Contribution of K_{V1.5} Channel to Hydrogen Peroxide–Induced Human Arteriolar Dilation and Its Modulation by Coronary Artery Disease

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Rationale: Hydrogen peroxide (H_{2}O_{2}) regulates vascular tone in the human microcirculation under physiological and pathophysiological conditions. It dilates arterioles by activating large-conductance Ca^{2+}-activated K^{+} channels in subjects with coronary artery disease (CAD), but its mechanisms of action in subjects without CAD (non-CAD) when compared with those with CAD remain unknown.

Objective: We hypothesize that H_{2}O_{2}-elicited dilation involves different K^{+} channels in non-CAD versus CAD, resulting in an altered capacity for vasodilation during disease.

Methods and Results: H_{2}O_{2} induced endothelium-independent vasodilation in non-CAD adipose arterioles, which was reduced by paxilline, a large-conductance Ca^{2+}-activated K^{+} channel blocker, and by 4-aminopyridine, a voltage-gated K^{+} (K_{V1.3}) channel blocker. Assays of mRNA transcripts, protein expression, and subcellular localization revealed that K_{V1.5} is the major K_{V1} channel expressed in vascular smooth muscle cells and is abundantly localized on the plasma membrane. The selective K_{V1.5} blocker diphenylphosphine oxide-1 and the K_{V1.3/1.5} blocker 5-(4-phenylbutoxy)psoralen reduced H_{2}O_{2}-elicited dilation to a similar extent as 4-aminopyridine, but the selective K_{V1.3} blocker phenoxylalkoxypsoralen-1 was without effect. In arterioles from CAD subjects, H_{2}O_{2}-induced dilation was significantly reduced, and this dilation was inhibited by paxilline but not by 4-aminopyridine, diphenylphosphine oxide-1, or 5-(4-phenylbutoxy)psoralen. K_{V1.5} cell membrane localization and diphenylphosphate oxide-1-sensitive K^{+} currents were markedly reduced in isolated vascular smooth muscle cells from CAD arterioles, although mRNA or total cellular protein expression was largely unchanged.

Conclusions: In human arterioles, H_{2}O_{2}-induced dilation is impaired in CAD, which is associated with a transition from a combined large-conductance Ca^{2+}-activated K^{+}- and K_{V1.5}-mediated vasodilation toward a large-conductance Ca^{2+}-activated K^{+}-predominant mechanism of dilation. Loss of K_{V1.5} vasomotor function may play an important role in microvascular dysfunction in CAD or other vascular diseases. (Circ Res. 2017;120:658-669. DOI: 10.1161/CIRCRESAHA.116.309491.)

Key Words: arterioles • endothelium • hydrogen peroxide • potassium channels • vasodilation

Hydrogen peroxide (H_{2}O_{2}), a diffusible reactive oxygen species (ROS), has been recognized as an important regulator of vascular tone and homeostasis under physiological and pathophysiological conditions. As an endothelium-derived hyperpolarization factor, H_{2}O_{2} induces smooth muscle cell hyperpolarization and vasodilation in human coronary arterioles (HCAs) and adipose arterioles from subjects with coronary artery disease (CAD). Other studies have also demonstrated H_{2}O_{2}-induced hyperpolarization and dilation in normal human and animal arteries. The mechanisms of H_{2}O_{2}-induced vasodilation have not been fully elucidated; however, 2 main types of K^{+} channels in vascular smooth muscle cells (VSMCs), large-conductance Ca^{2+}-activated K^{+} (BK_{Ca}) channels and voltage-gated K^{+} (K_{v}) channels, have been variably implicated in different vascular beds. For instance, BK_{Ca} channels contribute to H_{2}O_{2}-induced dilation in porcine coronary arteries. In contrast, 4-aminopyridine (4-AP)–sensitive K_{v} channels mediate dilation to H_{2}O_{2} in canine coronary arteries, rat coronary and mesenteric arteries, and porcine coronary resistance arteries. Using coronary arterioles from CAD subjects, we found that H_{2}O_{2}...
What Is Known?

- The primary mediator of shear stress–induced endothelium-dependent vasodilation is NO in patients without coronary artery disease (CAD) and hydrogen peroxide (H$_2$O$_2$) in those with CAD.
- The large-conductance Ca$^{2+}$–activated K$^+$ (BK$_{Ca}$) channel and the voltage-gated K$^+$ (K$_V$) channel have been variably implicated in H$_2$O$_2$-induced dilation in both animals and humans.
- K$_V$1.5 family channels are known to be redox regulated.
- K$_V$.5 channels in preclinical models mediate the actions of H$_2$O$_2$ and connect cardiac metabolism to myocardial blood flow.

What New Information Does This Article Contribute?

- H$_2$O$_2$ induces potent smooth muscle–mediated vasodilation through BK$_{Ca}$ and K$_V$ (especially K$_V$.5) channels in adipose arterioles from human subjects without CAD.
- H$_2$O$_2$-elicited dilation is reduced in arterioles from patients with CAD and is accompanied by a loss of K$_V$.5-dependent dilation but not BK$_{Ca}$-dependent dilation.

Nonstandard Abbreviations and Acronyms

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>4-AP</td>
<td>4-aminopyridine</td>
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<tr>
<td>BK$_{Ca}$ channel</td>
<td>large-conductance Ca$^{2+}$–activated K$^+$ channel</td>
</tr>
<tr>
<td>CAD</td>
<td>coronary artery disease</td>
</tr>
<tr>
<td>DPO-1</td>
<td>diphenyl phosphine oxide-1</td>
</tr>
<tr>
<td>EC</td>
<td>endothelial cell</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HCA</td>
<td>human coronary artery</td>
</tr>
<tr>
<td>K$_V$ channel</td>
<td>voltage-gated K$^+$ channel</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>Psora-4</td>
<td>5-(4-phenylbutoxy)psoralen</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
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Opens smooth muscle BK$_{Ca}$ channels to elicit smooth muscle hyperpolarization and relaxation. The mechanisms of dilation by H$_2$O$_2$ in subjects without CAD (non-CAD) versus those with CAD, as well as the functional consequences, remain unknown, but there is evidence that BK$_{Ca}$ and K$_V$ constitute 2 major K$^+$ currents in VSMCs isolated from non-CAD human arteries. In addition to H$_2$O$_2$-induced activation of K$_V$ channels, excessive and prolonged elevation of ROS can exert differential effects on vascular K$^+$ channel function in disease. Depending on the sensitivity of individual K$^+$ channels and the oxidative species involved, ROS can activate, inhibit, or leave unaltered K$^+$ channel function. For example, impaired functions of K$_V$ channels have been shown in various animal models of cardiovascular disease, while BK$_{Ca}$ channels may exhibit either gain or loss of function under pathophysiological conditions. The disease-associated alteration of vascular K$^+$ channel function in humans is less well understood. In this study, we tested the hypothesis that H$_2$O$_2$-elicited dilation involves different K$^+$ channels in non-CAD versus CAD arterioles, resulting in an altered vasodilatory response in CAD. Using an integrated approach comprising isolated vascular reactivity measurement, molecular and immunohistochemical analyses, and electrophysiology, we assessed the role of 2 different types of K$^+$ channels (BK$_{Ca}$ versus K$_V$) in H$_2$O$_2$-induced dilation of human arterioles from non-CAD and CAD subjects. We further identified specific K$_V$.1 channels, the major vascular K$_V$ channel subfamily, in VSMCs and examined their functional contribution to H$_2$O$_2$-induced dilation. The impact of CAD on the function of K$_V$.1 channels and potential underlying mechanisms were also determined.

Methods

Tissue Acquisition

Fresh human adipose tissues (pericardial, visceral, and subcutaneous, n=24, 68, and 28, respectively) were obtained as discarded surgical specimens from a total of 120 patients undergoing abdominal surgeries or cardiopulmonary bypass procedures and unused whole hearts (n=14) acquired from Donor Network. Patient demographic information is summarized in Online Table I.

Videomicroscopy

Arterioles (internal diameter, 100–250 μm) were carefully dissected from human adipose tissues and cannulated with 2 glass micropipettes for measurements of diameter with a video system as previously described. Arterioles were preconstricted with endothelin-1 to ±30% to 50% of the baseline internal diameter. Relaxation responses to cumulative addition of H$_2$O$_2$ (1–100 μmol/L) to the vessel bath were determined in the absence and presence of 30 minutes preincubation with various modulators, including BK$_{Ca}$ and K$_V$ blockers. At the end of each experiment, papaverine (100 μmol/L) was added to determine the maximal internal diameter for normalization of dilator responses. Unless otherwise stated, experiments were performed on endothelium-intact arterioles and in the presence of N$^\omega$-nitro-L-arginine methyl ester (100 μmol/L) and indomethacin (10 μmol/L).

Enzymatic Isolation of Vascular Cells

VSMCs were enzymatically dissociated from arteries as previously described. Cells were placed on ice and used the same day.

Patch-Clamp Recording of K$^+$ Currents

Whole-cell K$^+$ currents were measured in freshly dissociated smooth muscle cells using the standard (ruptured patch) or perforated patch-clamp method as previously described. The pipette solution contained (in mmol/L) 90 potassium aspartate, 30 KCl, 20 NaCl, 1 MgCl$_2$, 1 Mg-ATP, 1 EGTA, and 10 HEPES (pH 7.2 with KOH). The bath solution was composed of (in mmol/L) 140 NaCl, 5 KCl, 0.1 CaCl$_2$, and 1.84 mmol/L MgCl$_2$.
1 MgCl₂, 5 glucose, and 10 HEPES (pH 7.4 with NaOH). Paxilline (100 nmol/L) was added in the bath solution to further minimize BKCa currents and thus allow relative isolation of KV currents. Unless otherwise stated, all chemicals were applied to the bath through perfusion. Experiments were performed at room temperature.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction
Total RNA from human adipose arteries and arterioles (200–500 μm) was extracted, and cDNA was synthesized. The cDNA was amplified using a touch-down polymerase chain reaction (PCR) protocol with gene-specific primers. Relative Kvα1.5 gene expression was quantified with real-time PCR using pooled sample cDNA from each non-CAD and CAD group. For primer sequences refer to Online Table III.

Immunoblotting
Human adipose arteries and arterioles (200–500 μm) and coronary arteries (200–2,000 μm) were dissected, and membrane proteins were prepared with a differential centrifugation method as described previously.22 Protein samples (20 μg) were separated by 10% SDS-PAGE. Membranes were blotted with a primary antibody against a specific Kvα, β-subunit, BKCa, and Na⁺/K⁺-ATPase (1:2000 dilution), followed by a horseradish peroxidase–conjugated secondary antibody (1:20,000 dilution). Membranes were developed using the ECL Prime reagent (Amersham).

Immunohistochemistry
Freshly dissected arteries were embedded in OTC compound, frozen on dry-ice, and cut into 10-μm sections.20 Sections were blocked with 5% normal goat serum and probed with a monoclonal or polyclonal antibody against a specific Kvα-subunit, BKCa, and Na⁺/K⁺-ATPase (1:200 dilution), followed by secondary probing with an Alexa Fluor 568–conjugated goat anti-rabbit or anti-mouse IgG antibody (1:400 dilution). Sections were counterstained with DAPI and mounted in SlowFade antifade medium (Invitrogen). Images were immediately captured using a confocal fluorescence microscope (model A1-R, Nikon).

Immunocytochemistry
Freshly isolated VSMCs were fixed with 4% paraformaldehyde and permeabilized with 0.1% saponin.7 Cells were then incubated either with a monoclonal or polyclonal antibody specific to a Kvα-subunit (1:200 dilution), followed by an appropriate goat secondary antibody conjugated with Alexa Fluor 488 (1:400 dilution). Cells were then mounted and images were captured using a confocal fluorescence microscope.

Chemicals
Diphenylphosphine oxide-1 (DPO-1), paxilline, and 5-(4-phenyl-butoxy)psoralen (Psora-4) were obtained from Tocris; stromatoxin from Alomone; and tris(2-carboxyethyl)phosphine from Thermo Scientific. All other chemicals were purchased from Sigma. Stock solutions were prepared in distilled water, except for the following: 1-benzyl-4-pentylimino-1,4-dihydroquinoline, DPO-1, phenoxalkoxypsoralen-1, paxilline, and Psora-4 (ethanol); 4-AP (HCl, pH readjusted to 7.4); and indomethacin (0.1 mol/L Na₂CO₃).

Statistical Analysis
All data are presented as mean±SEM. Comparisons of concentration–response curves of isolated vessels were performed using 2-way repeated-measures ANOVA, followed by the Student–Newman–Keuls multiple-comparison test. Other comparisons were made using 1-way ANOVA or Student t test. P values <0.05 were considered statistically significant.

Results
Kᵥ Channels Contribute to H₂O₂-Induced Vasodilation in Non-CAD but Not in CAD Human Arterioles
Previous studies have shown impaired function of smooth muscle Kv⁺ channels in animal models of vascular disease, such as hypertension and metabolic syndrome.9,16–18 It remains largely unknown whether Kv⁺ channel function is similarly altered in humans. We first examined the role of BKCa and Kv channels in H₂O₂-induced dilation using adipose arterioles

![Figure 1. Role of large-conductance Ca²⁺-activated K⁺ (BKCa) and voltage-gated K⁺ (Kᵥ) channels in hydrogen peroxide (H₂O₂)-induced dilation of human adipose arterioles from non-coronary artery disease (non-CAD) and CAD subjects. H₂O₂ induced dose-dependent dilation in adipose arterioles. The dilation was reduced by paxilline (100 nmol/L), a BKCa channel blocker, in both non-CAD (A, left) and CAD (B, left) arterioles. In contrast, 4-aminopyridine (4-AP; 10 mmol/L), a general Kv channel blocker, reduced the dilation in non-CAD (A, right) but not CAD (B, right) arterioles, suggesting a loss of Kv channel function in disease. *P<0.05 vs control; n=5 to 6 vessels per group.](http://circres.ahajournals.org/Downloaded/from/http://circres.ahajournals.org/Downloaded/from/...
from non-CAD and CAD subjects. Accumulating evidence indicates that peripheral arterioles such as those from adipose tissues, which are more readily available, can serve as a surrogate for assessing systemic and coronary arteriolar function.19

In non-CAD adipose arterioles (Figure 1A), H₂O₂ (1–100 μmol/L) induced vasodilation in a concentration-dependent manner (% maximal dilation, 96±2). Treatment of arterioles with paxilline (100 nmol/L), a potent BK<sub>Ca</sub> channel blocker, induced a rightward shift of H₂O₂-induced dilation (% dilation at 10 μmol/L H₂O₂, 19±13 versus 58±8 in control, and % dilation at 50 μmol/L H₂O₂, 66±12 versus 90±3 in control, n=5; P<0.05). The general K<sub>Ca</sub> channel blocker 4-AP (10 μmol/L) caused a similar rightward shift in the response to H₂O₂ (% dilation at 10 μmol/L H₂O₂, 19±5 versus 57±10 in control, % dilation at 50 μmol/L H₂O₂, 49±8 versus 85±4 in control, n=5; P<0.05). Combined blockade of BK<sub>Ca</sub> and K<sub>Ca</sub> channels abolished dilation up to 30 μmol/L H₂O₂ (Online Figure 1A), indicating that these 2 K<sup>+</sup> channels mediate a major portion of H₂O₂-induced dilation in non-CAD human adipose arterioles. The potency of H₂O₂ (EC₅₀, 12±1 μmol/L) induced vasoconstriction in non-CAD but not in CAD subjects (Online Figure II). Dilation to H₂O₂ was abolished by high K<sup>+</sup> in both endothelium-intact and endothelium-denuded arterioles (% dilation at 100 μmol/L, 8±4 and 4±6, respectively, n=3; P<0.05 versus control), indicating H₂O₂-induced dilation is dependent on membrane hyperpolarization of VSMCs.

Treatment of adipose arterioles with exogenous catalase (1000 U/ml), an H₂O₂ metabolizing enzyme, also completely abolished H₂O₂-induced dilation (Online Figure II). An important mechanism by which H₂O₂ elicits biological effects is through oxidizing thiol groups of its target proteins.21 Indeed, dithiothreitol (3 mmol/L), a membrane-permeant and thiol-specific reducing agent, quickly reversed H₂O₂-induced dilation within 3 to 5 minutes (% dilation at 100 μmol/L H₂O₂, 1±3 versus 83±4 before dithiothreitol, n=3; P<0.05). However, tris(2-carboxyethyl)phosphine (5 mmol/L), a membrane-impermeable thiol reducing agent, had no effect. These data further confirm that H₂O₂ is a potent, specific, and reversible smooth muscle relaxant, a characteristic consistent with its putative important signaling role in human arterioles.

Detection of K<sub>Ca</sub>1 Subunit mRNA and Protein Expression in Non-CAD Human Arteries

The specific type(s) of K<sub>Ca</sub> channels expressed in human arteries remains largely unexplored. We focused on K<sub>Ca</sub>1 channels (shaker-related family), which are functionally significant K<sub>Ca</sub> channels in most animal vascular beds studied22 and are 4-AP sensitive. Figure 3 shows mRNA expression of different K<sub>Ca</sub>1 channel subunits in non-CAD adipose arterioles as assessed by reverse transcription-PCR. Several K<sub>Ca</sub>1 α (pore forming)-subunits, including 1.1, 1.2, 1.4, 1.5, and BK<sub>Ca</sub> α-subunits, were consistently found in different samples, including denuded vessels (n=5), with K<sub>Ca</sub>1.5 being the most abundantly expressed K<sub>Ca</sub>1 α-subunit. The 2 other K<sub>Ca</sub>1 α-subunits (1.3 and 1.6) were variably expressed in some but not all samples. We also detected the α-subunits that form a second type of vascular K<sub>Ca</sub> channels (2.1 and 9.3), K<sub>Ca</sub>1 β (accessory)-subunits (1.1–1.3), although these subunits were not further pursued in the following immunoblotting assays.

Using subunit-specific antibodies, we examined the protein expression of K<sub>Ca</sub>1.1 to 1.6 and BK<sub>Ca</sub> α-subunits in the membrane fraction prepared from non-CAD adipose arteries/arterioles (n=2). Consistent with mRNA expression data, K<sub>Ca</sub>1.4 and 1.5 and BK<sub>Ca</sub> channels were readily detected at the protein level (Figure 4). K<sub>Ca</sub>1.3 and 1.6 proteins were also found in non-CAD arteries/arterioles, whereas K<sub>Ca</sub>1.1 and 1.2

**Figure 2. Effects of nitric oxide synthase (NOS) and cyclooxygenase inhibition, endothelium denudation, and high K⁺ on hydrogen peroxide (H₂O₂)–induced dilation in non-coronary artery disease (non-CAD) human adipose arterioles. A.** The dilation was not affected by the NOS inhibitor N⁷-nitro-L-arginine methyl ester (L-NAME; 100 μmol/L), the cyclooxygenase inhibitor indomethacin (Indo; 10 μmol/L), alone or in combination. n=8 vessels per group. B. The dilation was not affected by removal of the endothelium but was abolished by high K⁺ (60 mmol/L), n=3 vessels per group; “P<0.05 vs control.”
proteins were not detected. The absence of Kᵥ1.2 in human vascular samples was unexpected because it is a dominant Kᵥ1α-subunit that forms channel complex with Kᵥ1.5 in rat cerebral arteries. The failure to detect Kᵥ1.1 or 1.2 in arterial samples does not seem to result from nonreactivity of the antibodies used because abundant expression of these 2 proteins was observed in human brain parallel controls.

Kᵥ1 channels (except Kᵥ1.6) are expressed as mature N-glycosylated proteins in native tissues such as brain. As shown in Figure 4, the apparent molecular mass values of Kᵥ1 proteins in human brain or adipose arterial tissues were ≈75 kDa (1.1 and 1.2), doublet around 75 kDa (1.3 and 1.5), doublet around 110 kDa (1.4), and 60 kDa (1.6). Whereas Kᵥ1.6 seems to be unmodified, Kᵥ1.1 to 1.5 channels are of higher apparent molecular weights corresponding to glycosylated or other post-translationally modified forms as previously reported in the brain or vascular tissues.

Detection of Kᵥ1 Subunit Protein Localization in Non-CAD Human Arteries

To determine the cell type–specific localization of Kᵥ1 α-subunits in human arteries, we performed frozen-section immunofluorescence staining of human adipose arterioles. KV1 proteins were labeled with red Alexa-568-conjugated secondary antibody and images were captured with a confocal fluorescence microscope. Thus, autofluorescence intrinsic to the internal elastic lamina can be detected in the green fluorescein isothiocyanate channel and used to visually divide the endothelium and smooth muscle layers. As shown in Figure 5A, Kᵥ1.5 and to a less amount Kᵥ1.4 proteins were readily detected in the smooth muscle layer. Surprisingly, these 2 proteins were also abundantly expressed in endothelial cells (ECs). A small amount of Kᵥ1.3 and 1.6 was also detected in ECs.

The limited resolution power of immunohistochemistry did not allow individual VSMCs to be distinguished even at x600 magnification. To further examine the subcellular localization of Kᵥ1 proteins, we freshly dissociated VSMCs from adipose arterioles for immunocytochemistry. As shown in Figure 5B, Kᵥ1.5 protein was predominantly localized on the plasma membrane of dissociated smooth muscle cells. Interestingly, Kᵥ1.5 was also detected on the nuclei envelope, a cellular structure that is continuous with endoplasmic reticulum and serves as a site of initial protein synthesis for some membrane proteins. Kᵥ1.4 protein was also detected in VSMCs, however, the expression of Kᵥ1.4 protein was lower than that of Kᵥ1.5 and also seemed largely intracellular.

Kᵥ1.5 as a Major Functional Kᵥ1 Channel in H₂O₂-Induced Dilation of Non-CAD but Not CAD Human Arterioles

Given that Kᵥ1.5 is 4-AP sensitive and abundantly expressed in VSMCs of human adipose arteries/arterioles, we subsequently...
examined whether this K_1 channel is functionally involved in human arteriolar dilation. Adipose arterioles from non-CAD subjects were pretreated with subtype-specific K_v channel blockers and examined for H_2O_2-induced vasodilation. We found that selective K_v1.5 channel blocker DPO-1 (1 μmol/L) markedly reduced H_2O_2-induced dilation (Figure 6A; % dilation at 10 μmol/L, 13±6 versus 55±7 in control, % dilation at 50 μmol/L, 53±9 versus 86±1 in control, n=5; P<0.05), to a similar extent as after 4-AP treatment (Figure 1). Selective K_v1.3/1.5 blocker Psora-4 (30 nmol/L) caused a similar rightward shift in H_2O_2-induced dilation, but further reduced the maximal dilation to 100 μmol/L H_2O_2 (59±5% versus 95±2% in control, n=5; P<0.05; Figure 6B). In contrast, selective K_v1.3 blocker phenoxalkoxypsoralen-1 (10 nmol/L) did not affect H_2O_2-induced dilation (Figure 6C; n=5). The K_v1.3/1.4 blocker benzyl-4-pentylimino-1,4-dihydroquinoline (3 μmol/L) slightly attenuated the dilation only at 10 μmol/L H_2O_2 (Figure 6D), suggesting a minor contribution of K_v1.4 to H_2O_2-induced dilation. In addition, H_2O_2-induced and DPO-1–inhibitable dilation was significantly reduced in arterioles treated with K_v1.5 blocker CP339818 (3 μmol/L, Figure 6D), suggesting a role of K_v1.5 in H_2O_2-induced dilation.
siRNA (Online Figure III). Together, these results suggest that \( K_v1.5 \) serves as a major 4-AP-sensitive \( K_v \) channel contributing to \( H_2O_2 \)-induced dilation in non-CAD adipose arterioles.

Compared with non-CAD arterioles, there was a slight but statistically significant reduction of \( H_2O_2 \)-induced dilation in CAD (% dilation at 10 \( \mu \)mol/L \( H_2O_2 \); 40±4 vs 59±4 in non-CAD, % dilation at 50 \( \mu \)mol/L; 71±3 vs 86±2 in non-CAD, n=18 per group; \( P<0.05 \); Figure 7A). In contrast to non-CAD arterioles (Figure 6), \( K_v1.5 \) channel blockade by either DPO-1 or Psora-4 did not alter \( H_2O_2 \)-induced dilation in CAD (Figure 7B and 7C), a finding consistent with ineffectiveness of 4-AP in CAD arterioles. These results indicate a loss of functional \( K_v \) channels, smooth muscle \( K_v1.5 \) in particular, in human arterioles during CAD.

Potential Mechanism of Impaired \( K_v1.5 \) Function in CAD Human Arteries

We further examined the potential mechanisms responsible for impaired \( K_v1.5 \) function in CAD vessels. By immunostaining of freshly dissociated VSMCs, we found that the plasma membrane staining of \( K_v1.5 \) protein was markedly reduced in CAD subjects (Figure 8A). Analysis of plasma membrane/cytoplasmic ratio of \( K_v1.5 \) fluorescence further confirmed reduced plasma membrane distribution of this protein in VSMCs from CAD subjects when compared with non-CAD subjects (1.83±0.12 and 2.62±0.15, respectively; \( P<0.05 \)). Intriguingly, \( K_v1.5 \) immunofluorescence from the nuclear envelope was comparable in CAD versus non-CAD samples.

In contrast to \( K_v1.5 \), \( BK_{Ca} \) protein localization on the plasma membrane was maintained in VSMCs from CAD subject when compared with non-CAD subjects (Online Figure IVA; calculated plasma membrane/cyttoplasmic ratio, 4.03±0.32 and 3.07±0.18, respectively). We also examined \( K_v1.5 \) and \( BK_{Ca} \) immunofluorescence in ECs isolated from adipose arterioles of non-CAD and CAD subjects (Online Figure IVA and IVB). Whole-cell immunofluorescence of \( K_v1.5 \) in ECs seemed comparable between CAD and non-CAD, although further analysis of plasma membrane/cyttoplasmic ratio is difficult because of small cell size of isolated ECs. The immunoreactivity for \( BK_{Ca} \) channel \( \alpha \)-subunits in ECs was minimal, which is in line with our previous findings that mRNA transcripts of \( BK_{Ca} \) were not detected in isolated ECs from HCAs. The specificity of \( K_v1.5 \) antibodies was confirmed by using human embryonic kidney 293 cell lines with and without \( K_v1.5 \)-DDK transfection and double-staining technique (Online Figure V).

Using end point reverse transcription-PCR analysis, the mRNA level of \( K_v1.5 \) in CAD vessels was comparable to that of non-CAD tissues when normalized to the housekeeping gene \( \beta \)-actin (Figure 8B). These results were further confirmed by the quantitative analysis of mRNA expression using real-time PCR (Figure 8B, bar graph). Analysis of relative mRNA expression of \( K_v1.5 \) normalized to the mean of 2 housekeeping genes (\( \beta \)-actin and ATP5o) did not show marked change in non-CAD and CAD vessels (intact n=6 per group and denuded n=4 per group). A slight increase (7%) in intact CAD vessels but a moderate decrease (26%) in denuded CAD vessels was observed.

We also compared the expression of \( K_v1.5 \) protein in the membrane fraction of HCAs from non-CAD and CAD patients (Figure 8C). Because vessels collected from CAD adipose tissues were usually limited and were not sufficient for membrane protein preparation, HCAs were used instead for these experiments. Our recent studies have shown that human conduit and resistance arteries express a similar profile of \( K_v1.5 \) channels at both mRNA and protein levels. The average protein expression level of \( K_v1.5 \) subunits normalized to \( Na^+/(K^+\text{-ATPase}) \) showed a trend toward an increase (but not statistically significant) in patients with CAD compared with those without (n=4 non-CAD and CAD subjects, respectively), although in 1 sample (out of 4) from CAD patients the level of \( K_v1.5 \) channel expression was much lower than average. The protein expression of \( BK_{Ca} \) did not differ in the 2 groups.

We next examined whole-cell \( K^+ \) currents in VSMCs freshly dissociated from non-CAD and CAD adipose arterioles (Figure 8D). In non-CAD VSMCs, progressive depolarizing steps from a holding potential of −70 mV elicited outward currents at membrane potentials positive to −40 mV that were subsequently reduced by DPO-1 (1 \( \mu \)mol/L). Compared with non-CAD, baseline \( K^+ \) currents in CAD were lower. Although \( K^+ \) currents were further reduced by DPO-1 in CAD, DPO-1-sensitive current density was markedly reduced when compared with non-CAD. Because \( K^+ \) currents were recorded with low-Ca\(^{2+}\) pipette and bath solutions and in the presence of the \( BK_{Ca} \) channel blocker paxilline (100 nmol/L), the contribution of \( BK_{Ca} \) to whole-cell \( K^+ \) currents was minimal. Recording of \( K_v \) currents was also confirmed by the findings that 4-AP concentration dependently.
Figure 8. Comparison of K$_V$1.5 subcellular localization, total mRNA and protein expression, and K$^+$ current in non-coronary artery disease (non-CAD) and CAD human arterioles. A, Immunofluorescence detection of K$_V$1.5 subunit proteins (green) in freshly dissociated vascular smooth muscle cells (VSMCs) from non-CAD and CAD human adipose arterioles. The plasma membrane expression of K$_V$1.5 was markedly reduced in CAD when compared with non-CAD VSMCs. Lower, Cross-sectional views of top images vertically sectioned along red lines. Images are representative of results obtained from 3 to 4 each of non-CAD and CAD tissues. B, reverse transcription-polymerase chain reaction (RT-PCR) analysis of K$_V$1.5 mRNA. Top, End point PCR gel images of K$_V$1.5, as well as β-actin control from non-CAD and CAD human adipose arterioles (n=3 per group). Lower, Relative abundance of K$_V$1.5 mRNA in non-CAD and CAD, normalized to the mean of β-actin and ATP5o by quantitative PCR (qPCR). Tissues were processed individually for mRNA extraction and cDNA synthesis before an equal amount of individual cDNA samples was pooled within each group for PCR analysis (n=6, 6, 4, and 4 for non-CAD intact, CAD intact, non-CAD denuded, and CAD denuded groups, respectively). C, Western blot detection of K$_V$1.5, large-conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) and Na$^+$/K$^+$-ATPase proteins in non-CAD (tissue number in black) and CAD (tissue number in gray) human coronary arteries (n=4 tissues per group). Right, Summarized data. D, Effect of diphenylphosphine oxide-1 (DPO-1) on voltage-elicited whole-cell K$^+$ currents in VSMCs freshly isolated from non-CAD and CAD human adipose arterioles. (Continued)
Discussion

The major new findings of this study are 3-fold. First, H₂O₂ induces potent endothelium-independent vasodilation in adipose arterioles from non-CAD subjects that is largely mediated by BKCa and KV channels. Second, KV1.5 is the major type of smooth muscle KV1 channel responsible for KV-dependent H₂O₂ dilation in non-CAD arterioles. Third, the H₂O₂-elicited response is reduced in CAD arterioles and is accompanied by a loss of KV1.5 (especially KV1.5, 1.5), but not BKCa-dependent dilation. The impaired function of KV1.5 may result from reduced cell surface localization of the channel protein without significant change of mRNA or total protein expression. Together, these results demonstrate a transition from BKCa, and KV-mediated vasodilation toward a BKCa-predominant mechanism of dilation in the human microcirculation during CAD. Although the pathophysiological significance of this K⁺ channel transition remains to be explored, the loss of KV1.5-mediated dilation to H₂O₂ or other vasodilators may represent an important mechanism contributing to microvascular dysfunction in humans with CAD or other vascular diseases.31–33

Smooth Muscle K⁺ Channels in H₂O₂-Induced Dilation

Using HCAs from subjects with CAD, we previously reported that H₂O₂ induces vasodilation by activating smooth muscle BKCa channels, an effect secondary to H₂O₂-induced dimerization of protein kinase G.7 BKCa channels have also been implicated in H₂O₂-induced dilation in different vascular beds such as porcine coronary arteries.10,11 However, other studies indicate that KV channels but not BKCa channels contribute to the dilatory effect of H₂O₂ in canine and rat coronary arteries.12 In this study, we found that H₂O₂ induces high-K⁺–sensitive dilation in non-CAD adipose arterioles that is inhibited by blockers of both BKCa (paxilline and iberiotoxin) and KV channels (4-AP). Furthermore, a combination of BKCa and KV channel blockers almost completely abolished H₂O₂-induced dilation (≤30 µmol/L H₂O₂), indicating that both BKCa and KV mediate H₂O₂-induced dilation in non-CAD human arterioles. The mechanisms by which H₂O₂ dilation involves distinct K⁺ channels in different species or vascular beds remain to be established.

There is evidence that endothelial pathways contribute to vasomotor effects of H₂O₂, including cyclooxygenase-derived prostacyclin.34 However, in human adipose arterioles, H₂O₂-induced dilation was not affected by inhibition of either NO and cyclooxygenase or endothelial denudation, confirming that the dilation is mostly smooth muscle dependent.

Membrane-permeant thiol reducing agent dithiothreitol completely blocked H₂O₂-induced dilation in human adipose arterioles, whereas membrane-impermeable thiol reagent failed to reverse the dilation. These data support an H₂O₂-mediated redox modification of K⁺ channels proposed earlier12 but further suggest an intracellular site of action. Indeed, H₂O₂ can alter the activities of ion channels such as L-type Ca²⁺ channel15 and ATP-sensitive K⁺ channel.36 H₂O₂ also activates smooth muscle KV2.1 channels in rat mesenteric arteries through a similar mechanism via S-glutathionylation.13 It remains to be determined whether H₂O₂-induced dilation involves redox modification of KV channels or alternatively other intermediate signaling proteins as we reported previously for protein kinase G in H₂O₂-induced BKCa activation.7

KV1 Channel Subtypes in Human Vasculature

The expression of specific KV subunit channel gene products and proteins varies greatly among species and vascular beds,24 and the molecular identity of KV channels, especially 4-AP–sensitive KV1 channels, in the human vasculature remains largely unknown. Using reverse transcription-PCR analysis of mRNA transcripts, we consistently detected KVα1.1, 1.2, 1.4, 1.5, 2.1, 9.3, and KVβ1.1 to 1.3 in both intact and denuded adipose arterioles from non-CAD subjects, although sample-to-sample variations were noted for KVα1.3 and 1.6 (Figure 3). Among 6 KVα1 subunits, KV1.3 to 1.6 but not KV1.1 to 1.2 were also detected at the protein level (Figure 4). Immunofluorescence assay of vessel sections and freshly dissociated VSMCs further revealed that KV1.5 is the main subunit expressed on the plasma membrane of VSMCs. KV1.4 seems mainly intracellular in adipose VSMCs. We also found KV1.5, 1.4, and to a much less extent, KV1.3 and 1.6, in the endothelium of adipose arterioles.

Pharmacological studies further demonstrated that KV1.5 is a major functional KV1 channel responsible for H₂O₂-induced dilation in non-CAD adipose arterioles (Figure 6). This is based on the findings that KV1.5-selective blocker DPO-1 reduced H₂O₂-induced dilation by the same extent as the general KV1 blocker 4-AP. KV1.5 siRNA also significantly reduced H₂O₂-induced dilation in non-CAD arterioles (Online Figure III). Selective KV1.3/1.5 blocker Psora-4 caused a similar rightward shift, whereas KV1.3 blocker failed to affect the dilation. Psora-4 further reduced the maximal dilation to 100 µmol/L H₂O₂, which may be because Psora-4 is ≥20-fold more potent for KV1.5 (IC₅₀ 7.7 µmol/L) than DPO-1 (IC₅₀ 0.2–0.3 µmol/L). Finally, KV1.3/1.4 blocker 1-benzyl-4-pentyl-imino-1,4-dihydroquinoline slightly reduced the vasodilator response to H₂O₂, suggesting a minor role of KV1.4.

In non-CAD adipose arterioles, the dilation induced by the highest concentration of H₂O₂ (100 µmol/L) was blocked by high K⁺ but only slightly blocked by the combination of BKCa and KV channel blockers, indicating the potential involvement of other K⁺ channels. Several studies reported that KV1.2 α-subunits or KV2.1/9.3 heterotetramers contribute to the dilation.
regulation of vascular tone in rodent arteries.\(^9\) In this study, we detected vascular \(K_{\text{C3.1}}\) mRNA (Figure 3) and protein expression (data not shown); however, selective \(K_{\text{C3.1}}\) and \(K_{\text{C3.1/9.3}}\) blocker stromatoxin did not alter H\(_2\)O\(_2\)-induced dilation in non-CAD arterioles (Online Figure I). Redox-sensitive \(K_{\text{C7}}\) channels represent another potential candidate, but a recent study indicate that they do not contribute to H\(_2\)O\(_2\) dilation in porcine coronary arteries.\(^9\) Further investigations are needed to elucidate the role of other \(K^+\) channels in human arterioles.

### Alteration of \(K^+\) Channel Function in CAD

Compared with non-CAD arterioles, H\(_2\)O\(_2\)-induced dilation was significantly reduced in CAD arterioles (Figure 7A). Furthermore, H\(_2\)O\(_2\)-induced dilation of CAD adipose arterioles was inhibited by only BK\(_{\text{Ca}}\) but not \(K_v\) channel blockers. To the best of our knowledge, this is the first report on the recruitment of different smooth muscle \(K^+\) channels in H\(_2\)O\(_2\)-induced vasodilation from health to disease. The expression of BK\(_{\text{Ca}}\), mRNA and protein, as well as protein localization, was not altered by CAD; a finding consistent with that of our previous study in HCA.\(^7\) We, thus, conclude that BK\(_{\text{Ca}}\) channels remain functional in CAD arterioles. In other vascular beds or species, the effect of disease on the expression and function of BK\(_{\text{Ca}}\) channels remains complex or controversial.\(^9\) For example, BK\(_{\text{Ca}}\) channel activity is increased in human VSMCs obtained from coronary atherosclerotic lesions.\(^42\) Animal studies support that BK\(_{\text{Ca}}\) channel expression is increased in hypertension, possibly as a compensatory mechanism for the downregulation of \(K_v\) channel expression.\(^18\) However, BK\(_{\text{Ca}}\) activity is reduced by exposure to high glucose or high concentrations of H\(_2\)O\(_2\) and in the porcine model of metabolic syndrome.\(^43\)

The reduction of H\(_2\)O\(_2\)-induced dilation in CAD arterioles can be mainly attributed to a loss of \(K_v\) especially \(K_v_{\text{1.5}}\), channel function in VSMCs. In contrast to non-CAD adipose arterioles, blocking of \(K_v_{\text{1.5}}\) or \(K_v_{\text{1.1}}\) channels did not affect H\(_2\)O\(_2\)-induced dilation in CAD vessels (Figures 1 and 7). Alternations in vascular \(K_v\) channel function and expression have also been reported in other pathological conditions such as pulmonary\(^45\) and systemic\(^46\) hypertension, metabolic syndrome,\(^47\) and diabetes mellitus,\(^48\) but the identities of individual \(K_v\) channels involved have not been well established. In human atrial myocytes, chronic atrial fibrillation, which is associated with oxidative stress or elevated ROS, reduces \(K_v_{\text{1.5}}\) (\(I_{\text{Kw}}\)) expression.\(^49\)

The precise mechanisms of impaired \(K_v\) function under pathological conditions remain poorly understood. By examining \(K_v_{\text{1.5}}\) mRNA/protein expression and subcellular localization and \(K_v\) currents, we provide initial evidence that a reduced plasma membrane expression of \(K_v_{\text{1.5}}\) in VSMCs, rather than a change of mRNA and total protein synthesis, may be mainly responsible for the reduced \(K_v_{\text{1.5}}\) channel function in CAD. There is accumulating evidence that ion channels can recycle between the cytosol and cell membrane, and this dynamic recycling determines the number of functional channels present in the plasma membrane.\(^50\)\(^-\)\(^52\) For example, a fraction of \(K_v_{\text{1.5}}\) channels on the cell membrane is rapidly internalized with a half time of \(\sim 10\) minutes and some return to the surface with a half time of \(\sim 30\) minutes.\(^52\) In a HL-1 cell line, elevated ROS induces fairly rapid (within \(60\) minutes) reduction of surface expression of \(K_v_{\text{1.5}}\).\(^53\) It remains to be tested in future studies whether prolonged elevation of ROS during CAD modulates cellular trafficking of smooth muscle \(K_v_{\text{1.5}}\) channels to reduce their membrane expression in human arterioles.

### Potential Study Limitations

We found that \(K_v_{\text{1.5}}\) and \(K_v_{\text{1.4}}\) channels are expressed in ECs of human adipose arterioles. It is thus possible that H\(_2\)O\(_2\) may activate endothelial \(K_v\) channels to induce endothelial hyperpolarization and subsequent vasodilation. Although the expression and function of endothelial \(K_v\) channels are poorly understood,\(^17\) there is evidence on the role of \(K_v\) channels in endothelium-dependent hyperpolarization and dilation in arteries such as porcine coronary arteries\(^54\) and guinea pig coronary and carotid arteries.\(^55\)\(^,\)\(^56\) However, H\(_2\)O\(_2\)-induced dilation of adipose arterioles is largely smooth muscle dependent, and, therefore, the contribution of endothelial \(K_v\) channels seems unlikely, at least in the present experimental settings. Nevertheless, we have not excluded a potential role for endothelial \(K_v\) channels in the dilation to H\(_2\)O\(_2\) under other conditions (eg, endogenous H\(_2\)O\(_2\) generated in ECs).

This study used arterial tissues from human subjects with a variety of conditions that can affect vasodilator responses. By necessity, the non-CAD tissue samples are often collected from subjects with diverse diseases and thus are not true normal controls. To minimize potential confounding effects of underlying disease, only subjects with no more than \(1\) risk factor for CAD and no evidence of CAD were classified as non-CAD for this study. We further addressed this limitation with a statistical approach, identifying and controlling the influence of individual risk factors. Analysis of risk factors for CAD did not show an impact because of hypertension, hyperlipidemia, sex, or age on H\(_2\)O\(_2\)-induced dilation. There is an interaction between CAD and body mass index on EC\(_50\) of H\(_2\)O\(_2\) response (Online Figure VIII); however, this interaction may require further investigation in another cohort with a larger sample size. We also used adipose arterioles from several regions of the body; however, pilot studies indicate that H\(_2\)O\(_2\)-induced dilation was similar among visceral, subcutaneous, or pericardial adipose arterioles within non-CAD and CAD subject groups (Online Figure VII).

A limitation of the functional studies with regard to H\(_2\)O\(_2\)-induced dilation is that we use a largely pharmacological approach, which may have off-target effects on other proteins such as other families of \(K_v\) channels. To mitigate this possibility, we used several chemically distinct blockers of \(K_v\) channels, and the results obtained with these blockers invariably pointed toward an important role of \(K_v_{\text{1.5}}\) in H\(_2\)O\(_2\)-induced dilation of non-CAD adipose arterioles. In addition, we found that \(K_v_{\text{1.5}}\)-targeted blockers had no significant effects on CAD arterioles, suggesting that any nonspecific effects should be minimal in this study. A recent study also reported a preferential inhibition of \(K_v_{\text{1.5}}\) by DPO-1 using VSMCs dissociated from wild-type versus \(K_v_{\text{1.5}}\) knockout mice.\(^57\) Nevertheless, a molecular approach using \(K_v_{\text{1.5}}\) siRNA was also included to determine the specific role of \(K_v_{\text{1.5}}\) in H\(_2\)O\(_2\)-induced dilation of non-CAD arterioles.

### Clinical Implications

Microvascular dysfunction has been implicated in a wide variety of pathologies, including obesity-associated insulin resistance, inflammation in visceral fat, and ischemic heart
In the absence of CAD or its risk factors, traditional vasodilator factors (ie, NO and prostacyclin) are important for vasodilation in HCA and adipose arterioles. With the onset of CAD, the dilation is switched to a new mechanism requiring the release of H$_2$O$_2$ from ECs and subsequent smooth muscle hyperpolarization. In this study, we demonstrate a reduced smooth muscle–mediated dilation resulting from a functional transition from BK$_{Ca}$- and K$_v$-mediated vasodilation to predominantly BK$_{Ca}$-mediated mechanism of dilation in human arterioles during CAD. These results, thus, reveal another potentially important aspect of microvascular dysfunction where cardiovascular disease not only changes the primary endothelial vasodilators (NO to H$_2$O$_2$) but also affects smooth muscle K$^+$ channels that respond to vasodilator factors. Our unpublished observations also show altered kinetics of dilation in healthy vessels in the presence of 4-AP, suggesting that in addition to reduced overall peak dilation in CAD versus health, the time-to-peak dilation may be altered because of changes in the expression/function of K$_v$ channels.

Impaired K$_v$ channel function will negatively impact local blood flow regulation in response to not only endothelial factors but also to tissue factors such as β-adrenergic transmitters and other metabolic factors. This may induce deficit in regional blood supply and have detrimental effect on the function of tissues such as the heart where a tight coupling of blood perfusion and cell metabolism is essential. Regional organ perfusion, especially in the heart where near-maximal extraction of oxygen occurs at rest, requires tight, beat-to-beat regulation of blood flow to nearly instantaneously match oxygen supply with tissue’s metabolic demand. In health, expression of different types and subtypes of K$^+$ channels and their axillary subunits with varying activation/inactivation kinetic properties contribute to this precise vasoregulatory control. With the onset/progression of disease, changes in function/expression of these channels would result in a dysregulation of blood flow and, in consequence, lead to a mismatch in oxygen supply and demand. Over time, these brief but repetitious states of tissue hypoxia could induce local inflammation, fibrosis, and eventually adverse tissue remodeling. The molecular mechanisms of impaired K$_v$ function in CAD, as well as exact causal factors responsible for the K$^+$ channel remodeling, remain to be determined. A better understanding of these mechanisms may provide new strategies to improve or even restore normal K$_v$ channels and cardiovascular function.

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**Disclosures**

None.

**References**


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Contribution of Kv1.5 Channel to Hydrogen Peroxide–Induced Human Arteriolar Dilation and Its Modulation by Coronary Artery Disease
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Supplemental Material

Supplemental Methods

Tissue Acquisition
Fresh human adipose tissues (pericardial, visceral and subcutaneous, \(n=24, 68\) and \(28,\) respectively.) were obtained as discarded surgical specimens from a total of 120 patients undergoing abdominal surgeries or cardiopulmonary by-pass procedures, and unused whole hearts (\(n=14\)) from Donor Network. After surgical removal, specimens were placed in ice-cold HEPES buffer or in a cardioplegic buffer solution (for heart tissue) and immediately transferred to the laboratory for isolated vessel studies. De-identified patient demographic data were collected using the Generic Clinical Research Database (GCRD) at the Medical College of Wisconsin. All protocols were approved by the Institutional Review Board of the Medical College of Wisconsin and Froedtert Hospital. Patient demographic information is summarized in Online Table I.

Cell Culture and Transfection
HEK-293 cells were provided by Dr. David Wilcox (Medical College of Wisconsin) and grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin G, and 100 µg/mL streptomycin at 37 °C and 5% CO\(_2\). HEK-293 cells were used for the present study between passages 6 and 10.

The full-length human \(K\alpha_1.5\) cDNA clone was obtained from Origene and subcloned into pCMV6 mammalian expression vectors as a fusion protein with the COOH-terminus of \(K\alpha_1.5\) tagged with a FLAG tag. The nucleic sequence of \(K\alpha_1.5\)-FLAG constructs was verified by direct DNA sequencing. \(K\alpha_1.5\)-FLAG coding plasmids were isolated by an endotoxin-free plasmid kit (Qiagen), and transiently transfected into HEK-293 cells using the lipid-mediated lipofectamine 2000 kit (Invitrogen) according to the manufacturer’s protocols. In brief, cells were plated in 60-mm petri dishes 16-24 h before transfection. Cells were transfected with 2 µg plasmid DNA/60-mm dish and used 24-48 h after transfection.

Enzymatic Isolation of Vascular Cells
Smooth muscle cells (SMCs) were enzymatically dissociated from arteries as previously described.\(^1,2\) In brief, artery segments were cut into small rings and incubated for 10 min at room temperature in a low-Ca\(^{2+}\) dissociation solution consisting of (in mmol/L) 140 NaCl, 5.0 KCl, 0.05 CaCl\(_2\), 1.0 MgSO\(_4\), 10 glucose, and 10 HEPES, with 0.1% BSA (pH 7.4). The solution was carefully removed and vessel segments were incubated with papain (1.0 mg/mL) and dithiothreitol (0.5 mg/mL) in dissociation solution for 15 min at 37°C, followed by incubation with collagenase (Sigma blend H; 1.0 mg/mL), trypsin inhibitor (1 mg/mL), and elastase (0.5 mg/mL) in dissociation solution for 15-30 min at 37°C. All enzymes and chemicals were purchased from Sigma. Artery segments were then gently triturated to release smooth muscle cells. The dissociated cells were washed twice in dissociation solution by centrifugation at 250 \(\times\) g for 5 min. Cells were stored on ice or at 4°C and used the same day for immunocytochemistry as described below.

Videomicroscopy
Small arterioles (internal diameter 100-250 µm) from fresh human adipose tissue were carefully dissected. Vessels were cannulated with two glass micropipettes for continuous measurements of diameter with a video system as previously described.\(^1\) Vessels were pressurized to an intramural pressure of 60 mmHg under no-flow conditions and equilibrated for 1 hour at 37°C in modified Krebs-physiological saline solution (in mmol/L; NaCl 123, KCl 4.7, MgSO\(_4\) 1.2, CaCl\(_2\) 2.5, NaHCO\(_3\) 19, KH\(_2\)PO\(_4\) 1.2, EDTA 0.026, and glucose 11) continuously gassed with a mixture of 21% O\(_2\), 5%, CO\(_2\) and 74% N\(_2\) to maintain pH 7.4.

To study vasodilator response, arterioles were preconstricted with endothelin-1 (0.1-0.5 nmol/L) to approximately 30% to 50% of the baseline internal diameter. After the contraction
reached steady state, relaxation responses to cumulative concentrations of H$_2$O$_2$ (1-100 μmol/L) were determined in the absence and presence of 20-30 minutes preincubation with one of the following modulators alone or in combination: N$^2$-nitro-L-arginine methyl ester (L-NNAME, 100 μmol/L), a nitric oxide synthase (NOS) inhibitor; indomethacin (10 μmol/L), a cyclooxygenase inhibitor; 4-aminopyridine (4-AP, 10 mmol/L), a non-selective voltage-dependent K$^+$-channel blocker; paxilline (100 nmol/L), a specific and cell-permeable BK$_{ca}$ channel blocker; iberiotoxin (100 nmol/L), a specific but not cell-permeable BK$_{ca}$ channel blocker; diphenyl phosphine oxide-1 (DPO-1, 1 μmol/L), selective a K$_{v}α1.5$ channel blocker; 5-(4-phenylbutoxy)psoralen (Psora-4, 30 nmol/L), a selective K$_{v}α1.3/α1.5$ channel blocker; phenoxyalkyoxypsoralen-1 (PAP-1, 10 nmol/L), a selective K$_{v}α1.3$ channel blocker; 1-benzyl-4-pentylimino-1,4-dihydroquinoline (CP339818, 3 μmol/L), a selective K$_{v}α1.3/α1.4$ channel blocker; Stromotaxin (100 nmol/L), a selective K$_{v}α2.1$, K$_{v}α2.1/α9.3$ blocker; DL-dithiothreitol (DTT, 3 mmol/L), a membrane-permeant thiolspecific reducing agent; Tris(2-carboxyethyl)phosphine (TCEP, 5 mmol/L), a membrane-impermeable thiol-reducing agent; and catalase (1000 U/mL), a H$_2$O$_2$ metabolizing enzyme. To examine the role of smooth muscle hyperpolarization in H$_2$O$_2$-induced dilation, vessels were constricted with a High-K$^+$ (60 mmol/L) Krebs-PSS.

In some vessels, the endothelium was removed by perfusing 3-5 mL of air through the lumen. The endothelium was considered intact if acetylcholine (10 μmol/L) caused >80% relaxation of endothelin-1-constricted vessels and effectively denuded if acetylcholine induced <10% relaxation. At the end of each experiment, papaverine (100 μmol/L), an endothelium independent vasodilator, was added to the vessel bath to determine the maximal internal diameter for normalization of dilator responses. Unless otherwise stated, experiments were performed on endothelium-intact arterioles and in the presence of L-NNAME and Indomethacin.

**RNA extraction and reverse transcription-polymerase chain reaction**

Human adipose arteries and arterioles (200-500 μm) freshly dissected were snap-frozen in liquid nitrogen and store at -80°C until use. In some vessels, the endothelium was removed by gentle scraping of the lumen of vessels. Total RNA was extracted with RNeasy Fibrous Tissue Mini Kit (Qiagen) and cDNA synthesized using SuperScript III reverse transcriptase (Invitrogen), according to the manufacturer’s instructions. Unless otherwise stated, a total of 2-5 ng input RNA (cDNA) was amplified in a Peltier thermal cycler (MJ Research, model PTC-200) using Platinum PCR Supermix (Invitrogen) and a 40-45-cycle touch-down protocol. The gene-specific primers were synthesized by IDT Integrated DNA Technologies, Inc. (Coralville, Iowa), with detailed sequence information in Supplemental Online Table II. Two negative controls, without reverse transcription (RT-) and without template (H$_2$O), were amplified in parallel by using K$_{v}α9.3$ primers. Human brain total RNA samples from a normal donor (Agilent Technologies, Santa Clara, CA) were included as a positive control. Human brain tissue is known to express all 11 subunits of K$_{v}$ channels and BK$_{ca}$ channel examined in this study.

Quantitative PCR was performed using CFX96 C1000 Thermal Cycler (BioRad). The reactions were conducted in a 20 μL volume containing 10 μL 2x All-in-One SYBR Green qPCR mix (GeneCopoeia) or SsoAdvanced Universal SYBR Green Supermix (for siRNA qPCR, BioRad), 2 μL (each for forward and reverse) of K$_{v}α1.5$-specific primers, nuclease-free water and 2 μL of pooled or individual (for siRNA qPCR) sample cDNA (2 ng). Pooled sample cDNA was prepared by extracting mRNA and synthesizing cDNA from each tissue sample individually. An equal amount of cDNA from each sample was then added to create pooled samples for each non-CAD and CAD group. Cycle threshold (Ct) values of products were determined using Bio-Rad CFX Manager 3.1 software (BioRad). Relative K$_{v}α1.5$ gene expression was normalized to average of β-actin and ATP5o genes. For primer sequences refer to Online Table III. The thermal profile employed 1 cycle of initial denaturation at 95°C for 5 min, 40 cycles of denaturation at 95°C for 10 sec, annealing at 60°C for 30 sec and extension at 72°C for 15 sec. Melting curve analysis was performed immediately after the 40th cycle at 72 to 95°C with 0.5 degree increments for 5
sec. Comparative Ct Method was used to calculate relative expression of Kvα1.5 subunit, where CAD was normalized to non-CAD for both intact and denuded pooled samples.

**Preparation of membrane protein**
Human adipose arteries/arterioles (200-500 µm) were dissected out from adipose tissues, cleaned off fat and connective tissue, and placed in ice-cold HEPES buffer. Vessel segments were frozen in liquid nitrogen if not used immediately. For some experiments, coronary arteries (200-2,000 µm) were dissected from donor hearts using a similar procedure. Arterial membrane proteins were prepared with a differential centrifugation method as described previously. All procedures were performed on ice or at 4°C. Briefly, vascular tissues were homogenized with a Tenbroeck glass homogenizer in 2-5 volumes of an ice-cold homogenization buffer (in mmol/L; 20 HEPES pH 7.4, 250 sucrose, 1 EDTA, supplemented with protease and phosphatase inhibitor cocktail), and sonicated at a power setting of 2-3 with a 3-mm microtip at 10 seconds pulses x 3 with 1-2 minute(s) pause on ice (Fisher Scientific Sonci Dismembrator model D100). The homogenates were centrifuged at 3,000 × g for 10 minutes, the supernatants were transferred to a new tube and centrifuged at 10,000 × g for 20 minutes, and the supernatants were then centrifuged at 100,000 × g for 90 minutes (Beckman TLA-100.3 rotor, 50,000 rpm). The pellets containing crude membrane fraction were re-solubilized in a detergent-containing extraction buffer containing (in mmol/L) 25 Tris-HCl pH 7.4, 150 NaCl, 1 EDTA, 1% NP40, 0.5% sodium deoxycholate, and 5% glycerol, supplemented with protease and phosphatase inhibitors. Protein samples were aliquoted, frozen in liquid N₂, and stored at −80°C until use. Human brain membrane proteins extracted from a normal donor were obtained from BioChain (Newark, CA).

**Immunoblotting**
Protein samples (20 µg) were separated by SDS-PAGE on 10% gels and transferred to PVDF membranes. Membranes were blocked by 5% NFDM or BSA for 1 hour at room temperature, and incubated overnight at 4°C with one of the following primary antibodies: Kvα1.1 (75-007, NeuroMab K20/48 clone), Kvα1.2 (73-008, NeuroMab K14/16 clone), Kvα1.3 (75-009, NeuroMab L23/27 clone), Kvα1.4 (75-010, NeuroMab K13/31 clone), Kvα1.5 (APC-150, Alomone), Kvα1.6 (75-012, NeuroMab K19/36 clone), BKCa (75-022, NeuroMab L6/60 clone), and Na+/K+-ATPase (ANP-001, Alomone); 1:2,000 dilution of each antibody in TBST with 5% NFDM or BSA. Blots were then washed with TBST prior to the addition of a HRP-conjugated secondary antibody (1:20,000 dilution in TBST with 5% NFDM or BSA) for 1 hour at room temperature. Membranes were developed using the ECL Prime reagent (Amersham).

**Immunohistochemistry**
Freshly dissected arteries were embedded in OTC compound, frozen on dry-ice, and cut into 10-µm sections. Some tissue sections were stained with hematoxylin and eosin to confirm the intact vascular structure before further processing for immunofluorescence staining. Tissue sections were washed in PBS and blocked with 5% normal goat serum in PBS containing 0.3% Triton X-100. Sections were then probed with a monoclonal or polyclonal antibody against a specific Kvα subunit (1:200 dilution) overnight at 4°C, followed by secondary probing with an Alexafluor 568-conjugated goat anti-rabbit or goat anti-mouse IgG antibody for 1 h at room temperature. After several washes with PBS, sections were counterstained with DAPI, a fluorescence nuclear probe, and mounted in SlowFade antifade medium (Invitrogen). Images were immediately captured using a confocal fluorescence microscope (model A1-R, Nikon) with a 60× (NA 1.40) oil objective.

**Immunocytochemistry**
Freshly isolated SMCs, along with sheets of isolated ECs, were allowed to attach to poly-L-lysine-coated 12-mm coverslips for 30 min at 4°C as previously reported. After several washes with phosphate buffered saline (PBS), cells were fixed with 4% paraformaldehyde for 10 min at room
temperature. Cells were then rinsed 3 times with PBS containing 50 mmol/L NH₄Cl, and blocked with 1% BSA/5% normal goat serum/0.1% saponin in PBS for 30 min. For immunodetection of Kᵥ1α subunits, cells were incubated overnight at 4°C with one of the following monoclonal or polyclonal antibodies (1:200 dilution): Kᵥα1.3 (75-009, NeuroMab L23/27 clone), Kᵥα1.4 (75-010, NeuroMab K13/31 clone), Kᵥα1.5 (APC-150, Alomone), Kᵥα1.6 (75-012, NeuroMab K19/36 clone), and BKᵥCa (75-022, NeuroMab L6/60 clone). Afterward, cells were rinsed with 1% BSA/0.1% saponin in PBS, followed by incubation for 1 h at room temperature with an appropriate goat secondary antibody conjugated with Alexa Fluor 488 (Invitrogen). The labeled cells were post-fixed with 4% paraformaldehyde for 10 min, and counterstained with the nuclear dye DAPI (300 nmol/L) for 5 min. After several washes with PBS, cells were mounted in SlowFade antifade medium (Invitrogen) and images immediately captured using a confocal fluorescence microscope (model A1-R, Nikon) with a 100× (NA 1.45) oil objective.

To confirm the specificity of Kᵥα1.5 antibodies, HEK293 cells transiently expressing hKᵥ1.5-DDK fusion protein and non-transfected HEK293 cells were incubated with rabbit anti-Kᵥα1.5 (1:200 dilution; APC-150, Alomone) and mouse anti-DDK/FLAG (1:500 dilution; TA50011-100, Origene 4C5 clone) followed by Alexa 488-labeled goat anti-rabbit and Alexa 561-labeled goat anti-mouse secondary antibodies, using a immunostaining procedure similar as described above for freshly isolated vascular cells.

Fluorescence data were analyzed using ImageJ (version 1.45s), a public domain program developed at the NIH. Background fluorescence taken just outside of the cells was first subtracted from each image. The fluorescence intensities of eight rectangular user-defined regions of interest (ROIs) both over the plasma membrane (5 x 30 pixels) and in adjacent cytoplasmic areas (5 x 30 pixels) in each cell were measured and averaged to calculate the ratio of plasma membrane to cytoplasmic intensity. Only those ROIs that are free of artifactual staining were included in the analysis. For the analysis of Kᵥ1.5 and BKᵥCa protein localization, the fluorescence ratio was calculated from 6-10 cells each from non-CAD (n=5 and 3 for Kᵥ1.5 and BKᵥCa, respectively) and CAD (n=5 and 3 for Kᵥ1.5 and BKᵥCa, respectively) subjects. Cells were freshly dissociated from 5-6 pooled segments of arterioles from each subject. The measurement of this ratio is a commonly used method to assess membrane accumulation of protein molecules in cells and also has been used in our previous studies.

**Kᵥ1.5 siRNA transfection with reversible permeabilization**

Human arteries/arterioles (200-500 μm) freshly dissected from each non-CAD subject were divided into two groups for transfection with control siRNA (ON-TARGETplus non-targeting control pool, D-001810-10-05, GE/Dharmacon) or siRNA against Kᵥ1.5 (ON-TARGETplus KCNA5 siRNA smart pool, L-006215-00-0005, GE/Dharmacon). Transfection of smooth muscle with control siRNA or Kᵥ1.5-specific siRNA were performed using a reversible permeabilization method as previously described. In brief, arterial segments were sequentially incubated in the following solutions using a 6-well plate with gentle rocking: 1) 20 min at 4°C in Solution I (in mmol/L; 10 EGTA, 120 KCl, 5 ATP, 2 MgCl₂, 20 TES, pH 6.8 at 4°C); 2) 3 h at 4°C in Solution II (in mmol/L; 120 KCl, 5 ATP, 2 MgCl₂, 20 TES, pH 6.8 at 4°C) containing 100 nmol/L siRNA; 3) 30 min at 4°C in Solution III (in mmol/L; 120 KCl, 5 ATP, 10 MgCl₂, 20 TES, pH 6.8 at 4°C) containing 100 nmol/L siRNA. To reverse permeabilization, arterioles were then incubated for 15 min at room temperature in Solution IV (in mmol/L; 140 NaCl, 5 KCl, 10 MgCl₂, 5.6 glucose, 2 MOPS, pH 7.1, 22°C) containing 100 nmol/L siRNA. After which, the Ca²⁺ concentration of Solution IV was gradually increased from nominally Ca²⁺-free to 0.01, 0.1, and 1.6 mmol/L every 15 min. Using sterile technique, arterioles were then transferred to DMEM/F-12 (1:1 mixture) culture medium supplemented with 1 mmol/L L-glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin, and incubated for additional 24-48 hours in a cell culture incubator (at 30°C in 95% air, 5% CO₂). Arterial samples were assessed for Kᵥ1.5 mRNA and protein knockdown using RT-
PCR and immunofluorescence as described above. Separated arterial segments were examined for DPO-sensitive and H$_2$O$_2$-induced vasodilator response.

**Patch-Clamp Recording of K$^+$ Currents**

Whole-cell K$^+$ currents were measured in freshly dissociated smooth muscle cells using the standard (ruptured-patch) or perforated patch-clamp method as previously described. Patch-clamp was performed with an Axopatch 200B amplifier and pClamp 10 software (Molecular Devices). Patch pipettes were fabricated from borosilicate glass capillaries (glass type 7052) and fire-polished to produce 2 to 4 MΩ tip resistance. Outward K$^+$ currents were elicited by progressive 10-mV depolarizing voltage steps (300-ms duration, 5-s intervals) from a holding potential of -70 mV to potentials ranged between -70 and +60 mV. In some experiments, cells were held at -70 mV and linear voltage ramps from -100 to +100 mV (300-ms duration) were applied at a frequency of 0.2 Hz. Currents were filtered at 1 kHz and sampled at 5 kHz. Total cell capacitance and series resistance were estimated by integrating the uncompensated capacitative transients generated by a 10-mV step pulse, and by adjusting the amplifier whole-cell capacitance and series resistance controls to eliminate the resulting current transitions. Series resistance in ruptured whole-cell recordings was <8 MΩ and was not corrected. Amplitude of currents in this configuration was always <1000 pA, resulting in a voltage error of <8 mV. Series resistance in perforated-patch recording was <20 MΩ, and was compensated when currents were >400 pA. All data were corrected for a 10-mV liquid junction potential after the experiment. For each experimental condition in a cell, at least 3 trials were performed and currents were then averaged during analysis. To account for differences in cell membrane surface area, current densities (pA/pF) were calculated by normalization of average whole-cell current to cell capacitance.

The bath solution was composed of (in mmol/L) 140 NaCl, 5 KCl, 0.1 CaCl$_2$, 1 MgCl$_2$, 5 glucose, and 10 HEPES (pH 7.4 with NaOH). The pipette solution contained (in mmol/L) 90 potassium aspartate, 30 KCl, 20 NaCl, 1 MgCl$_2$, 1 Mg-ATP, 1 EGTA, and 10 HEPES (pH 7.2 with KOH). The concentration of free Ca$^{2+}$ in the pipette solution is estimated to be around 10 nM. Contaminating currents through large-conductance, Ca$^{2+}$-activated K$^+$ channels (BK$_{Ca}$) were minimized by lowering free Ca$^{2+}$ concentrations in the pipette and bath solutions, and by including the BK$_{Ca}$ channel blocker paxilline (100 nmol/L) in the bath solution. Perforated patches were used in some experiments by backfilling amphotericin B (100-150 µg/ml in the pipette solution) up to 500-µm distance from the tip of the pipette. Currents were recorded first under control conditions and then after bath perfusion of the drug of interest at the indicated final concentrations. All chemicals were applied to the bath solution by a gravity perfusion system at a flow rate of 1 ml/min. Experiments were performed at room temperature.

**Chemicals**

DPO-1, paxilline, and Psora-4 were obtained from Tocris, stromatoxin from Alomone, and TCEP from Thermo Scientific. All other chemicals were purchased from Sigma. Stock solutions were made in distilled water, except for the following: CP339818, DPO-1, PAP-1, paxilline, and Psora-4 (ethanol); 4-AP (HCl, pH readjusted to 7.4); and indomethacin (0.1 mol/L Na$_2$CO$_3$).

**Statistical Analysis**

All data are presented as mean±SEM. Comparisons of concentration-response curves of isolated vessels were performed using 2-way repeated measures ANOVA, followed by the Student-Newman-Keuls multiple-comparison test. Other comparisons were made using 1-way ANOVA or Student t-test. P values <0.05 were considered statistically significant.

For analysis of selected vasodilator responses (e.g., H$_2$O$_2$-induced dilation in non-CAD versus CAD group), individual concentration-response curves were fitted to the following 4-parameter sigmoidal logistic equation: $Y_i = P1_i + P2/(1 + \exp(P3 \times (\log(X_i) - P4))) + e_i$, where $Y_i$ = vasodilator response, $X_i$ = molar concentration of vasodilator, $P1_i$ = lower plateau response subject random
effect \{Normal(\mu_1, \sigma_1^2)\}, P2 = \text{difference between the lower and the maximal plateau responses},
P3 = \text{slope of concentration-response curve}, P4 = \text{logarithm of the molar concentration of}
vasodilator required to produce 50\% of the maximal response (logEC_{50}), and \(\epsilon_i\) = independent
error \{Normal(0, \sigma_e^2)\}.^{11} EC_{50} values were calculated as \(-\log EC_{50}\). Nonlinear regression analysis
with mixed effects were performed to assess whether coronary artery disease (CAD), risk factors
for CAD, age, or gender influenced EC_{50}. Statistical analyses were performed with SAS version
9.4 (SAS Institute Inc., Cary, NC, USA). All other analyses were conducted using the statistical
programs in SigmaPlot, version 12.

Supplemental Results

Online Figure I. H\textsubscript{2}O\textsubscript{2}-induced dilation of non-CAD human adipose arterioles. A, Effect of
BK\textsubscript{Ca} blockade alone and in combination with KV blockade on H2O\textsubscript{2}-induced dilation. BK\textsubscript{Ca} channel
blocker iberiotoxin (IbTX; 100 nmol/L) also reduced the dilation, and the combination of IbTX and
4-AP (10 mmol/L), a KV channel blocker, markedly diminished the dilation except for the highest
dose of H\textsubscript{2}O\textsubscript{2} (100 μmol/L). \(n=6-7\) vessels/group. *\text{P}<0.05 versus control, *\text{P}<0.05 versus IbTX. B, 
Effect of 4-AP on H\textsubscript{2}O\textsubscript{2}-induced dilation of arterioles. Over-constriction of arterioles (70\%±3\% from
baseline) by 4-AP plus a higher concentration of ET-1 did not further enhance the inhibitory effect
of 4-AP on H\textsubscript{2}O\textsubscript{2}-induced dilation (\(n=5, *\text{P}<0.05\) versus control). C, H\textsubscript{2}O\textsubscript{2}-induced concentration-
dependent dilation in non-CAD arterioles was fitted with a 4-parameter sigmoidal equation. EC_{50}
of H\textsubscript{2}O\textsubscript{2} was then calculated from the fitted curve. \(n=18\) vessels. D, In non-CAD arterioles, selective KV2.1 and KV2.1/9.3 blocker stromatoxin (100 nmol/L) did not affect H\textsubscript{2}O\textsubscript{2}-induced dilation. \(n=4\) vessels/group.

Online Figure II. Effects of catalase and thiol-reducing agents on H\textsubscript{2}O\textsubscript{2}-induced dilation in
non-CAD human adipose arterioles. A, The dilation was abolished by H\textsubscript{2}O\textsubscript{2} metabolizing enzyme catalase (1000 U/mL) in both intact and denuded vessels. B, The dilation was completely reversed by DTT (3 mmol/L), a membrane-permeable thiol-specific reducing agent, but not by
TCEP (5 mmol/L), a membrane-impermeable thiol-reducing agent. DTT had no effect on the
dilation induced by papaverine (Pap; 100 μmol/L), which was added after H\textsubscript{2}O\textsubscript{2}. *\text{P}<0.05 versus
control (A) or 100 μmol/L H\textsubscript{2}O\textsubscript{2} (B); \(n=3-5\) vessels/group.

Online Figure III. Effects of KV\textsubscript{1.5} siRNA on H\textsubscript{2}O\textsubscript{2}-induced dilation in non-CAD human
adipose arterioles. A, H2O\textsubscript{2}-induced dilation was significantly reduced in arterioles treated with
KV\textsubscript{1.5} siRNA (% dilation at 10 μmol/L H\textsubscript{2}O\textsubscript{2}, 3±3 vs. 28±5 with control siRNA, % dilation at 50 μmol/L H\textsubscript{2}O\textsubscript{2}, 18±8 vs. 63±5 with control siRNA, and % dilation at 100 μmol/L H\textsubscript{2}O\textsubscript{2}, 53±5 vs. 83±4 in control-siRNA, \(n=4\) vessels/group, *\text{P}<0.05). B, In arterioles treated with control siRNA, the
selective KV\textsubscript{1.5} blocker DPO-1 significantly reduced the dilation confirming KV\textsubscript{1.5} channels were
functional in these vessels (% dilation at 10 μmol/L H\textsubscript{2}O\textsubscript{2}, 12±4 vs. 28±5 with control siRNA, %
dilation at 50 μmol/L H\textsubscript{2}O\textsubscript{2}, 31±2 vs. 63±5 with control siRNA, and % dilation at 100 μmol/L H\textsubscript{2}O\textsubscript{2},
62±6 vs. 83±4 in control siRNA, \(n=4\) vessels/group, *\text{P}<0.05). C, In contrast, the selective KV\textsubscript{1.5}
blocker DPO-1 did not affect the dilation in KV\textsubscript{1.5} siRNA-treated arterioles. \(n=4\) vessels/group. D,
Three representative gel images of end-point RT-PCR analysis of KV\textsubscript{1.5} gene knockdown (left). Summarized quantitative RT-PCR data (right) indicate that KV\textsubscript{1.5} mRNA expression (normalized
to average of β-actin and ATP5α genes) was significantly reduced in KV\textsubscript{1.5} siRNA-treated vessels
(\(n=4\) vessels/group, *\text{P}<0.05). Input RNA (cDNA), 20 pg for end-point PCR (left) and 2 ng for
quantitative PCR (right), respectively.
Online Figure IV. Immunofluorescence detection of BK<sub>Ca</sub> and K<sub>V</sub>1.5 α-subunits in vascular cells freshly dissociated from human adipose arterioles. A, Expression of BK<sub>Ca</sub> channel-forming α-subunits (green) in freshly dissociated smooth muscle cells (SMCs) and a sheet of endothelial cells (ECs) from non-CAD and CAD human adipose arterioles. Left, SMCs; right, ECs. B, Expression of K<sub>V</sub>1.5 channel-forming α-subunits (green) in freshly dissociated endothelial cells (ECs) from non-CAD and CAD human adipose arterioles. For panels A and B, cell nuclei was stained with DAPI (blue). Scale bar, 10 µm. Data are representative of >3 independent tissues.

Online Figure V. Immunostaining with K<sub>V</sub>1.5 and DDK antibodies. HEK293 cells transiently expressing K<sub>V</sub>1.5-DDK (top) and non-transfected cells (lower) were immunolabeled with rabbit polyclonal anti-K<sub>V</sub>1.5 antibodies (green) and mouse monoclonal anti-DDK antibodies (red). Cell nuclei was stained with DAPI (blue). Scale bar represents 10 µm.

Online Figure VI. 4-AP-sensitive voltage-gated K<sup>+</sup> currents recorded in smooth muscle cells freshly isolated from human adipose arterioles. A, Representative whole-cell currents recorded before (control) and after development of stable inhibition by 5 mmol/L 4-AP, a K<sub>V</sub> channel blocker. Currents were elicited by progressive 10 mV depolarizing steps from a holding potential of -70 mV to +60 mV. Cell capacitance was 28 pF. B, Averaged I-V relationships for end-pulse K<sup>+</sup> currents (normalized to cell capacitance) before and after 5 mmol/L 4-AP (n=5 cells each). *P<0.05 versus control. C, Concentration-dependent effect of 4-AP (1-10 mmol/L) on outward currents (average of 5-10 current traces) evoked by a ramp depolarization from -100 to +100 mV using the same cell as in panel A.

Online Figure VII. Comparison of H<sub>2</sub>O<sub>2</sub>-induced dilation in adipose arterioles obtained from different regions of the body. There was no significant difference in H<sub>2</sub>O<sub>2</sub>-induced dilation among visceral, subcutaneous, or pericardial adipose arterioles within non-CAD (A) or CAD (B) subject groups (n=4-11 vessels/group).

Online Figure VIII. Interaction of coronary artery disease (CAD) with body mass index (BMI) in influencing EC<sub>50</sub> values of H<sub>2</sub>O<sub>2</sub> concentration-response curves. H<sub>2</sub>O<sub>2</sub> (1-100 µmol/L)-induced vasodilation were examined in isolated adipose arterioles from non-CAD and CAD subjects. Individual concentration-response curves were statistically fitted to a 4-parameter sigmoidal logistic equation and modeled by non-linear regression analysis with mixed effects to assess the influence of CAD, risk factors for CAD (e.g., BMI), or their interactions on EC<sub>50</sub>. Plotted EC<sub>50</sub> (Log M) data represent fitted values ± 95% confidence intervals (CI).
References


Online Figure II

A

- Control (intact)
- Catalase (intact)
- Control (denuded)
- Catalase (denuded)

% Max. Dilation

$\text{H}_2\text{O}_2$ (μmol/L)

1 10 50 100

B

% Max. Dilation

$\text{H}_2\text{O}_2$ (100 μM) + + + +
DTT (3 mM) - + + -
Pap (100 μM) - - + -
TCEP (5 mM) - - - +
Online Figure III

**A**

- **Control siRNA**
- **Kv1.5 siRNA**

![Graph showing the effect of H2O2 on % Max. Dilation for Control siRNA and Kv1.5 siRNA](image)

**B**

- **Control siRNA**
- **Control siRNA + DPO-1**

![Graph showing the effect of H2O2 on % Max. Dilation for Control siRNA and Control siRNA + DPO-1](image)

**C**

- **Kv1.5 siRNA**
- **Kv1.5 siRNA + DPO-1**

![Graph showing the effect of H2O2 on % Max. Dilation for Kv1.5 siRNA and Kv1.5 siRNA + DPO-1](image)

**D**

- **Kv1.5**
- **β-actin**

![Gel electrophoresis images and bar graph showing Kv1.5 expression by qPCR](image)
Online Figure IV

A

B

Non-CAD

BKCa

SMC

EC

CAD

BKCa

SMC

EC

Non-CAD

Kv1.5

EC

CAD

SMC

EC
Online Figure V

KV1.5 DDK Merge
KV1.5-DDK Non-transfected

KV1.5
DDK
Merge

Kv1.5-DDK

Non-transfected
Online Figure VI

A

Control

4-AP

50 pA

4-AP

60 mV

Membrane potential (mV)

Current (pA)

-80 -60 -40 -20 0 20 40 60 80

50

100 150 200

B

Current density (pA/pF)

0

2

4

6

-80 -60 -40 -20 0 20 40 60 80

Control

4-AP

C

Current (pA)

0

50

100

150

200

-80 -60 -40 -20 0 20 40 60 80

Membrane potential (mV)

a. Control

b. 4-AP

c. 4-AP

d. 4-AP
Online Figure VII

A  Non-CAD

B  CAD

% Max. Dilation

Visceral
Subcutaneous

H₂O₂ (µmol/L)

1 10 50 100

% Max. Dilation

Visceral
Subcutaneous
Pericardial

H₂O₂ (µmol/L)
### Online Table I.

**Patient demographics** (n=134)

<table>
<thead>
<tr>
<th></th>
<th>Adipose (n=120)</th>
<th>Donor heart (n=14)</th>
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<tbody>
<tr>
<td></td>
<td>non-CAD (n=79*)</td>
<td>CAD (n=41†)</td>
</tr>
<tr>
<td>Gender, Male/Female</td>
<td>22(28%)/57(72%)</td>
<td>31(76%)/10(24%)</td>
</tr>
<tr>
<td>Age, yr (mean ± SEM)</td>
<td>49±16</td>
<td>66±9</td>
</tr>
<tr>
<td>Body mass index (mean ± SEM)</td>
<td>30±7‡</td>
<td>30±7</td>
</tr>
<tr>
<td>Underlying diseases/Risk factors</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coronary artery disease</td>
<td>0</td>
<td>41(100%)</td>
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<tr>
<td>Hypertension</td>
<td>15(19%)</td>
<td>21(51%)</td>
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<tr>
<td>Hyperlipidemia</td>
<td>8(10%)</td>
<td>17(42%)</td>
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<tr>
<td>Diabetes mellitus</td>
<td>4(5%)</td>
<td>10(24%)</td>
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<td>Atrial fibrillation</td>
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<td>4(10%)</td>
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<td>Congestive heart failure</td>
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<tr>
<td>Myocardial infarction</td>
<td>0</td>
<td>5(12%)</td>
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<tr>
<td>(Tobacco use)</td>
<td>6(8%)</td>
<td>9(22%)</td>
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<tr>
<td>None of the above</td>
<td>45(57%)</td>
<td>11(27%)</td>
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</tbody>
</table>

* pericardial n=2, visceral n=52, subcutaneous n=25
† pericardial n=22, visceral n=16, subcutaneous n=3
‡ Total of 2 patients with no information on height and weight.
## Online Table II

Characteristics of isolated adipose arterioles

<table>
<thead>
<tr>
<th>Non CAD</th>
<th>Internal Diameter, μm</th>
<th>Preconstriction (%)</th>
<th>Number of arteries</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Passive (without blocker)</td>
<td>Active (with blocker)</td>
<td>Endothelin-1</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>145 ± 10</td>
<td>145 ± 9</td>
<td>144 ± 10</td>
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<tr>
<td>10 mM 4-AP</td>
<td>159 ± 13</td>
<td>154 ± 11</td>
<td>99 ± 16*</td>
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<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>160 ± 24</td>
<td>147 ± 25</td>
<td>145 ± 28</td>
</tr>
<tr>
<td>10 mM 4-AP (over-constriction)</td>
<td>160 ± 24</td>
<td>152 ± 22</td>
<td>104 ± 27*</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>183 ± 20</td>
<td>180 ± 20</td>
<td>179 ± 21</td>
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<tr>
<td>100 nM paxilline</td>
<td>178 ± 24</td>
<td>174 ± 24</td>
<td>159 ± 31</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>247 ± 14</td>
<td>229 ± 25</td>
<td>233 ± 22</td>
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<tr>
<td>100 nM IbTX</td>
<td>250 ± 16</td>
<td>242 ± 15</td>
<td>242 ± 15</td>
</tr>
<tr>
<td>100 nM IbTX + 10 mM 4-AP</td>
<td>247 ± 14</td>
<td>226 ± 19</td>
<td>152 ± 27*</td>
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<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>146 ± 10</td>
<td>143 ± 9</td>
<td>146 ± 10</td>
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<tr>
<td>1 μM DPO-1</td>
<td>147 ± 8</td>
<td>144 ± 8</td>
<td>117 ± 18</td>
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<td>148 ± 12</td>
<td>146 ± 11</td>
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<tr>
<td>30 nM Psora-4</td>
<td>148 ± 12</td>
<td>145 ± 12</td>
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<td>Control (+L-NAME &amp; Indo)</td>
<td>150 ± 13</td>
<td>149 ± 12</td>
<td>149 ± 13</td>
</tr>
<tr>
<td>10 mM PAP-1</td>
<td>147 ± 12</td>
<td>142 ± 14</td>
<td>141 ± 13</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>163 ± 16</td>
<td>162 ± 13</td>
<td>160 ± 15</td>
</tr>
<tr>
<td>3 μM CP-339818</td>
<td>163 ± 16</td>
<td>162 ± 16</td>
<td>157 ± 14</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>166 ± 21</td>
<td>155 ± 21</td>
<td>157 ± 20</td>
</tr>
<tr>
<td>100 nM stromatoxin</td>
<td>166 ± 21</td>
<td>152 ± 21</td>
<td>151 ± 20</td>
</tr>
<tr>
<td>CAD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>165 ± 28</td>
<td>164 ± 28</td>
<td>163 ± 28</td>
</tr>
<tr>
<td>10 mM 4-AP</td>
<td>183 ± 26</td>
<td>181 ± 26</td>
<td>134 ± 12</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>134 ± 14</td>
<td>133 ± 14</td>
<td>126 ± 15</td>
</tr>
<tr>
<td>100 nM paxilline</td>
<td>134 ± 14</td>
<td>131 ± 12</td>
<td>117 ± 10</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>139 ± 15</td>
<td>137 ± 13</td>
<td>137 ± 16</td>
</tr>
<tr>
<td>1 μM DPO-1</td>
<td>136 ± 21</td>
<td>134 ± 17</td>
<td>127 ± 19</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>178 ± 23</td>
<td>176 ± 22</td>
<td>173 ± 23</td>
</tr>
<tr>
<td>30 nM Psora-4</td>
<td>177 ± 22</td>
<td>173 ± 22</td>
<td>166 ± 18</td>
</tr>
</tbody>
</table>

Values are means ± SEM of H₂O₂-induced dilation. Indo, indomethacin; 4-AP, 4-aminopyridine; IbTX, iberiotoxin.

*P < 0.05 indicates significant difference in active diameters with and without inhibitors as determined using paired t-test.

All experiments shown in this table were performed on endothelium-intact arterioles and in the presence of L-NAME and Indomethacin.
### Online Table III.

#### Nucleotide sequence of Primers used for PCR

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Forward and Reverse primers</th>
<th>Amplicon size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For traditional end-point PCR</strong></td>
<td></td>
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</tr>
<tr>
<td>KV α1.1</td>
<td>5'-AGT CGC ACT TCT CCA GTA TCC-3'</td>
<td>424</td>
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<tr>
<td></td>
<td>5'-TCG GTC AGT AGC TTG CTC TTA T-3'</td>
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<tr>
<td>KV α1.2</td>
<td>5'-TCA AGT TGT CCA GAC ACT CCA A-3'</td>
<td>548</td>
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<td>5'-TGT GTT AGC CAA GGT ACA GTT G-3'</td>
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<tr>
<td>KV α1.3</td>
<td>5'-CTT CAG GTT TCA GCA GCA TCC-3'</td>
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<td>5'-GCA GGT AGT GGA ATT G-3'</td>
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<td>KV α1.4</td>
<td>5'-GAG GCG GAT GAA CCT ACT ACC-3'</td>
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<td>5'-CCC TTT CCC TGA CAC TCC TCC-3'</td>
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<td>KV α1.5</td>
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<td>5'-CCA GGC AGA GGG CAT AAA GG-3'</td>
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<td>KV α1.6</td>
<td>5'-AGA GGC TGA CGA TGA CGA TTC-3'</td>
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<td>KV α9.3</td>
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<td>5'-ACT GGT CCA CAT CAA TGT CCT T-3'</td>
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<tr>
<td>KV β1.1</td>
<td>5'-CAG CAG CCT TAG TCC CTC AG-3'</td>
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<td>5'-GCC GTT CAG CAA CCT CAT CT-3'</td>
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<tr>
<td>KV β1.2</td>
<td>5'-GTG GAG CAG CCG AAC AGA A-3'</td>
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<td>5'-GCC GTT CAG CAA CCT CAT CT-3'</td>
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<tr>
<td>KV β1.3</td>
<td>5'-ACC TCT CGT GAC TGC CTG T-3'</td>
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<td>5'-GCC GTT CAG CAA CCT CAT CT-3'</td>
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<td>BKCa α</td>
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<td>5'-TCG CCA AAG ATG CAG ACC AC-3'</td>
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<tr>
<td>β-actin</td>
<td>5'-GCT CGT CGT CGA CAA CGG CTC-3'</td>
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<td></td>
<td>5'-CAA ACA TGA TCT GGG TCA TCT TCT C-3'</td>
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<td><strong>For quantitative PCR (qPCR)</strong></td>
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<tr>
<td>Transcript</td>
<td>Forward and Reverse primers</td>
<td>Amplicon size, bp</td>
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<td>-----------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>KV α1.5</td>
<td>5'-CGTCATCGTCTCCAACCTCAA-3'</td>
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<td></td>
<td>5'-TCGCCAATCCTCTCAGCTCA-3'</td>
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<tr>
<td>ATP synthase O subunit</td>
<td>5'-TCTCCTTAAAGAGCAGCCTACT-3'</td>
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<td>5'-GACAAGTCCATCGCAACGAGAT-3'</td>
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<tr>
<td>β-actin</td>
<td>5'-GACTCTTCAGCCTTCT-3'</td>
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<td>5'-TGTTGCGTACAGGCTT-3'</td>
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