Integrative Physiology

Contribution of KV1.5 Channel to Hydrogen Peroxide–Induced Human Arteriolar Dilation and Its Modulation by Coronary Artery Disease

Yoshinori Nishijima, Sheng Cao,* Dawid S. Chabowski,* Ankush Korishettar, Alyce Ge, Xiaodong Zheng, Rodney Sparapani, David D. Gutterman, David X. Zhang

**Rationale:** Hydrogen peroxide (H2O2) regulates vascular tone in the human microcirculation under physiological and pathophysiological conditions. It dilates arterioles by activating large-conductance Ca2+-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 H2O2-elicited dilation involves different K+ channels in non-CAD versus CAD, resulting in an altered capacity for vasodilation during disease.

**Methods and Results:** H2O2-induced endothelium-independent vasodilation in non-CAD adipose arterioles, which was reduced by paxilline, a large-conductance Ca2+-activated K+ channel blocker, and by 4-aminopyridine, a voltage-gated K+ (Kv) channel blocker. Assays of mRNA transcripts, protein expression, and subcellular localization revealed that Kv1.5 is the major Kv1 channel expressed in vascular smooth muscle cells and is abundantly localized on the plasma membrane. The selective Kv1.5 blocker diphenylphosphine oxide-1 and the Kv1.3/1.5 blocker 5-(4-phenylbutoxy)psoralen reduced H2O2-elicited dilation to a similar extent as 4-aminopyridine, but the selective Kv1.3 blocker phenoxalkoxypsoralen-1 was without effect. In arterioles from CAD subjects, H2O2-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. Kv1.5 cell membrane localization and diphenylphosphine 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, H2O2-induced dilation is impaired in CAD, which is associated with a transition from a combined large-conductance Ca2+-activated Kv1- and Kv1.5-mediated vasodilation toward a large-conductance Ca2+-activated Kv1-predominant mechanism of dilation. Loss of Kv1.5 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 (H2O2), 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, H2O2 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 H2O2-induced hyperpolarization and dilation in normal human and animal arteries. The mechanisms of H2O2-induced vasodilation have not been fully elucidated; however, 2 main types of K+ channels in vascular smooth muscle cells (VSMCs), large-conductance Ca2+-activated K+ (BKCa) channels and voltage-gated K+ (Kv) channels, have been variably implicated in different vascular beds. For instance, BKCa channels contribute to H2O2-induced dilation in porcine coronary arteries. In contrast, 4-aminopyridine (4-AP)–sensitive Kv1 channels mediate dilation to H2O2 in canine coronary arteries, rat coronary and mesenteric arteries, and porcine coronary resistance arteries. Using coronary arterioles from CAD subjects, we found that H2O2...
K⁺ Channels in H₂O₂-Induced Dilation

Novelty and Significance

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 (H₂O₂) in those with CAD.
- The large-conductance Ca²⁺-activated K⁺ (BKCa) channel and the voltage-gated K⁺ (Kᵥ) channel have been variably implicated in H₂O₂-induced dilation in both animals and humans.
- K₁ family channels are known to be redox regulated.
- K₁.5 channels in preclinical models mediate the actions of H₂O₂ and connect cardiac metabolism to myocardial blood flow.

What New Information Does This Article Contribute?
- H₂O₂ induces potent smooth muscle–mediated vasodilation through BKCa and Kᵥ (especially Kᵥ1.5) channels in adipose arterioles from human subjects without CAD.
- H₂O₂-elicited dilation is reduced in arterioles from subjects with CAD and is accompanied by a loss of Kᵥ1.5-dependent dilation but not BKCa-dependent dilation.

Nonstandard Abbreviations and Acronyms

4-AP 4-aminopyridine
BKCa channel large-conductance Ca²⁺-activated K⁺ channel
CAD coronary artery disease
DPO-1 diphenylphosphine oxide-1
EC endothelial cell
H₂O₂ hydrogen peroxide
HCA human coronary artery
Kᵥ channel voltage-gated K⁺ channel
PCR polymerase chain reaction
Psora-4 5-(4-phenylbutoxy)psoralen
ROS reactive oxygen species
VSMC vascular smooth muscle cell

opens smooth muscle BKCa channels to elicit smooth muscle hyperpolarization and relaxation. The mechanisms of dilation by H₂O₂ in subjects without CAD (non-CAD) versus those with CAD, as well as the functional consequences, remain unknown, but there is evidence that BKCa and Kᵥ constitute 2 major K⁺ currents in VSMCs isolated from non-CAD human arteries.

In addition to H₂O₂-induced activation of K⁺ 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 Kᵥ channels have been shown in various animal models of cardiovascular disease, while BKCa 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 H₂O₂-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 (BKCa versus Kᵥ) in H₂O₂-induced dilation of human arterioles from non-CAD and CAD subjects. We further identified specific Kᵥ1 channels, the major vascular Kᵥ channel subfamily, in VSMCs and examined their functional contribution to H₂O₂-induced dilation. The impact of CAD on the function of Kᵥ1 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 H₂O₂, (1–100 μmol/L) to the vessel bath were determined in the absence and presence of 30 minutes preincubation with various modulators, including BKCa and Kᵥ blockers. At the end of each experiment, papaverine (100 μmol/L) was added to determine the maximal internal diameter for normalization of dilation. Relaxation responses were compared with the responses to amyl nitrite (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 MgCl₂, 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 CaCl₂, 0.5 MgCl₂, 10 HEPES (pH 7.2 with KOH), and 0.45 glucose.

...
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. Protein samples (20 μg) were separated by 10% SDS-PAGE. Membranes were blotted with a primary antibody against a specific Kᵥ1α-subunit, BKCa, and Na/K⁺-ATPase (1:2000 dilution), followed by a horseradish peroxidase–conjugated secondary antibody (1:20000 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. Sections were blocked with 5% normal goat serum and probed with a monoclonal or polyclonal antibody against a specific Kᵥ1α-subunit (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. Cells were then incubated either with a monoclonal or polyclonal antibody specific to a Kᵥ1α-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-phenylbutoxy)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, phenoxyalkoxypsoralen-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 K⁺ channels in animal models of vascular disease, such as hypertension and metabolic syndrome. It remains largely unknown whether K⁺ 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.

A. Non-CAD
- Control
- Paxilline

B. CAD
- Control
- Paxilline

P values <0.05 vs control; n=5 to 6 vessels per group.
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.

In non-CAD adipose arterioles (Figure 1A), H2O2 (1–100 μmol/L) induced vasodilatation in a concentration-dependent manner (% maximal dilation, 96±2). Treatment of arterioles with paxilline (100 μmol/L), a potent BKCa 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+ channel blocker 4-AP (10 μmol/mL) 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 BKCa and KV channels abolished dilation up to 30 μmol/L H2O2 (Online Figure IA), indicating that these 2 K+ channels mediate a major portion of H2O2-induced dilation in non-CAD human adipose arterioles. The potency of H2O2 (EC50, 12±1 μmol/L; n=18, Online Figure IC) is similar to those reported in canine11 and human coronary arterioles.

In CAD arterioles (Figure 1B), H2O2-induced dilation was also blocked by paxilline (% dilation at 10 μmol/L, 1±6 vs 41±8 in CAD control, % dilation at 50 μmol/L, 40±7 vs 76±6 in CAD control, n=5; P<0.05). However, the dilation was not affected by 4-AP. These results indicate that KV channel–dependent dilation in response to H2O2 is impaired in non-CAD human adipose arterioles. The potency of H2O2 (EC50, 12±1 μmol/L; n=18, Online Figure IC) is similar to those reported in canine11 and human coronary arterioles.

Incubation of arterioles with 4-AP induced significant vasoconstriction in non-CAD but not in CAD subjects (Online Table II), indicating that KV channels regulate basal vascular tone in non-CAD subjects.

**H2O2-Induced Dilation Involves Smooth Muscle Hyperpolarization and Is Redox Sensitive**

H2O2-induced dilation was not affected by Nω-nitro-L-arginine methyl ester (100 μmol/L), a nitric oxide synthase inhibitor, indomethacin (Indo, 10 μmol/L), a cyclooxygenase inhibitor, alone or in combination, suggesting that endothelial NO and prostacyclin do not contribute to H2O2-induced dilation. In addition, H2O2 induced similar dilation in endothelium-intact and denuded arterioles (Figure 2B), further inferring that the dilation is mediated via an endothelium-independent, likely smooth muscle mechanism. Next, the role of smooth muscle hyperpolarization in H2O2-induced dilation was examined using high K+ (60 mmol/L)-Krebs-physiological salt solution in the presence of Nω-nitro-L-arginine methyl ester and indomethacin (Figure 2B). Dilation to H2O2 was abolished by high K+ in both endothelium-intact and endothelium-denuded arterioles (% dilation at 100 μmol/L, 8±4 and 4±2, 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.23 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 8±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 KV1 Subunit mRNA and Protein Expression in Non-CAD Human Arteries**

The specific type(s) of KV channels expressed in human arteries remains largely unexplored. We focused on KV1 channels (shaker-related family), which are functionally significant KV channels in most animal vascular beds studied24 and are 4-AP sensitive. Figure 3 shows mRNA expression of different KV1 channel subunits in non-CAD adipose arterioles as assessed by reverse transcription-PCR. Several KV1 α (pore forming)-subunits, including 1.1, 1.2, 1.4, 1.5, and BKCa α-subunits, were consistently found in different samples, including denuded vessels (n=5), with KV1.5 being the most abundantly expressed KV1 α-subunit. The 2 other KV1 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 KV channels (2.1 and 9.3), KV1 β (accessory)-subunits (1.1–1.3), although these subunits were not further pursued in the following immunoblots.

Using subunit-specific antibodies, we examined the protein expression of KV1.1 to 1.6 and BKCa α-subunits in the membrane fraction prepared from non-CAD adipose arterioles (n=2). Consistent with mRNA expression data, KV1.4 and 1.5 and BKCa channels were readily detected at the protein level (Figure 4). KV1.3 and 1.6 proteins were also found in non-CAD arteries/arterioles, whereas KV1.1 and 1.2
proteins were not detected. The absence of Kᵥ1.2 in human vascular samples was unexpected because it is a dominant Kᵥα₁-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 ×600 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\textsubscript{v1} channel is functionally involved in human arteriolar dilation. Adipose arterioles from non-CAD subjects were pretreated with subtype-specific K\textsubscript{v} channel blockers and examined for H\textsubscript{2}O\textsubscript{2}-induced vasodilation. We found that selective K\textsubscript{v1.5} channel blocker DPO-1 (1 \(\mu\)mol/L) markedly reduced H\textsubscript{2}O\textsubscript{2}-induced dilation (Figure 6A; % dilation at 10 \(\mu\)mol/L, 13\(\pm\)6 versus 55\(\pm\)7 in control, n=5; \(P<0.05\)), to a similar extent as after 4-AP treatment (Figure 1). Selective K\textsubscript{v1.3/1.5} blocker Psora-4 (30 nmol/L) caused a similar rightward shift in H\textsubscript{2}O\textsubscript{2}-induced dilation, but further reduced the maximal dilation to 100 \(\mu\)mol/L H\textsubscript{2}O\textsubscript{2} (59\(\pm\)5% versus 95\(\pm\)2% in control, n=5; \(P<0.05\); Figure 6B). In contrast, selective K\textsubscript{v1.3} blocker phenoxalkoxypsoralen-1 (10 nmol/L) did not affect H\textsubscript{2}O\textsubscript{2}-induced dilation (Figure 6C; n=5). The K\textsubscript{v1.3/1.4} blocker 1-benzyl-4-pentylimino-1,4-dihydroquinoline (3 \(\mu\)mol/L) slightly attenuated the dilation induced by 10 \(\mu\)mol/L H\textsubscript{2}O\textsubscript{2} only. * \(P<0.05\) vs control; n=5 vessels per group. KV indicates voltage-gated K\textsuperscript{+}.

**Figure 6.** Role of K\textsubscript{v1.5} in hydrogen peroxide (H\textsubscript{2}O\textsubscript{2})-induced dilation of non-coronary artery disease (non-CAD) human adipose arterioles. The dilation was blocked by diphenylphosphine oxide-1 (DPO-1; 1 \(\mu\)mol/L, A), a selective K\textsubscript{v1.5} channel blocker, and 5-(4-phenylbutoxy)psoralen (Psora-4; 30 nmol/L, B), a K\textsubscript{v1.3/1.5} blocker. However, the dilation was not affected by phenoxalkoxypsoralen (PAP-1; 10 nmol/L, C), a K\textsubscript{v1.3} blocker. 1-benzyl-4-pentylimino-1,4-dihydroquinoline (CP-339818; 3 \(\mu\)mol/L, D), a K\textsubscript{v1.3/1.4} blocker, slightly attenuated the dilation induced by 10 \(\mu\)mol/L H\textsubscript{2}O\textsubscript{2} only. * \(P<0.05\) vs control; n=5 vessels per group. K\textsubscript{v} indicates voltage-gated K\textsuperscript{+}. 

**Figure 5.** Immunofluorescence localization of voltage-gated K\textsuperscript{+} (K\textsubscript{v1}) \(\alpha\)-subunits in human adipose arterioles. K\textsubscript{v1.5} is the major K\textsubscript{v1} channel protein expressed in human adipose arteriolar smooth muscle cells. A, Confocal immunofluorescence images of K\textsubscript{v1} \(\alpha\)-subunit proteins (red) in cross tissue sections (10 \(\mu\)m) of an intact human adipose arteriole. K\textsubscript{v1.5} and 1.4 of a lower level were detected in smooth muscle cells (SMCs) and endothelial cells (ECs). K\textsubscript{v1.3} and 1.6 subunits were also faintly visible in ECs. Cell nuclei were stained with DAPI (blue), IEL, internal elastic lamina (green autofluorescence). B, Confocal immunofluorescence images of corresponding K\textsubscript{v1} \(\alpha\)-subunit proteins (green) in freshly dissociated SMCs from a human adipose arteriole. K\textsubscript{v1.5} protein was mainly localized on the cell membrane of SMCs. Cell nuclei was stained with DAPI (blue). Data are representative of >3 independent tissues.
siRNA (Online Figure III). Together, these results suggest that 
Kv1.5 serves as a major 4-AP–sensitive Kv1 channel contrib-
uting to H$_2$O$_2$–induced dilation in non-CAD adipose arterioles.

Compared with non-CAD arterioles, there was a slight but
statistically significant reduction of H$_2$O$_2$–induced dilation in
CAD (% dilation at 10 μmol/L H$_2$O$_2$, 40±4 versus 59±4 in
non-CAD, % dilation at 50 μmol/L, 71±3 versus 86±2 in non-
CAD, n=18 per group; P<0.05; Figure 7A). In contrast to non-
CAD arterioles (Figure 6), Kv1.5 channel blockade by either
DPO-1 or Psora-4 did not alter H$_2$O$_2$–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 Kv1 channels, smooth muscle Kv1.5 in particular, in
human arterioles during CAD.

**Potential Mechanism of Impaired Kv1.5 Function in
CAD Human Arteries**

We further examined the potential mechanisms responsible for
impaired Kv1.5 function in CAD vessels. By immunostain-
ing of freshly dissociated VSMCs, we found that the plasma
membrane staining of Kv1.5 protein was markedly reduced
in CAD subjects (Figure 8A). Analysis of plasma membrane/
cytoplasmic ratio of Kv1.5 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, Kv1.5 immunofluorescence from the nuclear en-
velope was comparable in CAD versus non-CAD samples.

In contrast to Kv1,5, BK$_{Ca}$ protein localization on the plas-
ma membrane was maintained in VSMCs from CAD subject
when compared with non-CAD subjects (Online Figure IVA;
calculated plasma membrane/cytoplasmic ratio, 4.03±0.32 and
3.07±0.18, respectively). We also examined Kv1,5 and BK$_{Ca}$
immunofluorescence in ECs isolated from adipose arterioles
of non-CAD and CAD subjects (Online Figure IVB and IVB).
Whole-cell immunofluorescence of Kv1,5 in ECs seemed com-
parable between CAD and non-CAD, although further analysis of
plasma membrane/cytoplasmic ratio is difficult because of
small cell size of isolated ECs. The immunoreactivity for BK$_{Ca}$
channel α-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 Kv1,5
antibodies was confirmed by using human embryonic kidney
293 cell lines with and without Kv1,5-DDK transfection and
double-staining technique (Online Figure V).

Using end point reverse transcription-PCR analysis, the
mRNA level of Kv1,5 in CAD vessels was comparable to that
of non-CAD tissues when normalized to the housekeeping gene
β-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
Kv1,5 normalized to the mean of 2 housekeeping genes (β-
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 mod-
erate decrease (26%) in denuded CAD vessels was observed.

We also compared the expression of Kv1,5 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 preparation, HCAs were used instead for
these experiments. Our recent studies have shown that hu-
man conduit and resistance arteries express a similar profile of
Kv1 channels at both mRNA and protein levels. The average
protein expression level of Kv1,5 subunits normalized to Na+/K+
-ATPase showed a trend toward an increase (but not statisti-
cally 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
Kv1,5 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 μmol/L). Compared with non-CAD, base-
line 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 Kv1.5 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 Kv1.5 subunit proteins (green) in freshly dissociated vascular smooth muscle cells (VSMCs) from non-CAD and CAD human adipose arterioles. The plasma membrane expression of Kv1.5 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 Kv1.5 mRNA. Top, End point PCR gel images of Kv1.5, as well as β-actin control from non-CAD and CAD human adipose arterioles (n=3 per group). Lower, Relative abundance of Kv1.5 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 Kv1.5, large-conductance Ca2+-activated K+ (BKCa) 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)
decreased the outward K⁺ currents (Online Figure VI). Together, the above results suggest that mainly the reduced plasma membrane expression of smooth muscle Kᵥ1.5 subunits, rather than a change of mRNA or total protein, contributes to impaired vasmotor function of Kᵥ1.5 channels in human arteries with CAD.

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 Kᵥ (especially Kᵥ1.5), 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 Kᵥ1-mediated vasodilation toward a BKCa-predominant mechanism of dilation in the human microcirculation during CAD. Although the pathophysiological significance of this Kᵥ 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.

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 dilation 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.¹³ 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-impermeant thiol reagent failed to reverse the dilation. These data support an H₂O₂-mediated redox modification of Kᵥ 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.¹⁶ 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 Vascular
The expression of specific Kᵥ subunit channel gene products and proteins varies greatly among species and vascular beds,²⁴ and the molecular identity of Kᵥ1 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 Kᵥα1 α-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 has >20-fold more potent for Kᵥ1.5 (IC₅₀ 7.7 µmol/L) than DPO-1 (IC₅₀ 0.2–0.3 µmol/L).¹⁷ 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 Kᵥ1 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
regulation of vascular tone in rodent arteries. In this study, we detected vascular K<sub>2.1</sub> mRNA (Figure 3) and protein expression (data not shown); however, selective K<sub>2.1</sub> and K<sub>2.1/9.3</sub> blocker stromatoxin did not alter H<sub>2</sub>O<sub>2</sub>-induced dilation in non-CAD arterioles (Online Figure I). Redox-sensitive K<sub>7</sub> channels represent another potential candidate, but a recent study indicate that they do not contribute to H<sub>2</sub>O<sub>2</sub> dilation in porcine coronary arteries. Further investigations are needed to elucidate the role of other K<sup>+</sup> channels in human arterioles.

**Alteration of K<sup>+</sup> Channel Function in CAD**

Compared with non-CAD arterioles, H<sub>2</sub>O<sub>2</sub>-induced dilation was significantly reduced in CAD arterioles (Figure 7A). Furthermore, H<sub>2</sub>O<sub>2</sub>-induced dilation of CAD adipose arterioles was inhibited by only BK<sub>K<sub>C</sub></sub> but not K<sub>dec</sub> channel blockers. To the best of our knowledge, this is the first report on the recruitment of different smooth muscle K<sup>+</sup> channels in H<sub>2</sub>O<sub>2</sub>-induced vasodilation from health to disease. The expression of BK<sub>K<sub>C</sub></sub> mRNA and protein, as well as protein localization, was not altered by CAD; a finding consistent with that of our previous study in HCA. We, thus, conclude that BK<sub>K<sub>C</sub></sub> channels remain functional in CAD arterioles. In other vascular beds or species, the effect of disease on the expression and function of BK<sub>K<sub>C</sub></sub> channels remains complex or controversial. For example, BK<sub>K<sub>C</sub></sub> channel activity is increased in human VSMCs obtained from coronary atherosclerotic lesions. Animal studies support that BK<sub>K<sub>C</sub></sub> channel expression is increased in hypertension, possibly as a compensatory mechanism for the downregulation of K<sub> dec</sub> channel expression. However, BK<sub>K<sub>C</sub></sub> activity is reduced by exposure to high glucose or high concentrations of H<sub>2</sub>O<sub>2</sub> and in the porcine model of metabolic syndrome.

The reduction of H<sub>2</sub>O<sub>2</sub>-induced dilation in CAD arterioles can be mainly attributed to a loss of K<sub> dec</sub>, especially K<sub>1.5</sub>, channel function in VSMCs. In contrast to non-CAD adipose arterioles, blocking of K<sub>1.5</sub> or other K<sub>1</sub> channels did not affect H<sub>2</sub>O<sub>2</sub>-induced dilation in CAD vessels (Figures 1 and 7). Alternations in vascular K<sub> dec</sub> channel function and expression have also been reported in other pathological conditions such as pulmonary and systemic hypertension, metabolic syndrome, and diabetes mellitus, but the identities of individual K<sub> dec</sub> 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<sub>1.5</sub> (I<sub>K<sub>1,5</sub></sub>) expression.

The precise mechanisms of impaired K<sub> dec</sub> function under pathological conditions remain poorly understood. By examining K<sub>1.5</sub> mRNA/protein expression and subcellular localization and K<sub>1.5</sub> currents, we provide initial evidence that a reduced plasma membrane expression of K<sub>1.5</sub> in VSMCs, rather than a change of mRNA and total protein synthesis, may be mainly responsible for the reduced K<sub>1.5</sub> 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. For example, a fraction of K<sub>1.5</sub> channels on the cell membrane is rapidly internalized with a half time of ~10 minutes and some return to the surface with a half time of ~30 minutes. In a HL-1 cell line, elevated ROS induces fairly rapid (within 60 minutes) reduction of surface expression of K<sub>1.5</sub>. It remains to be tested in future studies whether prolonged elevation of ROS during CAD modulates cellular trafficking of smooth muscle K<sub>1.5</sub> channels to reduce their membrane expression in human arterioles.

**Potential Study Limitations**

We found that K<sub>1.5</sub> and K<sub>1.4</sub> channels are expressed in ECs of human adipose arterioles. It is thus possible that H<sub>2</sub>O<sub>2</sub> may activate endothelial K<sub> dec</sub> channels to induce endothelial hyperpolarization and subsequent vasodilation. Although the expression and function of endothelial K<sub> dec</sub> channels are poorly understood, there is evidence on the role of K<sub> dec</sub> channels in endothelium-dependent hyperpolarization and dilation in arteries such as porcine coronary arteries and guinea pig coronary and carotid arteries. However, H<sub>2</sub>O<sub>2</sub>-induced dilation of adipose arterioles is largely smooth muscle dependent, and, therefore, the contribution of endothelial K<sub> dec</sub> channels seems unlikely, at least in the present experimental settings. Nevertheless, we have not excluded a potential role for endothelial K<sub> dec</sub> channels in the dilation to H<sub>2</sub>O<sub>2</sub> under other conditions (eg, endogenous H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub>-induced dilation. There is an interaction between CAD and body mass index on EC<sub>50</sub> of H<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub>-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<sub>2</sub>O<sub>2</sub>-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<sub> dec</sub> channels. To mitigate this possibility, we used several chemically distinct blockers of K<sub>1</sub> channels, and the results obtained with these blockers invariably pointed toward an important role of K<sub>1.5</sub> in H<sub>2</sub>O<sub>2</sub>-induced dilation of non-CAD adipose arterioles. In addition, we found that K<sub>1.5</sub>-targeted blockers had no significant effects in CAD arterioles, suggesting that any nonspecific effects should be minimal in this study. A recent study also reported a preferential inhibition of K<sub>1.5</sub> by DPO-1 using VSMCs dissociated from wild-type versus K<sub>1.5</sub> knockout mice. Nevertheless, a molecular approach using K<sub>1.5</sub> siRNA was also included to determine the specific role of K<sub>1.5</sub> in H<sub>2</sub>O<sub>2</sub>-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...
Aurora St Luke's Medical Center, Wheaton Franciscan Healthcare's Medical Center in Milwaukee, Froedtert Memorial Lutheran Hospital, Surgery Institute, Cardiothoracic Surgery Division at the Zablocki V A Medical Center, American Heart Association (0830042 N to D.X. Zhang), and Advancing a Healthier Wisconsin Research and Educational program (9520111 to R. Sparapani).

Disclosures
None.

References

Sources of Funding
Support for this research was provided by National Heart, Lung, and Blood Institute (HL096647 to D.X. Zhang; HL113612 to D.D. Gutterman), American Heart Association (0830042 N to D.X. Zhang), and Advancing a Healthier Wisconsin Research and Educational program (9520111 to R. Sparapani).

Acknowledgments
We thank the Division of Cardiothoracic Surgery at the Medical College of Wisconsin, the Cardiothoracic Surgery Group of Milwaukee, the Cardiovascular Surgery Associates of Milwaukee, the Midwest Heart Surgery Institute, Cardiothoracic Surgery Division at the Zablocki VA Medical Center in Milwaukee, Froedtert Memorial Lutheran Hospital, Aurora St Luke’s Medical Center, Wheaton Franciscan Healthcare’s Elmbrook Memorial Hospital, St Joseph’s Hospital, and the Wisconsin Heart Hospital, as well as the Wisconsin Donor Network, for providing human tissues. We also thank Victoria Nasci for organizing tissue sample data, Dr Paul Goldspink for assistance with quantitative polymerase chain reaction, Dr Jian Zhang for assistance with immunohistochemistry, and Dr Yanping Liu for critical review.

disease. 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 H2O2 from ECs and subsequent smooth muscle hyperpolarization. In this study, we demonstrate a reduced smooth muscle–mediated dilation resulting from a functional transition from BKCa- and K+ -mediated vasodilation to predominantly BKCa-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 H2O2) 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+ channels.

Impaired K+ 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 auxiliary 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+ 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+ channels and cardiovascular function.

668 Circulation Research February 17, 2017
Nishijima et al

K⁺ Channels in H₂O₂-Induced Dilation

669


Contribution of Kᵥ1.5 Channel to Hydrogen Peroxide–Induced Human Arteriolar Dilatation and Its Modulation by Coronary Artery Disease
Yoshinori Nishijima, Sheng Cao, Dawid S. Chabowski, Ankush Korishettar, Alyce Ge, Xiaodong Zheng, Rodney Sparapani, David D. Gutterman and David X. Zhang

Circ Res. 2017;120:658-669; originally published online November 21, 2016; doi: 10.1161/CIRCRESAHA.116.309491

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/120/4/658

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2016/11/21/CIRCRESAHA.116.309491.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org/subscriptions/
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 Kvα1.5 cDNA clone was obtained from Origene and subcloned into pCMV6 mammalian expression vectors as a fusion protein with the COOH-terminus of Kvα1.5 tagged with a FLAG tag. The nucleic sequence of Kvα1.5-FLAG constructs was verified by direct DNA sequencing. Kvα1.5-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 H₂O₂ (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: N²-nitro-L-arginine methyl ester (L-NAME, 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 BKCa channel blocker;iberiotoxin (100 nmol/L), a specific but not cell-permeable BKCa channel blocker; diphenyl phosphine oxide-1 (DPO-1, 1 µmol/L), selective a Kvα1.5 channel blocker; 5-(4-phenylbutoxy)psoralen (Psora-4, 30 nmol/L), a selective Kvα1.3/α1.5 channel blocker; phenoxylalkoxyspsoralen-1 (PAP-1, 10 nmol/L), a selective Kvα1.3 channel blocker; 1-benzyl-4-pentylimino-1,4-dihydroquinoline (CP-339818, 3 µmol/L), a selective Kvα1.3/α1.4 channel blocker; Stromatoxin (100 nmol/L), a selective Kvα2.1, Kvα2.1/α9.3 blocker; DL-dithiothreitol (DTT, 3 mmol/L), a membrane-permeant thiol-specific reducing agent; Tris(2-carboxyethyl)phosphine (TCEP, 5 mmol/L), a membrane-impermeable thiol-reducing agent; and catalase (1000 U/mL), a H₂O₂ metabolizing enzyme. To examine the role of smooth muscle hyperpolarization in H₂O₂-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-NAME 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 RNaseasy 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.¹ 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 (H₂O), were amplified in parallel by using Kvα9.3 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 Kv and BKCa channel examined in this study.²

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 Greeen Supermix (for siRNA qPCR, BioRad), 2 µL (each for forward and reverse) of Kvα1.5-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 Kvα1.5 gene expression was normalized to average of β-actin and ATP5o genes.³ 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 \( K_{\alpha}1.5 \) 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 \( \times \) g for 10 minutes, the supernatants were transferred to a new tube and centrifuged at 10,000 \( \times \) g for 20 minutes, and the supernatants were then centrifuged at 100,000 \( \times \) 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 N\( _2 \), 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: \( K_{\alpha}1.1 \) (75-007, NeuroMab K20/48 clone), \( K_{\alpha}1.2 \) (73-008, NeuroMab K14/16 clone), \( K_{\alpha}1.3 \) (75-009, NeuroMab L23/27 clone), \( K_{\alpha}1.4 \) (75-010, NeuroMab K13/31 clone), \( K_{\alpha}1.5 \) (APC-150, Alomone), \( K_{\alpha}1.6 \) (75-012, NeuroMab K19/36 clone), \( BK_{\alpha} \) (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 \( K_{\alpha}1 \) 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 100x (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 an 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 artificial 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\(^6\,^7\) and also has been used in our previous studies.\(^2\)

**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.\(^8\) 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 Ca\(^{2+}\) concentration of Solution IV was gradually increased from nominally Ca\(^{2+}\)-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 H₂O₂-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.⁸,¹⁰ 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 CaCl₂, 1 MgCl₂, 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 MgCl₂, 1 Mg-ATP, 1 EGTA, and 10 HEPES (pH 7.2 with KOH). The concentration of free Ca²⁺ in the pipette solution is estimated to be around 10 nM. Contaminating currents through large-conductance, Ca²⁺-activated K⁺ channels (BK₉Ca) were minimized by lowering free Ca²⁺ concentrations in the pipette and bath solutions, and by including the BK₉Ca 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 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.

For analysis of selected vasodilator responses (e.g., H₂O₂-induced dilation in non-CAD versus CAD group), individual concentration-response curves were fitted to the following 4-parameter sigmoidal logistic equation: 

\[ Y_i = P_1 + \frac{P_2}{1 + \exp(P_3 \times (\log(X_i) - P_4))} + e_i \]

where \( Y_i \) = vasodilator response, \( X_i \) = molar concentration of vasodilator, \( P_1 \) = lower plateau response subject random...
Supplemental Results

Online Figure I. H$_2$O$_2$-induced dilation of non-CAD human adipose arterioles. A, Effect of BK$_{Ca}$ blockade alone and in combination with K$_V$ blockade on H$_2$O$_2$-induced dilation. BK$_{Ca}$ channel blocker iberiotoxin (IbTX; 100 nmol/L) also reduced the dilation, and the combination of IbTX and 4-AP (10 mmol/L), a K$_V$ channel blocker, markedly diminished the dilation except for the highest dose of H$_2$O$_2$ (100 μmol/L). n=6-7 vessels/group. *P<0.05 versus control, *P<0.05 versus IbTX. B, Effect of 4-AP on H$_2$O$_2$-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$_2$O$_2$-induced dilation (n=5, *P<0.05 versus control). C, H$_2$O$_2$-induced concentration-dependent dilation in non-CAD arterioles was fitted with a 4-parameter sigmoidal equation. EC$_{50}$ of H$_2$O$_2$ was then calculated from the fitted curve. n=18 vessels. D, In non-CAD arterioles, selective K$_V$2.1 and K$_V$2.1/9.3 blocker stromatoxin (100 nmol/L) did not affect H$_2$O$_2$-induced dilation. n=4 vessels/group.

Online Figure II. Effects of catalase and thiol-reducing agents on H$_2$O$_2$-induced dilation in non-CAD human adipose arterioles. A, The dilation was abolished by H$_2$O$_2$ 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$_2$O$_2$. *P<0.05 versus control (A) or 100 μmol/L H$_2$O$_2$ (B); n=3-5 vessels/group.

Online Figure III. Effects of K$_V$1.5 siRNA on H$_2$O$_2$-induced dilation in non-CAD human adipose arterioles. A, H$_2$O$_2$-induced dilation was significantly reduced in arterioles treated with K$_V$1.5 siRNA (% dilation at 10 μmol/L H$_2$O$_2$, 3±3 vs. 28±5 with control siRNA, % dilation at 50 μmol/L H$_2$O$_2$, 18±8 vs. 63±5 with control siRNA, and % dilation at 100 μmol/L H$_2$O$_2$, 53±5 vs. 83±4 in control-siRNA, n=4 vessels/group, *P<0.05). B, In arterioles treated with control siRNA, the selective K$_V$1.5 blocker DPO-1 significantly reduced the dilation confirming K$_V$1.5 channels were functional in these vessels (% dilation at 10 μmol/L H$_2$O$_2$, 12±4 vs. 28±5 with control siRNA, % dilation at 50 μmol/L H$_2$O$_2$, 31±2 vs. 63±5 with control siRNA, and % dilation at 100 μmol/L H$_2$O$_2$, 62±6 vs. 83±4 in control siRNA, n=4 vessels/group, *P<0.05). C, In contrast, the selective K$_V$1.5 blocker DPO-1 did not affect the dilation in K$_V$1.5 siRNA-treated arterioles. n=4 vessels/group. D, Three representative gel images of end-point RT-PCR analysis of K$_V$1.5 gene knockdown (left). Summarized quantitative RT-PCR data (right) indicate that K$_V$1.5 mRNA expression (normalized to average of β-actin and ATP5o genes) was significantly reduced in K$_V$1.5 siRNA-treated vessels (n=4 vessels/group, *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 BKCa and Kv1.5 α-subunits in vascular cells freshly dissociated from human adipose arterioles. A, Expression of BKCa 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 Kv1.5 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 Kv1.5 and DDK antibodies. HEK293 cells transiently expressing Kv1.5-DDK (top) and non-transfected cells (lower) were immunolabeled with rabbit polyclonal anti-Kv1.5 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+ 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 Kv 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+ 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 H2O2-induced dilation in adipose arterioles obtained from different regions of the body. There was no significant difference in H2O2-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 EC50 values of H2O2 concentration-response curves. H2O2 (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 EC50. Plotted EC50 (Log M) data represent fitted values ± 95% confidence intervals (CI).
References

Online Figure I

(A) % Max. Dilation vs. $H_2O_2$ (Log M) with control, IbTX, and IbTX+4-AP.

(B) % Max. Dilation vs. $H_2O_2$ (Log M) with control and 4-AP.

(C) % Max. Dilation vs. $H_2O_2$ (Log M) with control.

(D) % Max. Dilation vs. $H_2O_2$ (μmol/L) with control and Stromatoxin.
Online Figure IV

A  BK\textsubscript{Ca}  
Non-CAD  SMC  BK\textsubscript{Ca}  EC  
CAD  SMC  EC

B  K\textsubscript{V}1.5  
Non-CAD  EC  
CAD  EC
Online Figure V

<table>
<thead>
<tr>
<th>K_v1.5</th>
<th>DDK</th>
<th>Merge</th>
</tr>
</thead>
<tbody>
<tr>
<td>K_v1.5-DDK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-transfected</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Online Figure VII

A  Non-CAD

<table>
<thead>
<tr>
<th>H₂O₂ (µmol/L)</th>
<th>% Max. Dilation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

B  CAD

<table>
<thead>
<tr>
<th>H₂O₂ (µmol/L)</th>
<th>% Max. Dilation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>50</td>
<td>60</td>
</tr>
<tr>
<td>100</td>
<td>80</td>
</tr>
</tbody>
</table>

- Visceral
- Subcutaneous
- Pericardial

Non-CAD vs. CAD comparison of % Max. Dilation with varying H₂O₂ concentrations.
Online Figure VIII

Graph showing the relationship between BMI and EC50 for CAD and non-CAD groups.
### Online Table I.

**Patient demographics (n=134)**

<table>
<thead>
<tr>
<th></th>
<th>Adipose (n=120)</th>
<th>Donor heart (n=14)</th>
</tr>
</thead>
<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>
</tr>
<tr>
<td>Hypertension</td>
<td>15(19%)</td>
<td>21(51%)</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>8(10%)</td>
<td>17(42%)</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>4(5%)</td>
<td>10(24%)</td>
</tr>
<tr>
<td>Atrial fibrillation</td>
<td>0</td>
<td>4(10%)</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>0</td>
<td>3(7%)</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>0</td>
<td>5(12%)</td>
</tr>
<tr>
<td>(Tobacco use)</td>
<td>6(8%)</td>
<td>9(22%)</td>
</tr>
<tr>
<td>None of the above</td>
<td>45(57%)</td>
<td>11(27%)</td>
</tr>
</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>Internal Diameter, μm</th>
<th>Preconstriction (%)</th>
<th>Number of arteries</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Passive (without blocker)</td>
<td>Active (with blocker)</td>
</tr>
<tr>
<td>Non CAD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>145 ± 10</td>
<td>145 ± 9</td>
</tr>
<tr>
<td>10 mM 4-AP</td>
<td>159 ± 13</td>
<td>154 ± 11</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>160 ± 24</td>
<td>147 ± 25</td>
</tr>
<tr>
<td>10 mM 4-AP (over-constriction)</td>
<td>160 ± 24</td>
<td>152 ± 22</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>183 ± 20</td>
<td>180 ± 20</td>
</tr>
<tr>
<td>100 nM paxilline</td>
<td>178 ± 24</td>
<td>174 ± 24</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>247 ± 14</td>
<td>229 ± 25</td>
</tr>
<tr>
<td>100 mM 1bTX</td>
<td>250 ± 16</td>
<td>242 ± 15</td>
</tr>
<tr>
<td>100 mM 1bTX + 10 mM 4-AP</td>
<td>247 ± 14</td>
<td>226 ± 19</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>146 ± 10</td>
<td>143 ± 9</td>
</tr>
<tr>
<td>1 μM DPO-1</td>
<td>147 ± 8</td>
<td>144 ± 8</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>149 ± 12</td>
<td>148 ± 12</td>
</tr>
<tr>
<td>30 nM Psora-4</td>
<td>148 ± 12</td>
<td>145 ± 12</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>150 ± 13</td>
<td>149 ± 12</td>
</tr>
<tr>
<td>10 mM PAP-1</td>
<td>147 ± 12</td>
<td>142 ± 14</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>163 ± 16</td>
<td>162 ± 13</td>
</tr>
<tr>
<td>3 μM CP-339818</td>
<td>163 ± 16</td>
<td>162 ± 16</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>166 ± 21</td>
<td>155 ± 21</td>
</tr>
<tr>
<td>100 nM stromatoxin</td>
<td>166 ± 21</td>
<td>152 ± 21</td>
</tr>
<tr>
<td>CAD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>165 ± 28</td>
<td>164 ± 28</td>
</tr>
<tr>
<td>10 mM 4-AP</td>
<td>183 ± 26</td>
<td>181 ± 26</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>134 ± 14</td>
<td>133 ± 14</td>
</tr>
<tr>
<td>100 nM paxilline</td>
<td>134 ± 14</td>
<td>131 ± 12</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>139 ± 15</td>
<td>137 ± 13</td>
</tr>
<tr>
<td>1 μM DPO-1</td>
<td>136 ± 21</td>
<td>134 ± 17</td>
</tr>
<tr>
<td>Control (+L-NAME &amp; Indo)</td>
<td>178 ± 23</td>
<td>176 ± 22</td>
</tr>
<tr>
<td>30 nM Psora-4</td>
<td>177 ± 22</td>
<td>173 ± 22</td>
</tr>
</tbody>
</table>

Values are means ± SEM of H₂O₂-induced dilation. Indo, indomethacin; 4-AP, 4-aminopyridine; 1bTX, 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.

<table>
<thead>
<tr>
<th>Nucleotide sequence of Primers used for PCR</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>For traditional end-point PCR</strong></td>
<td><strong>Forward and Reverse primers</strong></td>
<td><strong>Amplicon size, bp</strong></td>
</tr>
<tr>
<td>Transcript</td>
<td>5'-</td>
<td>3'</td>
</tr>
<tr>
<td>Kv α1.1</td>
<td>AGT CGC ACT TCT CCA GTA TCC-3'</td>
<td>424</td>
</tr>
<tr>
<td></td>
<td>TCG GTC AGT AGG TTG CTC TTA T-3'</td>
<td></td>
</tr>
<tr>
<td>Kv α1.2</td>
<td>TCA AGT TGT CCA GAC ACT CCA A-3'</td>
<td>548</td>
</tr>
<tr>
<td></td>
<td>TGT GTT AGCCAA GGT ACA GAC ATT G-3'</td>
<td></td>
</tr>
<tr>
<td>Kv α1.3</td>
<td>CTT CAG GTT TCA GCA GCA TCC-3'</td>
<td>395</td>
</tr>
<tr>
<td></td>
<td>GCA GGT AGT GGA ATT G-3'</td>
<td></td>
</tr>
<tr>
<td>Kv α1.4</td>
<td>GAG GCG GAT GAA CCT ACT ACC-3'</td>
<td>401</td>
</tr>
<tr>
<td></td>
<td>CCC TTT CCC TGA CAC TCC TCC-3'</td>
<td></td>
</tr>
<tr>
<td>Kv α1.5</td>
<td>CGT ACT TCG CAG AGG CT-3'</td>
<td>465</td>
</tr>
<tr>
<td></td>
<td>CGA AGG CAT AAG G-3'</td>
<td></td>
</tr>
<tr>
<td>Kv α1.6</td>
<td>AGA GGC TGA CGA TGA GTC TTC-3'</td>
<td>364</td>
</tr>
<tr>
<td></td>
<td>CCG ATG TGG TGT AGG AAG GTA G-3'</td>
<td></td>
</tr>
<tr>
<td>Kv α2.1</td>
<td>GCC TTG CTC ATC CTC TTC TCT-3'</td>
<td>289</td>
</tr>
<tr>
<td></td>
<td>CCT GCT TCT TCT GCT CCT T-3'</td>
<td></td>
</tr>
<tr>
<td>Kv α9.3</td>
<td>GTC TTC TCC TTC TCT TCC TCT-3'</td>
<td>297</td>
</tr>
<tr>
<td></td>
<td>ACT GGT CCA CAT CAA TGT CCT T-3'</td>
<td></td>
</tr>
<tr>
<td>Kv β1.1</td>
<td>CAG CAG CCT TAG TCC CTC AG-3'</td>
<td>239</td>
</tr>
<tr>
<td></td>
<td>GCC GTT CAG CAA CCT CAT CT-3'</td>
<td></td>
</tr>
<tr>
<td>Kv β1.2</td>
<td>GTG GAG CAG CCG AAC AGA A-3'</td>
<td>192</td>
</tr>
<tr>
<td></td>
<td>GCC GTT CAG CAA CCT CAT CT-3'</td>
<td></td>
</tr>
<tr>
<td>Kv β1.3</td>
<td>ACC TCT GTT GAC TGC CTG T-3'</td>
<td>378</td>
</tr>
<tr>
<td></td>
<td>GCC GTT CAG CAA CCT CAT CT-3'</td>
<td></td>
</tr>
<tr>
<td>BK&lt;sub&gt;α&lt;/sub&gt;</td>
<td>ATG CCG AAC TCA CCC AAC A-3'</td>
<td>224</td>
</tr>
<tr>
<td></td>
<td>TCG CCA AAG ATG CAG ACC AC-3'</td>
<td></td>
</tr>
<tr>
<td>β-actin</td>
<td>GCT GTT CCG CTG CCA CGG CTC-3'</td>
<td>353</td>
</tr>
<tr>
<td></td>
<td>CAA ACA TGA TCT GGG TCA TCT TCT C-3'</td>
<td></td>
</tr>
<tr>
<td><strong>For quantitative PCR (qPCR)</strong></td>
<td><strong>Forward and Reverse</strong></td>
<td><strong>Amplicon size, bp</strong></td>
</tr>
<tr>
<td>Transcript</td>
<td>5'-</td>
<td>3'</td>
</tr>
<tr>
<td>Kv α1.5</td>
<td>CGTCATCGTCTCCAACTTAAC-3'</td>
<td>126</td>
</tr>
<tr>
<td></td>
<td>TCCGCTGGACTCTCTGTT-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCTTCTAGAGAAGCCACT-3'</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>GCACAATCATTCACCCACAGAT-3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCACCTTCCAGCTTCCCT-3'</td>
<td>114</td>
</tr>
<tr>
<td></td>
<td>TGTGTTGGCCTACAGTGTT-3'</td>
<td></td>
</tr>
</tbody>
</table>