H$_2$O$_2$-Induced Dilation in Human Coronary Arterioles: Role of Protein Kinase G Dimerization and Large-Conductance Ca$^{2+}$-Activated K$^+$ Channel Activation

David X. Zhang, Lena Borbouse, Debebe Gebremedhin, Suelhem A. Mendoza, Natalya S. Zinkevich, Rongshan Li, David D. Gutterman

Rationale: Hydrogen peroxide (H$_2$O$_2$) serves as a key endothelium-derived hyperpolarizing factor mediating flow-induced dilation in human coronary arterioles (HCAs). The precise mechanisms by which H$_2$O$_2$ elicits smooth muscle hyperpolarization are not well understood. An important mode of action of H$_2$O$_2$ involves the oxidation of cysteine residues in its target proteins, including protein kinase G (PKG)-I$\alpha$, thereby modulating their activities.

Objective: Here we hypothesize that H$_2$O$_2$ dilates HCAs through direct oxidation and activation of PKG-I$\alpha$ leading to the opening of the large-conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) channel and subsequent smooth muscle hyperpolarization.

Methods and Results: Flow and H$_2$O$_2$ induced pressure gradient/concentration-dependent vasodilation in isolated endothelium-intact and -denuded HCAs, respectively. The dilation was largely abolished by iberiotoxin, a BK$_{Ca}$ channel blocker. The PKG inhibitor Rp-8-Br-PET-cGMP also markedly inhibited flow- and H$_2$O$_2$-induced dilation, whereas the soluble guanylate cyclase inhibitor ODQ had no effect. Treatment of coronary smooth muscle cells (SMCs) with H$_2$O$_2$ elicited dose-dependent, reversible dimerization of PKG-I$\alpha$, and induced its translocation to the plasma membrane. Patch-clamp analysis identified a paxilline-sensitive single-channel K$^+$ current with a unitary conductance of 246-pS in freshly isolated coronary SMCs. Addition of H$_2$O$_2$ into the bath solution significantly increased the probability of BK$_{Ca}$ single-channel openings recorded from cell-attached patches, an effect that was blocked by the PKG-I$\alpha$ inhibitor DT-2. H$_2$O$_2$ exhibited an attenuated stimulatory effect on BK$_{Ca}$ channel open probability in inside-out membrane patches.

Conclusions: H$_2$O$_2$ dilates HCAs through a novel mechanism involving protein dimerization and activation of PKG-I$\alpha$ and subsequent opening of smooth muscle BK$_{Ca}$ channels. (Circ Res. 2012;110:471-480.)

Key Words: endothelium-derived hyperpolarizing factor ■ hydrogen peroxide ■ protein kinase G ■ Ca$^{2+}$-activated K$^+$ channel ■ vasodilation

Hydrogen peroxide (H$_2$O$_2$), a membrane permeable and relatively stable reactive oxygen species (ROS), has emerged as an important signaling molecule in the regulation of physiological and pathophysiological processes in vascular cells.1-3 Our recent studies indicate that, in human coronary arterioles (HCAs), H$_2$O$_2$ serves as a key endothelium-derived hyperpolarizing factor (EDHF) responsible for both flow-mediated dilation (FMD) and to a lesser extent agonist-induced dilation.4-6 As an EDHF, H$_2$O$_2$ elicits vasodilation through a mechanism involving smooth muscle Ca$^{2+}$-activated K$^+$ channel (K$_{Ca}$) activation and subsequent membrane hyperpolarization.6 Other studies have also demonstrated that H$_2$O$_2$ induces potent K$_{Ca}$-mediated smooth muscle hyperpolarization and relaxation in porcine coronary,7-9 human10 and mouse mesenteric arteries.11 Intriguingly, the role of H$_2$O$_2$ as a vasodilator factor in HCAs is more prominent in disease states (eg, in coronary artery disease [CAD]), whereas other traditional factors (ie, nitric oxide [NO]) and prostacyclin [PGI$_2$]) play a more important role in vasodilation in the absence of CAD or its risk factors.6 Despite extensive data demonstrating the importance of H$_2$O$_2$ in mediating smooth muscle hyperpolarization and dilation, the precise mechanisms of action of H$_2$O$_2$ as an EDHF remain poorly understood. The lack of detailed analysis on the mechanism of action of H$_2$O$_2$ has raised some concerns regarding the proposed role of H$_2$O$_2$ as an EDHF.12 Accumulating evidence indicates that a conserved mechanism by which H$_2$O$_2$ activates intracellular signaling in cells involves the oxidation of key cysteine residues in its target proteins.13 A
more recent study suggests that, via this mechanism, $H_2O_2$ induces dimerization and subsequent activation of protein kinase G (PKG)-Iα in vascular smooth muscle cells.14 PKG-Iα is an important regulator of activity of various smooth muscle K+ channels, including the large-conductance KCa (BKCa) channel.15 We hypothesized that $H_2O_2$ induces relaxation of HCA through direct oxidation and activation of PKG-Iα leading to the opening of BKCa channels and subsequent smooth muscle hyperpolarization. Using an integrated approach comprising isolated vascular reactivity measurement, immunohistochemical and molecular biological analysis, and patch-clamp technique, we performed in-depth analysis of cellular mechanisms responsible for $H_2O_2$-induced dilation in HCA. Our results demonstrate that BKCa channel activation plays a key role in flow- and $H_2O_2$-induced dilation of HCA. We also demonstrate that the opening of BKCa channels requires an intermediate signaling event involving $H_2O_2$-induced protein dimerization of PKG-Iα.

**Methods**

A detailed Methods section is available in the Online Supplement available at http://circres.ahajournals.org.

**Tissue Acquisition**

Fresh human right atrial appendages were obtained as discarded surgical specimens from patients undergoing cardiopulmonary bypass procedures as described previously.6 Patient demographic data are summarized in Online Table I.

**Cell Culture**

Human coronary artery smooth muscle cells (HCASMCs) were obtained from Lonza (Walkersville, MD), and cultured in full growth medium according to the manufacturer’s instructions. Cells between passages 4 and 6 were used for experiments.

**Isometric Tension Recording**

Coronary arterioles (~100–200 μm) were carefully dissected from the endocardial surface of the atri, and mounted in multichannel wire myograph (model 610 M, Danish Myo Technology A/S, Denmark) as previously described.16,17 After contraction with endothelin-1, relaxation responses to cumulative concentrations of $H_2O_2$ ($10^{-6}$–$3 \times 10^{-4}$ mol/L), spermine NONOate ($10^{-7}$–$3 \times 10^{-5}$ mol/L), an NO donor, or 8-pCPT-cGMP ($10^{-6}$–$10^{-4}$ mol/L), a membrane-permeable analog of guanosine 3′,5′-cyclic monophosphate (cGMP), were determined in the absence or presence of the following inhibitors: iberiotoxin (IbTX; 100 nmol/L), a specific inhibitor of BKCa channels; catalase (1000 U/mL), a $H_2O_2$ metabolizing enzyme; ODQ (10 μmol/L), a selective inhibitor of soluble guanylate cyclase (sGC); Rp-8-Br-PET-cGMP (100 μmol/L), a competitive PKG inhibitor; or DT-2 (10 μmol/L), a specific peptide inhibitor of PKG-Iα. To examine the role of smooth muscle hyperpolarization in $H_2O_2$-induced dilation, arteries were preconstricted with high-$K^+$ (80 mmol/L) Krebs (K-PSS). Vasodilator responses are expressed as percentage maximal relaxation relative to endothelin-1 or K-PSS constriction, with 100% representing full relaxation to basal tension.

**Videomicroscopy**

Coronary arterioles (~100–200 μm) were cannulated with 2 glass micropipettes, and the internal diameter of arterioles was measured with a video system.5,16 Vessels were constricted with endothelin, −1% to 30% to 50% of the baseline internal diameter. In studies of flow-induced dilation, flow was produced by changing the heights of 2 syringe reservoirs in equal and opposite directions to generate a pressure gradient.16 Flow-mediated responses (5–100 cm H2O) were examined before and after 30 minutes incubation with ODQ (10 μmol/L), Rp-8-Br-PET-cGMP (100 μmol/L), or iberiotoxin (IbTX; 100 nmol/L). At the end of each experiment, papaverine (10−4 mol/L), an endothelin-1-dependent vasodilator, was added to determine the maximal internal diameter for normalization of dilator responses.

**Enzymatic Isolation of Vascular Cells**

Vascular endothelial cells (ECs) and SMCs were enzymatically dissociated from arteries as previously described.16,18 Cells were placed on ice or at 4°C and used the same day.

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

Single-channel $K^+$ currents were recorded from cell-attached and excised inside-out membrane patches of freshly isolated coronary SMCs using the patch-clamp method as previously described.16 For both cell-attached and inside-out patches, the pipette solution contained (in mmol/L) 125 KCl, 1.8 CaCl2, 1.0 MgCl2, 10 ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N′,N′,N′-tetraacetic acid (EGTA), and 5 HEPES (pH 7.2), and the bath solution contained (in mmol/L) 125 KCl, 1.8 CaCl2, 1.0 MgCl2, 10 glucose, 10 EGTA, and 5 HEPES (pH 7.2). Channel currents were recorded for at least 3 to 6 minutes under control conditions and after treatment with $H_2O_2$ (10–100 μmol/L) in the absence or presence of paxilline (100 μmol/L), a specific and cell-permeable inhibitor of BKCa channels). Unless otherwise stated, all chemicals were applied to the bath through perfusion. To determine the role of PKG-Iα in $H_2O_2$-induced BKCa channel activation, we preincubated cells for 45 to 60 minutes with DT-2 (10 μmol/L), and the effect of $H_2O_2$ was determined. Experiments were performed at room temperature.

**RNA Extraction and Reverse Transcription-Polymerase Chain Reaction**

Total RNA from vascular tissues was extracted with TRIzol, and cDNA was synthesized. For freshly isolated vascular cells, RNA
Role of BKCa Channels in H2O2-Induced Dilation

To further examine the mechanism by which H2O2 induces smooth muscle hyperpolarization through BKCa channel activation, we investigated the role of the sGC-cGMP-PKG pathway in H2O2- and NO-mediated dilation. As illustrated in Figure 2A, H2O2 dose-dependently dilated HCAs (dilation at 10–4 mol/L, 82%±3%, n=15). The dilation was attenuated by the PKG inhibitor Rp-8-Br-PET-cGMP (100 μmol/L) (5%±6%, n=6, P<0.05 versus control) but not by ODQ (10 μmol/L), an inhibitor of sGC (61%±8%, n=9), indicating that H2O2 acts downstream of sGC at the level of PKG. DT-2, a peptide inhibitor of PKG-Iα, further confirmed the downstream action of this transferrable dilator agent, as the H2O2-induced relaxation (81%±6%, n=3) was significantly reduced by administration of 10 μmol/L DT-2 (13%±9%, n=3, P<0.05 versus control) (data not shown). In contrast, the NO donor spermine NONOate-induced relaxation (dilation at 10–5 mol/L, 76%±6%, n=11) was inhibited by both Rp-8-Br-PET-cGMP (16%±1%, n=4, P<0.05 versus control) and ODQ (7%±6%, n=4, P<0.05 versus control), signifying NO action upstream of both sGC and PKG (Figure 2B). Furthermore, direct PKG activation by the stable cell-permeable cGMP analog 8-pCPT-cGMP (10–6–10–4 mol/L) dilated HCAs in a concentration-dependent manner (dilation at 10–4 mol/L, 42%±9%, n=7) as shown in Figure 2C. This response was blocked by iberiotoxin (15%±6%, n=5, P<0.05 versus control), confirming a role for BKCa channels in PKG-induced
smooth muscle hyperpolarization and relaxation. As summarized in Online Table II, no significant effect on baseline vascular tone was observed for ODQ, Rp-8-Br-PET-cGMP, and iberiotoxin, and similar dilation to the smooth muscle relaxant papaverine (100 μmol/L) was found in arterioles with or without pretreatment with these inhibitors.

**H₂O₂-Induced Protein Dimerization of PKG-Ια in Human Coronary Artery Smooth Muscle Cells**

Recent evidence suggests that H₂O₂ directly activates the PKG isoform PKG-Ια but not PKG-Ιβ by oxidizing a specific cysteine residue of PKG-Ια, leading to disulfide dimerization of this isoform.14 To elucidate the role of H₂O₂ in the dimerization and activation of PKG-Ια in HCAs, immunohistochemistry and Western blotting were used to assess protein expression of this kinase. Immunohistochemical analysis demonstrated expression of PKG-Ι protein in coronary arterioles, in particular within the smooth muscle cell layer (Figure 3A, left). This was further verified by Western blot, which revealed that under basal conditions, most of PKG-Ι exists as a 75-kDa monomer form (Figure 3A, right). Treatment of cultured coronary SMCs with H₂O₂ induced concentration-dependent dimerization of PKG-Ι, an effect that was abolished in the presence of the reducing agent β-mercaptoethanol. Given that only PKG-Ια undergoes disulfide dimerization in the presence of H₂O₂, these results indicate that PKG-Ι proteins detected in coronary SMCs are mostly the Ια isoform. Immunoblotting studies of cultured human vascular SMCs from multiple vascular beds using PKG-Ια- and PKG-Ιβ-specific antibodies also revealed that PKG-Ια is the predominant isoform of PKG-Ι.19 Immunofluorescence was used to

![Figure 2. Role of guanylate cyclase and PKG in H₂O₂- and NO-mediated relaxation of human coronary arterioles. A, H₂O₂-induced relaxation responses were inhibited by the PKG inhibitor Rp-8-Br-PET-cGMP (100 μmol/L) but not by the soluble guanylate cyclase inhibitor ODQ (10 μmol/L). B, The NO donor spermine NONOate-induced relaxations were inhibited by both Rp-8-Br-PET-cGMP and ODQ. C, The stable cGMP analog 8-pCPT-cGMP dose-dependently dilated coronary arterioles, a response inhibited by the BKCa channel blocker iberiotoxin (100 nmol/L). N=4 to 15 vessels/each group. *P<0.05 versus control.](image-url)

**Figure 3. H₂O₂-induced protein dimerization of PKG-Ι in human coronary artery smooth muscle cells. A, The protein expression of PKG-Ι in HCAs was detected by immunohistochemical analysis (left panel; scale bar=50 μm) and Western blot (right panel). Under basal conditions, PKG-Ι was primarily in monomeric form. B, Treatment of HCASMCs with H₂O₂ induced concentration-dependent dimerization of PKG-Ι, which was blocked by the reducing agent β-mercaptoethanol (β-ME). Data are representative of 3 independent experiments. C, Immunofluorescence detected diffuse cytosolic expression of PKG-Ι under control conditions (upper panel). H₂O₂ (100 μmol/L) induced punctate expression of PKG-Ι along the plasma membrane (middle panel). Scale bar=20 μm. Data are representative of 3 independent experiments with 5 to 10 cells/group/experiment.**
visualize the translocation of PKG-Iα in freshly isolated coronary artery smooth muscle cells. Diffuse cytosolic staining of PKG-Iα was observed under control conditions (Figure 3C, upper panel). Administration of 100 μmol/L H2O2 changed the staining pattern to one punctuated by expression along the plasma membrane (Figure 3C, middle panel), suggesting translocation of the protein from the cytosol to the plasma membrane on activation by H2O2. Determination of plasma membrane/cyttoplasmic ratio of PKG-Iα fluorescence confirmed the plasma membrane localization of this protein in H2O2-treated cells (1.30±0.04 versus 0.88±0.02 of control; P<0.05). The lack of signal in the preparations without primary antibodies (Figure 3C, lower) shows that this is an immunospecific response.

Expression of BKCa Channels in Coronary Smooth Muscle Cells
BKCa channel expression is known to be modulated by disease, but its expression in HCA from patients with CAD is not clear. Therefore, the expression of BKCa channels in coronary SMCs of patients with and without CAD was examined. Expression of BKCa channel α-subunit was detected at mRNA level in freshly isolated SMCs from patients with and without CAD, as indicated by a representative image of reverse transcription-polymerase chain reaction analysis. B, BKCa α-subunit protein was expressed in coronary arterioles from patients with (patient number in black) and without CAD (patient number in gray). Lower, summarized data; n=8 and 9 for CAD and non CAD, respectively. C, Presence of BKCa channel α-subunit protein (green) is confirmed with immunocytochemistry using freshly dispersed SMCs. Cell nuclei are stained in blue. Scale bar=20 μm. Data are representative of 3 independent experiments with 5 to 10 cells/group/experiment.

D, In inside-out patches of freshly isolated SMCs from human coronary arterioles, an increase in membrane potential enhanced BKCa channel open probability (left) that was abolished by 100 nmol/L paxilline, a specific BKCa channel inhibitor. The current–voltage relationship revealed a unitary conductance of 246 pS with a reversal potential of 0 mV in symmetrical (145 mmol/L) K+ solutions (right). Note that c, closed state, n=4 patches.
inside-out patches. As illustrated in Figure 4D (left), the channel open state probability (NPo) was enhanced with each sequential increase in patch potential. Channel openings were abolished by the addition to the bath of 100 nmol/L paxilline, a selective and cell-permeable BKCa channel blocker. The current–voltage relationship determined over the voltage range of –60 and 60 mV in 20-mV increments revealed a unitary conductance of 246 pS, and a reversal potential of 0 mV when recorded in symmetrical K solutions (145 mmol/L) solutions (Figure 4D, right). These findings indicate that the 246-pS single-channel K currents recorded from HCA smooth muscle cell membranes display electrophysiological and pharmacological properties consistent with those of BKCa single-channel currents recorded from conduit coronary SMCs.22

Effect of H2O2 on BKCa Channel Currents in Isolated Human Coronary Artery Smooth Muscle Cells

To investigate the activation of BKCa channel currents by exogenously applied H2O2, BKCa single-channel currents were recorded in cell-attached or inside-out membrane patches of freshly isolated smooth muscle cells of HCAs using the patch clamp technique. In cell-attached patches, addition of 50 μmol/L H2O2 to the bath induced activation of BKCa single-channel currents (NPo, 0.0050±0.0017 before versus 0.0495±0.0147 after H2O2; n=11, respectively; P<0.05) that was subsequently blocked by the addition of 100 nmol/L paxilline to the bath (NPo, 0.0039±0.0011, n=10, P<0.05 versus H2O2) (Figure 5A), indicating that H2O2 activates smooth muscle BKCa channels. The activation of BKCa channels by H2O2 was concentration dependent in the concentration range of 10 to 100 μmol/L (data not shown). Compared with cell-attached patches, the effect of H2O2 on BKCa single-channel opening in inside-out membrane patches was markedly reduced (NPo, 0.0048±0.0007 before and 0.0085±0.0028 after; n=12, respectively; P>0.05). Together, these results suggest that H2O2 activation of BKCa channels requires an intracellular second messenger signaling pathway. To determine whether PKG participates as an intracellular signaling molecule in the H2O2-induced BKCa activation, we measured the effects of H2O2 on BKCa single-channel currents in cell-attached patches in the presence of 10 μmol/L DT-2, a PKG-Iα inhibitor. As demonstrated in Figure 5C, inhibition of PKG-Iα by DT-2 attenuated the increase in BKCa single-channel activity elicited by H2O2 (NPo, 0.0077±0.0026 before versus 0.0103±0.0034 after; n=10, respectively; P>0.05) to a level similar to those observed in the inside-out membrane patches lacking intracellular signaling systems.
Role of sGC, PKG, and BKCa Channels in Human Coronary Arteriolar Relaxation to Flow

To verify the functional significance of the proposed signaling pathway, we examined the roles of guanylate cyclase, PKG, and BKCa in FMD. H2O2 has been demonstrated to be a key mediator of FMD in HCAs. Pretreatment with the sGC inhibitor ODQ had no effect on FMD (dilation at 100-cm H2O gradient, 62%±8% versus 68%±3% of control; n=3, respectively; P>0.05), indicating no role for sGC (Figure 6A). In contrast, inhibition of PKG by Rp-8-Br-PET-cGMP significantly attenuated flow-mediated dilation (30%±5% versus 58%±3% of control; n=5, respectively; P<0.05), indicating no role for sGC. Furthermore, flow-mediated dilation was significantly impaired in the presence of iberiotoxin (30%±5% versus 67%±4% of control; n=6, respectively; P<0.05), implicating no role for BKCa channel activity as an initiating mechanism of smooth muscle hyperpolarization and dilation. Treatment of arterioles with ODQ, Rp-8-Br-PET-cGMP and iberiotoxin did not significantly affect baseline vessel diameters (Online Table II).

Discussion

This study uncovers a novel mechanism of H2O2-mediated smooth muscle hyperpolarization and dilation in the human coronary microcirculation. The major new findings are 3-fold. First, exogenous H2O2-induced dilation of HCAs results from the opening of smooth muscle BKCa channels. Second, the opening of BKCa channels requires intracellular signaling through H2O2-dependent dimerization and translocation of PKG-1α. Third, shear stress, which releases endogenous endothelium-derived H2O2, dilates HCAs by activating a similar signaling pathway as exogenous H2O2. Collectively, these data indicate that H2O2-induced protein dimerization and activation of PKG-1α and subsequent opening of BKCa channels serves as an important mechanism responsible for H2O2-mediated dilation of HCAs. The findings of this study provide further support for the proposed role of H2O2 as a diffusible EDHF in the human coronary microvessels and may apply to other coronary stimuli that induce a H2O2-mediated dilation, such as metabolic coronary dilation.

PKG in H2O2-Induced Dilation

The role of cGMP as a second messenger for NO-induced vasodilation has been well established. This mediator is synthesized in SMCs by sGC, a cytosolic enzyme activated by NO donors and endogenous NO released from ECs in response to vasodilators such as acetylcholine and bradykinin. Once generated, cGMP activates its downstream target PKG (types Iα and Iβ) in SMCs, followed by the initiation of a variety of cellular processes leading to smooth muscle relaxation. Consistent with this general mechanism, both sGC and PKG inhibitors markedly reduced NO-mediated dilation in HCAs. In contrast, inhibition of PKG (specifically PKG-Iα), but not sGC, markedly reduced H2O2-mediated dilation in HCAs, indicating that H2O2 acts downstream of sGC signaling and at the level of PKG-Iα. Such lack of inhibition by the sGC inhibitor ODQ has also been reported previously by our laboratory.

The PKG-Iα and -Iβ are 2 splice-variant isoforms of PKG, differing in their N-terminus by ~100 amino acids. However, only the Iα isoform is redox sensitive and activated by H2O2 through disulfide dimerization. The activation of different targets within the cGMP-PKG pathway by H2O2 and NO is further supported by the findings that H2O2-mediated dilation was abolished by high K+ (data not shown). It is generally agreed that PKG-Iα mediates smooth muscle relaxation through both Ca2+-dependent (eg, K+ channel activation) and Ca2+-independent (eg, changes in the activity of myosin phosphatase) mechanisms, but 2 PKG-Iα isoforms may differ in their specific mechanisms of relaxation. Therefore, it is possible that the selective activation of PKG-Iα, but not PKG-Iβ, contributes at least partially to H2O2-induced high K+-sensitive dilation. It is also interesting to note that H2O2 activates PKG-Iα through a marked (>10-fold) increase in the affinity (indicated by a decrease in Km) for substrate, whereas cGMP activates PKG-Iα primarily by increasing its maximum velocity (Vmax) without changes in the Km for substrate. Thus, an increase in PKG-Iα affinity for selected substrates (eg, K+ channels) may serve as another potential mechanism underlying high K+-sensitive dilation to H2O2. The (patho)physiological significance of the specific signaling initiated by H2O2 versus NO in vascular smooth muscle remains to be clarified.

It has been well demonstrated that H2O2 relaxes bovine pulmonary and coronary arteries primarily through sGC activation. The mechanism by which H2O2 stimulates sGC activity has been associated with the formation of compound I, a short-lived oxidized intermediate form of catalase that occurs during the metabolism of H2O2 by this enzyme. Pretreatment of the reaction mixture with the catalese inhibitor 3-amino-1,2,4-
K⁺ Channels in H₂O₂-Induced Dilatation

There is substantial evidence that H₂O₂ applied exogenously produces membrane hyperpolarization and relaxation of smooth muscle in several vascular beds.⁴–¹¹,²⁹⁻³² However, disparate results have been reported for the specific K⁺ channel(s) involved in H₂O₂-induced hyperpolarization or dilation, and the signaling cascade leading to K⁺ channel activation remains largely unexplored. Activation of ATP-sensitive K⁺ (Kᵥ) channels has been implicated in H₂O₂-mediated dilation of porcine and cat cerebral arteries.⁹⁻¹⁰ A number of studies indicate that BKCa channels play an important role in H₂O₂-induced dilation of porcine coronary arteries and arterioles,⁷⁻⁹ whereas other studies show that voltage-gated K⁺ (Kᵥ) channels but not BKCa channels contribute to the dilatory effect of H₂O₂ in canine and rat coronary arterioles.³¹⁻³² By measuring both isolated vascular reactivity and single K⁺ -channel activity, we demonstrate that H₂O₂-induced dilation of human coronary arterioles depends on increased opening of BKCa channels. In particular, our patch-clamp studies showed that H₂O₂ activated BKCa channel currents recorded from the cell-attached patches (intracellular components present) but not from inside-out patches (intracellular components absent) and that the stimulatory effect of H₂O₂ on BKCa activity was markedly reduced by the PKG-I inhibitor DT-2. Taken together, these data provided strong evidence that H₂O₂-induced BKCa channel opening requires intracellular signaling, ie, the activation of PKG-I in smooth muscle cells.

Several recent studies indicate that BKCa channels are under complex but coordinated regulation by a variety of protein kinases, including protein kinase C (PKC), protein kinase A (PKA), and PKG, as well as by protein phosphatases, including protein phosphatase 1.¹³⁻¹⁵ Specifically, phosphorylation of S¹⁵¹ by PKC during receptor agonist stimulation renders the channel responsive to PKG but prevents its activation by PKA, whereas PKC phosphorylation of both S¹⁵¹ and S⁶⁹⁵ renders the channel insensitive to PKG and PKA. PKG sensitivity can be rescued by protein phosphatase 1, a BKCa-associated protein that is constitutively active against phosphorylation of S⁶⁹⁵. The balance between protein kinases and protein phosphatases may vary in different smooth muscle preparations, contributing to the sensitivity of BKCa channels and potentially other K⁺ channels to PKG and activators such as H₂O₂.

There are rather extensive data from experimental animal models indicating that different diseases can have divergent effects on the expression and/or channel activity of the vascular smooth muscle BKCa channel.²⁰ For example, an upregulation of BKCa channel expression associated with an increased K⁺ current and vasodilation has been reported in SHR and DOCA-salt-induced hypertensive rats.²⁰ In contrast, a loss of BKCa-mediated dilation with an unexpected increase in the expression of BKCa channel subunits has been described in the coronary microcirculation using a swine model of metabolic syndrome.²¹ In the present study, we found that there is no significant difference in the protein expression level of coronary smooth muscle BKCa α-subunits between patients with CAD and those without, and more importantly, the BKCa channel is functional and mediates the dilation of coronary arterioles to H₂O₂ and flow in disease. Thus, our data further underscore the complexity of BKCa channel regulation by cardiovascular pathologies.²⁰

It remains to be determined whether flow- and H₂O₂-induced activation of BKCa channels in HCAs represents a compensatory pathway for the loss or impaired function of other K⁺ channels in the presence of CAD and other risk factors. As discussed above, H₂O₂-induced dilation is mediated by Kᵥ channels in the coronary microcirculation of dogs and rats.³¹⁻³² Although the involvement of different K⁺ channels in H₂O₂-induced dilation in the human versus animals may simply reflect species-specific differences, there is evidence that Kᵥ channels may be importantly involved in H₂O₂-induced dilation in relatively healthy subjects without preexisting CAD or many other cardiovascular disorders (authors’ unpublished observations). Because functional BKCa channels are present in subjects both with and without CAD and risk factors, the activation of BKCa channel-mediated dilation in CAD may result from the modulation of signaling pathways (eg, protein phosphorylation) rather than from the change of the number of functional BKCa channels.

Interestingly, when applied to the cytosolic side of an excised membrane patch in human embryonic kidney (HEK)-293 cells overexpressing the BKCa α-subunit, H₂O₂ inhibits BKCa channel currents by oxidizing the cysteine residues on the α-subunit.³⁶⁻³⁸ In the present study, H₂O₂ had no inhibitory effect on BKCa channel currents in inside-out patches. The reasons for these discrepancies remain unclear but could be related to variation of channels in native cells versus overexpression systems or the H₂O₂ concentrations (50 μmol/L used in the current study, and >1 mmol/L H₂O₂ used in others).³⁶⁻³⁸ In HCAs, the dilation induced by the highest concentration of H₂O₂ (300 μmol/L) was blocked by high K⁺ but not affected by the BKCa channel blocker iberiotoxin, indicating the possible involvement of other K⁺ channels. The identities of such K⁺ channels remain to be determined.

In patch-clamp studies, we observed that in some cell-attached patches, BKCa channels exhibit smaller single-channel conductance (eg, 100–200 pS) even with high-K⁺ bath solution to nullify effects of cell resting membrane potential. Our results are comparable to those obtained in smooth muscle cells from large human coronary arteries.²² The reasons for the reduced conductance are unclear. Because the K⁺ currents recorded in cell-attached patches are sensitive to the specific BKCa blocker paxilline and the conduc-
tance of these currents increases to approximately 240 to 250 pS once the patch is detached from the cell to form an inside-out patch, we suggest that the properties of the currents studied are most consistent with those of BKCa channels.

**Study Limitations**

Exogenous H$_2$O$_2$ has been widely used to study the role of H$_2$O$_2$ in cell signaling. In the present study, EC$_{50}$ (the concentration of drug that produces a 50% maximal response) of exogenous H$_2$O$_2$-induced dilation in HCAs is approximately 3×10$^{-5}$ mol/L, with a minimum concentration required to induce a catalase-sensitive vasodilation of approximately 10$^{-5}$ mol/L. These concentrations, similar to or lower than those used in other studies, are higher than those reported for endogenously generated H$_2$O$_2$ ($<10^{-6}$ mol/L) by physiological stimuli such as shear stress and A23187. This could be explained by the fact that the intracellular H$_2$O$_2$ may only reach 1% to 15% of the exogenously applied concentration, probably due to endogenous antioxidant systems that protect cells from ROS. It is also possible that H$_2$O$_2$ is generated locally at higher concentrations than those measured, before it diffuses to the active site.

Using immunoblotting and immunocytochemistry, we found that H$_2$O$_2$ induced PKG-I association with a decrease in the enzyme’s K$_m$ value. It is presumed, though not directly tested, that H$_2$O$_2$-induced dimerization of PKG-I contributes to the opening of BKCa channels and smooth muscle dilation in HCAs. Because there are no specific pharmacological agents available to inhibit the dimerization of PKG-I by H$_2$O$_2$, testing this hypothesis would require genetic manipulation of cytosine residues of PKG-I, an approach not feasible for isolated human blood vessels. Similarly, we did not directly assess PKG-I-mediated protein phosphorylation of BKCa channels, the result of which could provide further support for the activation of BKCa by dimerized PKG-I. Since specific antibodies for PKG-targeted residue in BKCa (serine 1072) are not available, the results obtained with a general antiphosphoserine antibody may be difficult to interpret because other protein kinases (such as PKC) also phosphorylate serine residues of the BKCa channel.

**Clinical Implications**

Flow-induced dilation of coronary arterioles from patients with CAD is mediated by a unique mechanism requiring the release of H$_2$O$_2$ from endothelial cells and subsequent smooth muscle hyperpolarization. In the absence of CAD or its risk factors, other traditional mediators (i.e., NO and PG) may play a more prominent role in dilation of coronary arterioles. In the present study, we provide evidence that the dimerization of PKG-I and subsequent activation of BKCa channels are 2 key intracellular signaling events responsible for H$_2$O$_2$-induced smooth muscle hyperpolarization and relaxation, thus revealing a fundamental signaling mechanism responsible for the role of H$_2$O$_2$ in regulation of coronary vascular tone and blood flow. With near-maximal cardiac extraction of oxygen at rest, it is essential that myocardial perfusion is closely coupled to increases in myocyte metabolism. By serving as a potent vasodilator in the coronary microcirculation, H$_2$O$_2$ derived from the blood vessel itself as well as from beating myocytes, may be an important endogenous regulator of myocardial perfusion under normal conditions and in disease states.

**Acknowledgments**

The authors wish to thank Dr. Kathryn M. Gauthier for expert assistance with patch clamping. We also thank the Division of Cardiothoracic Surgery at the Medical College of Wisconsin and the Veterans Affairs Medical Center, the Cardiothoracic Surgery Group of Milwaukee, the Cardiovascular Surgery Associates of Milwaukee, the Midwest Heart Surgery Institute, the Wisconsin Heart Group, Froedtert Memorial Lutheran Hospital, and the Aurora St. Luke’s Medical Center in Milwaukee for providing surgical specimens.

**Sources of Funding**

This work was supported by the National Heart, Lung, and Blood Institute at the National Institutes of Health [R01 HL080704 and R01 HL094971 to David D. Gutterman, R01 HL096647 to David X. Zhang]; and the American Heart Association [SDG 083042N to David X. Zhang].

**Disclosures**

None.

**References**


What Is Known?

- Hydrogen peroxide (H2O2) is a relatively stable reactive oxygen species (ROS) that serves as a signaling molecule in diverse physiological and pathophysiological responses.
- H2O2 is a key transferable endothelium-derived mediator responsible for flow-induced dilation and to a lesser extent receptor agonist-induced dilation in the human coronary microcirculation, particularly in patients with or at risk for coronary artery disease.
- H2O2 dilates blood vessels through the activation of smooth muscle K+ channels, including the large-conductance Ca2+-activated K+ (BKCa) channel.

What New Information Does This Article Contribute?

- Exogenous H2O2 opens arterial smooth muscle BKCa channels, resulting in dilation of human coronary arterioles.
- The mechanism by which H2O2 opens BKCa channels involves dimerization and translocation of protein kinase G (PKG)-Iα.

Novelty and Significance

What Is Known?

- A similar signaling pathway, involving BKCa activation via PKG-Iα, mediates flow-induced and H2O2-dependent dilation of human coronary arterioles.

Accumulating evidence suggests that H2O2 serves as an important signaling molecule in the vascular system, although it is traditionally viewed as a harmful by-product of cell metabolism. Endothelial H2O2 is the key diffusible factor mediating flow-induced (and to a lesser extent bradykinin-induced) dilation in the human coronary microcirculation. However, the precise mechanisms by which H2O2 relaxes coronary arterioles remain unclear. In this study, we found that exogenous H2O2 dilates human coronary arterioles by opening smooth muscle BKCa channels through dimerization and translocation of PKG-Iα. Flow or shear stress, which releases endothelium-derived H2O2, dilates coronary arterioles by activating a signaling pathway similar to that induced by exogenous H2O2. Our findings provide further support for the proposed role of H2O2 as a diffusible endothelium-derived hyperpolarizing factor in both animals and humans and have implications for antioxidant treatment strategies in patients with coronary artery disease.
H$_2$O$_2$-Induced Dilation in Human Coronary Arterioles: Role of Protein Kinase G Dimerization and Large-Conductance Ca$^{2+}$-Activated K$^+$ Channel Activation
David X. Zhang, Lena Borbouse, Debebe Gebremedhin, Suelhem A. Mendoza, Natalya S. Zinkevich, Rongshan Li and David D. Gutterman

*Circ Res.* 2012; 110:471-480; originally published online December 8, 2011;
doi: 10.1161/CIRCRESAHA.111.258871

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 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/110/3/471

Data Supplement (unedited) at:
http://circres.ahajournals.org/content/suppl/2011/12/08/CIRCRESAHA.111.258871.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

H₂O₂-induced dilation in human coronary arterioles: role of protein kinase G dimerization and large-conductance Ca²⁺-activated K⁺ channel activation

David X. Zhang, Lena Borbouse, Debebe Gebremedhin, Suelhem A. Mendoza, Natalya S. Zinkevich, Rongshan Li, David D. Gutterman

Short title: Zhang et al. PKG and BKCa in H₂O₂-induced dilation

Address Correspondence to:
David X. Zhang, Ph.D.
Department of Medicine
Cardiovascular Center
Medical College of Wisconsin
8701 Watertown Plank Rd
Milwaukee, WI 53226
Phone: (414) 456-5633
Fax: (414) 456-6572
E-mail: xfzhang@mcw.edu
Detailed Methods

Tissue Acquisition
Fresh human right atrial appendages were obtained as discarded surgical specimens from patients undergoing cardiopulmonary bypass procedures, as described previously (1). After surgical removal, atrial tissues were immersed an ice-cold cardioplegia solution and immediately transported to laboratory for isolated vessel studies. Demographic data were obtained from hospital records at the time of surgery, and are summarized in supplemental Table S1. The study conforms to the principles outlined in the Declaration of Helsinki, and all protocols were approved by the appropriate local Institutional Review Boards on the use of human subjects in research.

Cell Culture
Human coronary artery smooth muscle cells (HCASMCs) were obtained from Lonza (Walkersville, MD), and cultured in full growth medium (SmGM-2, Lonza) at 37 °C in a humidified incubator with 5% CO₂ according to the manufacturer’s instructions. SMCs were subcultured when the cells reached 70-80% confluence, and cells between passages 4 and 6 were used for experiments.

Isometric Tension Recording
Human atrial tissues were placed in ice-cold HEPES-buffered physiological saline solution (HEPES-PSS) containing (in mM) 138 NaCl, 4.0 KCl, 1.6 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 5.5 glucose, and 10 HEPES (pH 7.4). Coronary arterioles (~100–200 µm) were carefully dissected from the endocardial surface of the atria, and prepared for isometric tension recording as previously described (2, 3). In brief, four to eight arterial segments of approximately 1.5 mm in length were cut and carefully mounted in multi-chamber wire myographs (model 610M, Danish Myo Technology). Arterial segments were bathed in a modified Krebs physiological saline solution (Krebs-PSS) of the following composition (in mM): 123 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 20 NaHCO₃, 1.2 KH₂PO₄, 0.026 Na₂EDTA, and 11 glucose. Krebs-PSS in the myograph chamber and buffer reservoir was gassed continuously with a mixture of 21% O₂-5% CO₂-74% N₂ at 37°C to maintain a pH of 7.4. Following a 20 min equilibration period, vessels were stretched in a stepwise manner to a resting tension of 1 mN. Arteries were stimulated two times with a high-K⁺ (80 mM) Krebs solution (K-PSS), in which NaCl was substituted with KCl of equal molar concentration, for 3 min each at 10 min intervals.

Vessels were then constricted to 50-75% of maximum K-PSS responses with endothelin-1 (5×10⁻¹⁰–10⁻⁹ M). After the contraction reached steady state, relaxation responses to cumulative concentrations of H₂O₂ (10⁻⁶–3×10⁻⁴ M), spermine NONOate (10⁻⁷–3×10⁻⁵ M), an NO donor, or 8-pCPT-cGMP (10⁻⁶–10⁻⁴ M), a membrane-permeable analogue of guanosine 3',5'-cyclic monophosphate (cGMP), were determined as paired rings in the absence or presence of following inhibitors: iberiotoxin (IbTX; 100 nM), a specific inhibitor of large-conductance Ca²⁺-activated K⁺ (BKCa) channels; catalase (1000 U/ml), a H₂O₂ metabolizing enzyme; ODQ (10 μM), a selective, irreversible inhibitor of soluble guanylate cyclase (sGC); Rp-8-Br-PET-cGMP (100 μM), a competitive, reversible protein kinase G (PKG) inhibitor; or DT-2 (10 μM), a specific, peptide-based inhibitor of PKG-Iα. For some experiments, responses were determined in the same ring before and after the application of an inhibitor as above. Arteries were incubated with the inhibitors for 30min unless indicated otherwise. To examine the role of smooth muscle hyperpolarization in H₂O₂-induced relaxation, arteries were constricted with K-PSS (80 mM K⁺).

To avoid the potential interference of endogenous prostacyclin and NO, arteries were treated throughout the experiment with indomethacin (10 μM, a cyclooxygenase inhibitor) and N⁵-nitro-L-arginine methyl ester (L-NAME; 100 μM, an NO synthase inhibitor). Where indicated, the endothelium was removed by gently rubbing the intimal surface of the arterial segment with a
wire or human hair. The endothelium was considered intact if bradykinin (10^{-6} M) caused >80% relaxation of endothelin-1-constricted arteries and effectively denuded if bradykinin induced <10% relaxation. Relaxation responses are expressed as percent maximal relaxation relative to endothelin-1 constriction, with 100% representing full relaxation to basal tension.

**Videomicroscopy**

Coronary arterioles (≈100–200 μm) were carefully dissected and prepared for continuous videomicroscopic measurement of diameter as previously described (2, 4). Briefly, coronary arterioles were cannulated with 2 glass micropipettes, pressurized to 60 mmHg under a no-flow state, and allowed to equilibrate at 37 °C in Krebs-PSS, bubbled with a mixture of 21% O₂, 5% CO₂, and 74% N₂. The internal diameter of arterioles was measured with video system composed of an inverted microscope (Olympus CK2; magnification ×200) coupled to a CCD camera (WV-BL200, Panasonic), a video monitor, and a calibrated video micrometer (VIA-100K, Boeckeler Instruments; 0.4-μm resolution). After 1 h equilibration, vessels were constricted with endothelin-1 (5×10^{-10}–10^{-9} M) to 30-50% of the baseline internal diameter if the spontaneous myogenic tone was not sufficient to achieve the target reduction in diameter.

In studies of flow-induced dilation, flow was produced by changing the heights of two syringe reservoirs in equal and opposite directions to generate a pressure gradient (2, 4). Flow-mediated responses (5–100 cm H₂O) were examined before and after 30min incubation with ODQ (10 μM), Rp-8-Br-PET-cGMP (100 μM), or iberiotoxin (IbTX, 100 nM). At the end of each experiment, papaverine (10^{-4} M), an endothelium-independent vasodilator, was added to determine the maximal internal diameter for normalization of dilator responses.

All pharmacological agents were added to the bath unless otherwise indicated, and the volume of drug added was <1% of the total organ bath volume. Final bath concentrations of ethanol and DMSO used to dissolve some drugs did not exceed 0.1%. Dilatory responses are expressed as percent relaxation relative to spontaneous tone or endothelin-1 constriction. The 100% relaxation represents the maximal diameter, which was usually the diameter following the application of papaverine, or the passive baseline diameter before the development of myogenic tone.

**Enzymatic Isolation of Vascular Cells**

Vascular endothelial cells (ECs) and SMCs were enzymatically dissociated from arteries as previously described (2, 5, 6). In brief, artery segments were cut into small rings and incubated for 10 min in a low-Ca²⁺ dissociation solution consisting of (in mM) 145 NaCl, 4.0 KCl, 0.05 CaCl₂, 1.0 MgSO₄, 10 glucose, and 10 HEPES, with 0.1% BSA (pH 7.4). The solution was carefully removed, followed by sequential incubation at 37°C with papain (1.0 mg/ml) and dithiothreitol (0.5 mg/ml) in dissociation solution for 15 min and then collagenase (Sigma blend H; 2.0 mg/ml), trypsin inhibitor (1 mg/ml), and elastase (0.5 mg/ml) for 15–30 min. All enzymes and chemicals were purchased from Sigma. Artery segments were gently triturated to release ECs and SMCs. The dissociated cells were washed twice by centrifugation at 450 g for 5 min and resuspended in fresh enzyme-free dissociation solution. Cells were placed on ice or at 4°C and used the same day for RT-PCR, immunocytochemistry, and patch-clamp studies as described below.

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

Single-channel K⁺ currents were recorded from cell-attached and excised inside-out membrane patches of freshly isolated human coronary SMCs using the patch-clamp method as previously described (5, 6). Drops of cell suspensions were placed in a 1-ml recording bath, and cells were allowed to adhere to the bottom of the bath for at least 10 min in a normal physiological saline solution containing (in mM) 140 NaCl, 5.0 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 10 glucose, and 5 HEPES,
with the final pH adjust to pH 7.4 with NaOH. For both cell-attached and inside-out patches, the pipette solution contained (in mM) 125 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 5 HEPES, and 10 ethylene glycol-bis(β-aminoethyl ether)-N,N′,N″,N‴-tetraacetic acid (EGTA), with the final pH adjusted to 7.2 with KOH. After the formation of high-resistance seals (>1 GΩ), the bath was switched to a high K⁺ bath solution composed of (in mM) 125 KCl, 1.8 CaCl₂, 1.0 MgCl₂, 10 glucose, 5 HEPES, and 10 EGTA, with pH adjusted to 7.2 with KOH, to allow for the optimum control of membrane potential of the cell. The addition of EGTA (10 mM) into the bath solution results in an estimated free [Ca²⁺] of 4×10⁻⁸ M according to the WEBMAXC computer program of Patton. Solutions were superfused through the bath by gravity at a flow rate of 1 ml/min. Channel currents from cell-attached or inside-out patches were recorded for least 3-6 min under control conditions and after treatment with H₂O₂ (10–100 μM) in the absence or presence of paxilline (100 nM, a specific and cell-permeable inhibitor of BKCa channels). Paxilline instead of iberiotoxin was used in these studies because iberiotoxin binds to the extracellular site of BKCa channel protein and thus has to be applied in the pipette solution for cell-attached and inside-out patches. Unless otherwise stated, all chemicals were applied to the bath through perfusion. To determine the role of PKG-Iα in H₂O₂-induced BKCa channel activation, cells were preincubated for 45-60 min with DT-2 (10 μM), a cell-permeable peptide inhibitor of PKG-Iα and the effect of H₂O₂ determined. Experiments were performed at room temperature. Channel currents were recorded with an Axopatch 200B amplifier (Axon Instruments) and pClamp 10 software (Axon instruments), or with an EPC-7 amplifier (List Biological Laboratories). Patch pipettes were fabricated from borosilicate glass capillaries using a horizontal Flaming-Brown pipette puller (model P-97, Sutter Instruments), and fire-polished under a microforge (model MF-83, Narishige) to produce 3 to 10 MΩ tip resistances. The recording pipette was mounted on a three-axis water hydraulic micromanipulator (Narishige), with the micromanipulator attached through an adaptor at the back of the stage of an inverted microscope (model Eclipse TE-200, Nikon). Signals were filtered at 1 kHz and sampled at 2.5-5 kHz. Single-channel currents were analyzed using the pClamp software. The open state probability (NPo) was determined by the event list analysis using criterion of 50% threshold crossing. Slope conductance was determined by fitting the unitary current-voltage relation using least-square linear regression analysis.

**RNA Extraction and RT-PCR**

Total RNA from vascular tissues was extracted with TRIzol, and cDNA was synthesized using iScript Reverse Transcriptase Kit (BioRad). For freshly isolated vascular cells, RNA extraction was performed as previously reported (2). In brief, ECs or SMCs were selectively aspirated into a large-bore glass pipette (10- to 20-μm tip). The pipette (containing 10–20 cells) was then placed tip-down in a sterile 0.5-ml PCR tube, and pipette tip broken by gentle pressure to release the cell-containing solution. The tubes were snap frozen in liquid N₂. The complete first-strand cDNA synthesis solution (Bio-Rad) except reverse transcriptase was added to each tube, followed by two rapid freeze-thaw cycles to rupture cells and allow access to cellular RNA. Reverse transcriptase was then added and samples incubated at 42°C for 1 h. The cDNA was divided into 3-4 aliquots and subjected to PCR amplification using a 38-cycle touch-down protocol with the following BKCa gene-specific primers: forward 5'- ATG CGG AAC TCA CCC AAC A-3' and reverse 5'- TCG CCA AAG ATG CAG ACC AC-3', [for a 224-bp fragment (NM_001014797)].

**Immunoblot Analysis**

Vascular tissues or cells were homogenized in ice-cold RIPA buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 1% deoxycholic acid, 0.5% Nonidet P-40, and 0.1% SDS] supplemented with a protease inhibitor cocktail (Roche), and centrifuged at 12,000 g for 10 min at 4°C. Protein samples (10–20 μg) were subjected to 10% SDS-PAGE and transferred to polyvinylidene
di fluoride or nitrocellulose membranes. Membranes were cut into strips and blotted each with a monoclonal mouse antibody specific to a conserved epitope of Slo1 (1:1000 dilution; 75-022, NeuroMab L6/60 clone) or with a monoclonal anti β-actin antibody (1:400,000 dilution; A5441, Sigma AC-15 clone), followed by horseradish peroxidase-conjugated secondary antibodies. After washing with Tris-buffered saline-Tween 20 (0.1%), immunoreactive complexed were visualized using the ECL chemiluminescence detection system (Amersham). Band density was quantitated using the Image J software. The protein expression of Slo1 was normalized to the β-actin loading control for each individual vascular tissue.

To detect disulfide dimerization of PKG-Iα, HCASMCs were treated with various concentrations of H2O2 in Krebs-PSS for 15 min at 37°C, and then homogenized in lysis buffer supplemented with 100 mM maleimide (7), a thiol-alkylating agent that prevents artifactual disulfide oxidation during homogenization of cells in air. Proteins were separated on a non-reducing SDS-PAGE, and detected with a polyclonal anti-human PKG-I (1:400 dilution; ab37709, Abcam). The reducing agent β-mercaptoethanol was added to some samples to confirm PKG-Iα dimers via disulfide formation.

**Immunohistochemistry**
Freshly dissected coronary arterioles were fixed with 10% buffered zinc formalin, embedded in paraffin wax, and cut into 4-μm sections. Tissue sections were deparaffinized in xylene and rehydrated through a graded series of ethanol and water. Sections were then treated with 3% H2O2 in phosphate buffered saline (PBS) to block endogenous peroxidase, followed by antigen retrieval by heating slides in citrate buffer (pH 6.0). After additional blocking with 3% BSA in PBS, sections were probed with a polyclonal antibody against a polyclonal rabbit antibody specific to human PKG-I (1:100 dilution; ab37709, Abcam). For immunodetection, sections were incubated with a polyclonal goat anti-rabbit, HRP-conjugated antibody (1:000 dilution; DAKO, Denmark), and then with a peroxidase-substrate solution (DAKO Liquid DAB-Substrate Chromogen System; DAKO, Denmark). Samples were then rinsed, counterstained with the nuclear dye hematoxylin (DAKO, Denmark), and mounted. Negative controls were obtained by omitting the primary antibody.

**Immunocytochemistry**
Freshly isolated SMCs were allowed to attach to poly-L-lysine-coated 12-mm coverslips for 30 min at 4°C. After several washes with PBS, cells were fixed with 4% paraformaldehyde for 10 min at room temperature, and then permeabilized with 0.1% saponin for 10 min. Cells were rinsed 3 times with PBS containing 50 mM NH₄Cl, and blocked with 1% BSA/5% normal goat serum/0.1% saponin in PBS in a humidified chamber for 30 min. For immunodetection of PKG-I and BKCa, cells were incubated either 1 h at room temperature or overnight at 4°C with one of the following antibodies: a polyclonal rabbit antibody specific to human PKG-I (1:50 dilution; ab37709, Abcam), and a monoclonal mouse antibody specific to a conserved epitope of Slo1 (1:200 dilution; 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 nM) for 5 min. After several rinses with PBS and then de-ionized H₂O, the coverslip were mounted on a 3-inch glass slide in SlowFade antifade medium (Invitrogen), and the edge of the coverslip sealed with a nail polish. Images were immediately taken using an epi-fluorescence microscope (model Eclipse TE-200, Nikon) with a 60x (NA 1.40) oil objective.

Fluorescence data were analyzed using ImageJ (version 1.37a), 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 six to eight rectangular user-
defined regions of interest (ROIs) both over the plasma membrane (2 x 10 pixels) and in adjacent cytoplasmic areas (5 x 10 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. The measurement of this ratio is a commonly used method to assess membrane accumulation of protein molecules in cells (8). Since we use a regular wide-field microscope to examine subcellular distribution of proteins in freshly isolated smooth muscle cells as previously described by others (9), the use of plasma membrane/cytoplasmic ratio may underestimate the plasma membrane localization of proteins of interest due to some out-of-focus fluorescence that is more likely to occur in the cytoplasm than around the plasma membrane.

**Chemicals**

Spermine NONOate was obtained from Cayman Chemical Company, paxilline from Tocris, and DT-2 from Axxora. All other chemicals were purchased from Sigma. Stock solutions were made in distilled water, except for paxilline (ethanol), ODQ (DMSO), spermine NONOate (0.01 N NaOH), and indomethacin (0.2 M Na₂CO₃).

**Statistical Analysis**

Data are presented as means ± SE. Significant differences between mean values were evaluated by Student t test or ANOVA followed by the Student-Newman-Keuls multiple-comparison test. To compare concentration-response between groups, a two-way repeated measures ANOVA was used. P values of p<0.05 were considered statistically significant.

**Supplemental References**


Supplemental Results

**Online Table I. Patient demographics (n=77)**

<table>
<thead>
<tr>
<th></th>
<th>n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male/female)</td>
<td>53/24</td>
</tr>
<tr>
<td>Age, yr (mean±SD)</td>
<td>66±10</td>
</tr>
<tr>
<td>Surgical procedure</td>
<td></td>
</tr>
<tr>
<td>MR</td>
<td>35 (45)</td>
</tr>
<tr>
<td>CABG</td>
<td>11 (14)</td>
</tr>
<tr>
<td>Valve replacement</td>
<td></td>
</tr>
<tr>
<td>Mitral</td>
<td>35 (45)</td>
</tr>
<tr>
<td>Aortic</td>
<td>23 (30)</td>
</tr>
<tr>
<td>Tricuspid</td>
<td>8 (10)</td>
</tr>
<tr>
<td>Other</td>
<td>7 (9)</td>
</tr>
<tr>
<td>Underlying diseases</td>
<td></td>
</tr>
<tr>
<td>CAD</td>
<td>52 (68)</td>
</tr>
<tr>
<td>HTN</td>
<td>54 (70)</td>
</tr>
<tr>
<td>HL</td>
<td>40 (52)</td>
</tr>
<tr>
<td>DM</td>
<td>17 (22)</td>
</tr>
<tr>
<td>AF</td>
<td>36 (47)</td>
</tr>
<tr>
<td>CHF</td>
<td>14 (18)</td>
</tr>
<tr>
<td>MI</td>
<td>7 (9)</td>
</tr>
<tr>
<td>(Tobacco use)</td>
<td>14 (18)</td>
</tr>
<tr>
<td>None of the above</td>
<td>2 (3)</td>
</tr>
</tbody>
</table>

n indicates the number of patients studied. AF, atrial fibrillation; CABG, coronary artery bypass graph; CAD, coronary artery disease; CHF, congestive heart failure; DM, diabetes mellitus; HL, hyperlipidemia; HTN, hypertension; MI, myocardial infarction; and MR, myocardial revascularization.
### Online Table II. Characteristics of isolated coronary arterioles

<table>
<thead>
<tr>
<th></th>
<th>Tension, mN</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal (-inhibitor)</td>
<td>Basal (+inhibitor)</td>
<td>ET-1</td>
<td>Papaverine (100 μM)</td>
<td>n</td>
</tr>
<tr>
<td><strong>$H_2O_2$-induced dilation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.2±0.2</td>
<td>8.8±1.2</td>
<td>ND</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>IbTX</td>
<td>1.9±0.4</td>
<td>10.2±1.9</td>
<td>ND</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
<td>0.9±0.2</td>
<td>13.1±5.1</td>
<td>ND</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Control (Denuded)</td>
<td>0.9±0.1</td>
<td>7.0±1.0</td>
<td>0.8±0.3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>IbTX (Denuded)</td>
<td>0.9±0.1</td>
<td>7.1±0.9</td>
<td>1.3±0.2</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.3±0.4</td>
<td>5.8±0.8</td>
<td>1.4±0.4</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>ODQ</td>
<td>1.7±0.7</td>
<td>6.1±0.9</td>
<td>1.9±0.7</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td>Rp-8-Br-PET-cGMP</td>
<td>1.3±0.5</td>
<td>5.7±1.0</td>
<td>1.7±0.5</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td><strong>Spermine NONOate-induced dilation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.5±0.1</td>
<td>3.9±0.5</td>
<td>0.5±0.1</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>ODQ</td>
<td>1.2±0.6</td>
<td>4.7±0.7</td>
<td>1.4±0.6</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Rp-8-Br-PET-cGMP</td>
<td>0.8±0.3</td>
<td>5.3±0.9</td>
<td>1.1±0.3</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td><strong>8-pCPT-cGMP-induced dilation</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.0±0.2</td>
<td>6.2±1.0</td>
<td>0.9±0.2</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>IbTX</td>
<td>0.7±0.2</td>
<td>6.8±1.7</td>
<td>0.9±0.4</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

|                          | Internal diameter, μm |            |           |           |        |
|--------------------------|                        | Passive (-inhibitor) | Passive (+inhibitor) | Active (-inhibitor) | Active (+inhibitor) | ET-1 | n  |
| **Flow-mediated dilation** |                        |            |           |           |        |
| Control                  | 128±22                 | 126±22     | 128±21    | 135±16    | 129±22 | 75±15 | 6   |
| ODQ                      | 128±22                 | 126±22     | 128±21    | 135±16    | 129±22 | 75±15 | 6   |
| Control                  | 138±15                 | 138±15     | 135±16    | 138±15    | 129±22 | 75±15 | 6   |
| Rp-8-Br-PET-cGMP         | 138±15                 | 125±9      | 125±9     | 125±9     | 129±22 | 74±15 | 6   |

Values are means ± SE; n indicates the number of arterioles studied. IbTX, iberiotoxin; ND, not determined.