodilation. Incubation with calpastatin significantly attenuated vasodilation to adenosine (from 3 μmol/L to 10 μmol/L) in lean arteries cleaned of PVAT (P<0.001; Figure 6A). In contrast, exposure to calpastatin had no effect on adenosine dilation in obese arteries cleaned of PVAT (P=0.30; Figure 6B).

Discussion

This investigation was designed to delineate the effects of lean and obese coronary PVAT on end-effector mechanisms of coronary vasodilation and to identify potential factors involved. The major new findings of this study are (1) diameters of adipocytes in epicardial coronary PVAT are similar in lean and obese swine; (2) factors derived from lean and obese coronary PVAT attenuate vasodilation in response to adenosine; (3) lean coronary PVAT inhibits KCa and Kv7 channel-mediated dilation but has no effect on KATP channel-mediated dilation in lean arteries; (4) coronary vasodilation to KCa and Kv7 channel activation is impaired in obese relative to lean arteries in the absence of PVAT; (5) obese PVAT has no effect on KCa or Kv7 channel activation in obese arteries; (6) obese PVAT inhibits KATP channel-mediated vasodilation in both lean and obese coronary arteries; and (7) calpastatin attenuates coronary vasodilation to adenosine in lean but not in obese arteries. These findings provide novel evidence that lean and obese PVAT-derived factors attenuate coronary vasodilation via differential inhibition of KCa and Kv7 channels and implicate a mechanistic link between alterations in PVAT-derived factors, such as calpastatin and diminished functional expression of coronary Kv7 channels in the setting of obesity.

Although the ability of PVAT to produce transferrable factors that influence the vasculature is well established, current understanding about the nature of this effect in specific adipose tissue depots remains rather limited. Although the majority of studies on peripheral (noncardiac) PVAT support the production of adipose-derived relaxing factor(s) and an overall anticontractile effect, recent data indicate that coronary PVAT is unique relative to these other adipose depots both in its expression profile and effects on the vasculature. In particular, factors derived from coronary PVAT have been found to attenuate endothelial-dependent dilation and potentiate coronary artery contractions, especially in the setting of obesity. In other studies in lean or hypercholesterolemic swine show little/no anticontractile effect of coronary PVAT in response to endothelin-1, angiotensin II, or the thromboxane A2 mimetic U46619. Results from this study further support the distinct vascular effects of coronary PVAT in that lean and obese coronary PVAT significantly attenuate coronary vasodilator responses to adenosine. This impaired dilator response is directly related to effects of PVAT-derived factors on smooth muscle K+ channels because adenosine-induced dilation was unaffected by endothelial denudation and was essentially abolished by preconstriction with KCl (Figure 2A and 2B). Inhibitory effects of PVAT on K+ channels have significant (patho)physiological implications as previous studies have clearly demonstrated the contribution of Kv and KATP channels to the regulation of coronary microvascular tone and KCa channels in endothelial-dependent dilation.

These findings provide novel evidence that lean and obese PVAT have distinct inhibitory effects on specific K+ channel subtypes in lean and obese coronary arteries. Specifically, factors derived from lean coronary PVAT impair KCa and Kv7 channel-mediated dilation, whereas factors derived from obese coronary PVAT attenuate KATP channel-mediated dilation (Figure 3). The lack of effect of obese coronary PVAT on KCa and Kv7 channels was observed in both obese (Figure 3) and lean (Figure 5) coronary arteries and is thus not related to intrinsic differences in smooth muscle phenotype of lean versus obese swine. Therefore, the cross-over studies in which lean arteries (ie, with normal vascular smooth muscle function) were incubated with lean and obese PVAT, strongly support that lean and obese PVAT-derived factors differentially affect KCa, Kv7, and KATP channels. This distinction is important because we found that coronary vasodilation in response to KCa and Kv7 channel agonists is attenuated in obese relative to lean arteries in the absence of PVAT (Figure 3). These data are consistent with previous work from our laboratory and others, which demonstrated the functional downregulation of BKCa and Kv channels in the coronary circulation and...
suggest the potential for PVAT-derived factors to contribute to the initiation and progression of coronary vascular dysfunction in the setting of obesity.

There are several potential mechanisms that could contribute to the effects of PVAT-derived factors on coronary K⁺ channels. In particular, it does not seem that differences in K⁺ channel expression levels are responsible for the divergent effects of PVAT because Western analyses revealed similar levels of K⁺,7 (KCNQ1) and K⁺ATP (K⁺,6.1) channels in coronary arteries of lean and obese swine (Figure 4). Previous studies from our group also found augmented expression of BKCa channel subunits in coronary arteries of obese swine.25 However, it is possible that expression of other channel subtypes or subunits could be altered in the setting of obesity. The potential for direct effects of PVAT-derived factors on specific coronary K⁺ channels is supported by previous evidence that NS-1619, L-364,373, and cromakalim directly open K⁺Ca, K⁺ATP, and K⁺,7 channels (ie, without activating transmembrane signaling pathways) because these agonists have been shown to bind to sites on channel subunits and increase the open probability of excised membrane patches.47–49 In addition, cellular signaling pathways also influence the response of these K⁺ channels to their respective agonists. For example, ischemic stimuli and Rho kinase signaling influence the response of K⁺Ca channels to NS-1619,7,63,65 whereas the effects of L-364,373 on K⁺,7 channels may interplay with extracellular signal-regulated kinase signaling52 and protein kinase C alters the K⁺ATP channel response to cromakalim.53 Therefore, it is possible that cellular signaling, including post-translational modifications, such as phosphorylation, influences the response of vascular smooth muscle K⁺ channel activation in the presence of PVAT.54 Such effects are in line with previous studies from our laboratory which demonstrated that coronary PVAT influences both protein kinase C and Rho kinase signaling.6,12 Whether coronary PVAT-derived factors interact with K⁺ channels directly or influence their function indirectly through intracellular signaling pathways warrants further investigation.

Identification of the precise factors responsible for the vascular effects of coronary PVAT remains a daunting task. Recent studies by the Weintraub laboratory have established that adipocytes from human coronary PVAT have a distinct phenotype and exhibit elevated expression of proinflammatory genes and genes associated with angiogenesis, coagulation, and vascular morphology,5,10,55,56 Evidence of macrophage infiltration (Figure 1I) and atheroma formation (Figure 1E) in obese arteries support a potential role for inflammatory cross-talk between PVAT and the artery wall in the pathogenesis of atherosclerosis. A previous global proteomic assessment revealed the upregulation of proteins associated with cellular growth, proliferation, and movement in obese versus lean coronary PVAT from swine.55 These differences seem to be independent of gross changes in morphology because similarities in adipocyte diameter were found between lean and obese PVAT (Figure 1A and 1B). Of particular interest is the endogenous calpain inhibitor, calpastatin, which we have shown to be significantly elevated in supernatant of obese coronary PVAT and to dose-dependently augment coronary artery contractions.12 Findings from the current investigation further support that calpastatin is capable of mimicking the effects of coronary PVAT in that it acts to impair smooth muscle dilation in response to adenosine in lean coronary arteries (Figure 6A). The loss of this effect in obese coronary arteries (Figure 6B) is consistent with the lack of effect of obese PVAT on K⁺Ca and K⁺,7 channel-mediated dilation in obese arteries (Figure 3B and 3D), and suggests that chronic local exposure of the coronary circulation to factors, such as calpastatin could contribute to the impairment of smooth muscle function in the setting of obesity.

It is important to recognize that although a differential effect of lean versus obese PVAT on vascular function was demonstrated in lean, healthy arteries, the effect of lean PVAT on obese arteries was not examined in this investigation. In addition, findings of this study were produced after short-term (=30 minutes) exposure to PVAT ex vivo. Thus, a critical question remains as to whether chronic exposure of the coronary vasculature to the PVAT milieu directly contributes to the altered functional expression of K⁺ channels in the setting of obesity. We propose that as the severity of obesity and other cardiovascular risk factors (insulin resistance, hypercholesterolemia, and hypertension) progresses, changes in the secretion profile of coronary PVAT adversely influences the function and expression of coronary ion channels. However, the extent to which phenotypic alterations in coronary PVAT causally contribute to mechanistic alterations in the obese coronary circulation merits further study.

In summary, these findings demonstrate that although coronary perivascular adipocytes from lean and obese swine share similar morphology, lean and obese PVAT-derived factors impair vasodilation via differential inhibition of vascular smooth muscle K⁺ channels. Specifically, our data are the first to demonstrate that lean coronary PVAT attenuates K⁺Ca and K⁺,7 channel-mediated dilation, whereas obese coronary PVAT impairs K⁺ATP channel-mediated dilation. These results further support the paradigm of distinct outside-to-inside signaling influences of coronary PVAT and that alterations in specific factors, such as calpastatin are capable of contributing to the initiation or progression of smooth muscle dysfunction in the setting of obesity.
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Disclosures
None.

References
Coronary perivascular adipose tissue (PVAT) normally surrounds the major coronary arteries of the heart. Evidence is mounting to support the potential for factors derived from coronary PVAT to influence the pathogenesis of coronary vascular disease. In particular, recent studies indicate that coronary PVAT-derived factors initiate/potentiate contraction of vascular smooth muscle, a property distinct from other adipose tissue depots, and that this effect is significantly augmented in the setting of obesity. However, the effects of coronary PVAT on vasodilation have not been clearly defined. Results from this investigation indicate that coronary PVAT attenuates dilation via inhibitory effects on vascular K^+ channels and that the mechanisms and factors involved in mediating these effects are markedly altered in the setting of obesity. These findings provide new insights into the unique vasoactive properties of coronary PVAT and the potential role of PVAT-derived factors in obesity-induced coronary disease.

Significance

Coronary perivascular adipose tissue (PVAT) normally surrounds the major coronary arteries of the heart. Evidence is mounting to support the potential for factors derived from coronary PVAT to influence the pathogenesis of coronary vascular disease. In particular, recent studies indicate that coronary PVAT-derived factors initiate/potentiate contraction of vascular smooth muscle, a property distinct from other adipose tissue depots, and that this effect is significantly augmented in the setting of obesity. However, the effects of coronary PVAT on vasodilation have not been clearly defined. Results from this investigation indicate that coronary PVAT attenuates dilation via inhibitory effects on vascular K^+ channels and that the mechanisms and factors involved in mediating these effects are markedly altered in the setting of obesity. These findings provide new insights into the unique vasoactive properties of coronary PVAT and the potential role of PVAT-derived factors in obesity-induced coronary disease.
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METHODS

Ossabaw Swine Model of Obesity

All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee in accordance with the *Guide for the Care and Use of Laboratory Animals*. Lean Ossabaw swine (n=30) were fed ~2000 kcal/day standard chow containing 18% kcal from protein, 71% kcal from complex carbohydrates, and 11% kcal from fat. Obese Ossabaw swine (n=28) were fed ≥8000 kcal/day atherogenic diet containing 16% kcal from protein, 41% kcal from complex carbohydrates, 43% kcal from fat, and supplemented with 2.0% cholesterol and 0.7% sodium cholate by weight (5L80 and KT324, Purina Test Diet, Richmond, IN). Swine were fed their respective diets for ~6 months prior to sacrifice.

Immunohistochemistry

Immunohistochemical analyses were performed in conjunction with Indiana University Health Pathology Laboratory (Indianapolis, IN). Briefly, hearts from lean and obese swine were excised upon sacrifice and immediately perfused with 4°C, Ca\(^{2+}\)-free Krebs buffer (131.5 mM NaCl, 5 mM KCl, 1.2 mM Na\(_2\)HPO\(_4\), 1.2 mM MgCl\(_2\), 25 mM NaHCO\(_3\), 10 mM glucose) via aortic cannulation. Segments of coronary arteries with perivascular adipose tissue (PVAT) intact were grossly dissected from the heart and placed in 10% Formalin (Fisher Scientific, Fair Lawn, NJ, SF98-4). Similar segments of coronary artery were harvested from a formalin fixed human heart obtained via Indiana University Health Pathology Laboratory (Indianapolis, IN) and with approval of the Institutional Review Board of Indiana University (IRB #1306011568). Fixed artery segments were embedded in paraffin and cross sectioned. Verhoeff-van Gieson (VVG) and Hematoxylin and Eosin (H&E) staining were performed. Additionally, sections were exposed to anti-CD163 antibody (1:100; Abcam, Cambridge, MA, ab87099), a marker for cells of the monocyte/macrophage lineage\(^1\) and anti-Rabbit IgG (1:100; Abcam, Cambridge, MA, ab172730) as an isotype control. Slides were imaged at 4X or 10X magnification, as indicated, on a Nikon Eclipse 80i microscope and images captured with a Nikon DS-Fi1 and associated Nikon Elements software. Linear adjustments of contrast, applied equally to all parts of an image, were made using ImageJ software Fiji.\(^2\) Diameters of adipocytes within 500 µm of the vessel wall were determined using Leica image processing system.

Functional Assessment of Isolated Coronary Rings

Functional studies on isolated coronary artery rings were performed as previously described.\(^3\)\(^,\)\(^4\) After the perfusion described above, coronary arteries from lean and obese swine were grossly dissected from the heart ([**Figure IA**]), removed from the myocardium ([**Figure IB**]) and cleaned of surrounding coronary PVAT ([**Figure IC**]). Subsequently, coronary PVAT was cut into ~50 mg pieces and stored in Ca\(^{2+}\)-free Krebs buffer at 4°C for later use. Cleaned coronary arteries were cut into 3 mm rings and mounted in organ baths filled with Ca\(^{2+}\)-containing Krebs buffer (131.5 mM NaCl, 5 mM KCl, 1.2 mM Na\(_2\)HPO\(_4\), 1.2 mM MgCl\(_2\), 25 mM NaHCO\(_3\), 10 mM glucose, 4mM CaCl\(_2\)) at 37°C. Once stabilized at optimal passive tension (~4 g), arteries were subjected to the experimental protocols outlined below.

Lean and obese coronary arteries were incubated with coronary PVAT from the same animal ([**Figure IIB and IIC**]), or left untreated as time-control ([**Figure IIA and IID**]) for 30 minutes at 37°C. For arteries incubated with PVAT, 0.3 g of PVAT (~50 mg pieces) was weighed and then added directly to the organ bath, as previously described.\(^3\)\(^,\)\(^4\) Arteries were then pre-constricted with the thromboxane A\(_2\) mimetic, U46619 (1 µM: Santa Cruz Biotechnology, Dallas, TX, sc-201242) or KCl (60 mM: Sigma Aldrich, St. Louis, MO, P9333), indicated as “KCl control” in ([**Figure 1**]). Active tension development (peak tension minus baseline tension) was recorded for each treatment group. Upon stabilization of contractions, arteries were exposed to increasing
concentrations of adenosine (10 nM – 30 µM: Sigma Aldrich, St. Louis, MO, A9251), the $K_{Ca}$ channel agonist NS-1619 (1 µM – 30 µM: Sigma Aldrich, St. Louis, MO, N170), the $K_{V7}$ channel agonist L-364,373 (10 nM – 10 µM: Tocris, Minneapolis, MN, Cat.No.2660), or the $K_{ATP}$ channel agonist cromakalim (30 nM – 1 µM: Sigma Aldrich, St. Louis, MO, C1055). For crossover experiments (Figure 5), lean arteries cleaned of PVAT, indicated as “control” were incubated with known quantities (0.3 g) of either lean PVAT from the same animal, indicated as “lean PVAT,” or obese PVAT from an obese animal sacrificed on the same day, indicated as “obese PVAT” (Figure III). For calpastatin studies, both lean and obese coronary arteries cleaned of PVAT were incubated with calpastatin (10 µM: Calbiochem, San Diego, CA, Cat#208902), or left untreated as control for 30 minutes at 37°C. Arteries were then pre-constricted with U46619 (1 µM) and exposed to increasing concentrations of adenosine (10 nM – 30 µM) in the presence or absence of calpastatin. Additional experiments were also conducted in endothelium denuded coronary arteries from lean swine. The endothelium was removed by gently rubbing fine-tip forceps along the lumen of the artery. Denudation was confirmed by <15% relaxation to bradykinin (1µM: Sigma Aldrich, St. Louis, MO, B3259). Results are reported as the percent relaxation for each animal and rings with the same treatment from the same animal were averaged for $n = 1$. One hundred percent relaxation is defined as a return to the level of baseline tension.

Western Analysis

Coronary arteries from lean (n=3) and obese (n=3) swine were cleaned of adipose tissue, frozen in liquid N$_2$ and stored at -80°C. Arteries were homogenized and total protein collected and quantified as previously described.$^5$ Equivalent amounts of protein were loaded onto 10% polyacrylamide gels (Life Technologies, Carlsbad, CA, NP0302) for electrophoresis and blotting. Membranes were incubated overnight at 4°C with primary antibodies directed against $K_{ir6.1}$ (1:200, Santa Cruz Biotechnology, Dallas, TX, sc-11224) or KCNQ1 (1:400, Sigma-Aldrich, St. Louis, MO, AV35529). The blots were washed and incubated with donkey anti-goat (1:5000, Santa Cruz Biotechnology, Dallas, TX, sc-2020) or goat anti-rabbit (1:1000, Santa Cruz Biotechnology, Dallas, TX, sc-2004) IgG-horseradish peroxidase secondary antibodies for 1.5 h at ambient temperature. To verify equal protein loading, membranes were washed and incubated with antibody to β-actin (1:200, Santa Cruz Biotechnology, Dallas, TX, sc-1616). Immunoreactivity was visualized using ECL (Thermo Scientific, Rockford, IL, Prod#32106) and the G:BOX system (Syngene). MagicMark XP Western Standard (Life Technologies, Carlsbad, CA, LC5602) was used as a protein ladder. Densitometry analyses were conducted using ImageJ. Protein levels of KCNQ1, $K_{ir6.1}$, and β-actin are reported as “% lean;” i.e. protein levels from each sample were normalized to the average level of the respective protein in lean arteries.

Statistical Analysis

Data are presented as mean ± SE. A t-test was used to compare phenotypic data (lean vs. obese) and densitometry of Western blot analyses. For isometric tension studies, a two-way ANOVA was used to test the effects of PVAT (Factor A) relative to doses of specific treatments (Factor B). If assumptions of normality and equal variance for parametric ANOVA were not met, a Kruskal-Wallis non-parametric ANOVA was performed. Importantly, results of non-parametric ANOVAs were consistent with those of the parametric ANOVA. When statistical differences were found with ANOVA ($P < 0.05$), a Student-Newman-Keuls multiple comparison test was performed. SigmaPlot version 11.0 (Systat Software Inc, San Jose, CA) was used for graphics and statistical analyses.
Reference List


Figure I. Representative pictures illustrating the isolation of coronary artery rings and perivascular adipose tissue (PVAT) from lean and obese hearts prior to isometric tension studies. Images adapted from Owen et al., with permissions from Wolters Kluwer Health Publishing. Copyright 2013, Circulation.
Figure II. Experimental design for isometric tension studies presented in Figure 2 and Figure 3.
Figure III. Experimental design for crossover isometric tension studies presented in Figure 5.