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

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Abstract

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)-Iα, thereby modulating their activities.

Objective—Here we hypothesize that H$_2$O$_2$ dilates HCAs through direct oxidation and activation of PKG-Iα 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-Iα, 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-Iα 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-Iα and subsequent opening of smooth muscle BK$_{Ca}$ channels.

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Keywords

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Introduction

Hydrogen peroxide (H_2O_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_2O_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_2O_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_2O_2 induces potent K_{Ca}-mediated smooth muscle hyperpolarization and relaxation in porcine coronary,[7–9] human[10] and mouse mesenteric arteries.[11] Intriguingly, the role of H_2O_2 as a vasodilator factor in HCAs is more prominent in disease states [e.g., in coronary artery disease (CAD)], whereas other traditional factors [i.e., 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_2O_2 in mediating smooth muscle hyperpolarization and dilation, the precise mechanisms of action of H_2O_2 as an EDHF remain poorly understood. The lack of detailed analysis on the mechanism of action of H_2O_2 has raised some concerns regarding the proposed role of H_2O_2 as an EDHF.[12]

Accumulating evidence indicates that a conserved mechanism by which H_2O_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 K_{Ca} (BK_{Ca}) channel.[15] We hypothesized that H_2O_2 induces relaxation of HCAs through direct oxidation and activation of PKG-Iα leading to the opening of BK_{Ca} 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 HCAs. Our results demonstrate that BK_{Ca} channel activation plays a key role in flow- and H_2O_2-induced dilation of HCAs. We also demonstrate that the opening of BK_{Ca} 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 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 ($\approx$100–200 µm) were carefully dissected from the endocardial surface of the atria, and mounted in multi-chamber wire myograph (model 610M, Danish Myo Technology) as previously described.[16,17] After contraction with endothelin-1, relaxation responses to cumulative concentrations of H_2O_2 (10^{-6}–3×10^{-4} mol/L), spermine NONOate (10^{-7}–3×10^{-5} mol/L), a NO donor, or 8-pCPT-cGMP (10^{-6}–10^{-4} mol/L), a membrane-permeable analogue of guanosine 3',5'-cyclic monophosphate (cGMP), were determined in the absence or presence of following inhibitors: iberiotoxin (IbTX; 100 nmol/L), a specific inhibitor of BK_{Ca} 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 pre-constricted with high-K^+ (80 mmol/L K^+) Krebs (K-PSS). Vasodilator responses are expressed as percent maximal relaxation relative to endothelin-1 or K-PSS constriction, with 100% representing full relaxation to basal tension.

Videomicroscopy

Coronary arterioles ($\approx$100–200 µm) were cannulated with 2 glass micropipettes, and the internal diameter of arterioles was measured with a video system.[6,16] Vessels were constricted with endothelin-1 to 30–50% of the baseline internal 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.[6,16] Flow-mediated responses (5–100 cm H_2O) were examined before and after 30 min 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 endothelium-independent 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.[18] For both cell-attached and inside-out patches, the pipette solution contained (in mmol/L) 125 KCl, 1.8 $CaCl_2$, 1.0 $MgCl_2$, 10 ethylene glycol-bis(β-aminoethyl ether)-*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 $CaCl_2$, 1.0 $MgCl_2$, 10 glucose, 10 EGTA, and 5 HEPES (pH 7.2). Channel currents were recorded for at least 3–6 min under control conditions and after treatment with H_2O_2 (10–100 µmol/L) in the absence or presence of paxilline (100 nmol/L, a specific and cell-permeable inhibitor of BK_{Ca} 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 BK_{Ca} channel activation, cells were preincubated for 45–60 min with DT-2 (10 µmol/L) and the effect of H_2O_2 determined. Experiments were performed at room temperature.

RNA Extraction and RT-PCR

Total RNA from vascular tissues was extracted with TRIzol, and cDNA was synthesized. For freshly isolated vascular cells, RNA extraction was performed as previously reported.[16] The cDNA was subjected to PCR amplification using a 38-cycle touch-down protocol with gene-specific primers.

Immunoblot Analysis

Protein samples (10–20 µg) were subjected to 10% SDS-PAGE. Membranes were blotted with a monoclonal mouse antibody specific to a conserved epitope of Slo1 (1:1000 dilution; NeuroMab L6/60 clone) or with a polyclonal rabbit anti-human PKG-I (1: 400 dilution; Abcam), followed by horseradish peroxidase-conjugated secondary antibodies.

Immunohistochemistry

Freshly dissected coronary arterioles were fixed with 10% formalin, embedded in paraffin wax, and cut into 4-µm sections. Sections were blocked for endogenous peroxidase and non-specific protein binding, and probed with a polyclonal rabbit antibody specific to human PKG-I (1:100 dilution; Abcam). For immunodetection, sections were incubated with a HRP-conjugated antibody, and then with a peroxidase-substrate solution.

Immunocytochemistry

Freshly isolated SMCs were fixed with 4% paraformaldehyde for 10 min, and then permeabilized with 0.1% saponin for 10 min. Cells were then incubated either with a polyclonal rabbit antibody specific to human PKG-I (1:50 dilution; Abcam), or a monoclonal mouse antibody specific to a conserved epitope of Slo1 (1:200 dilution; NeuroMab L6/60 clone), followed by an appropriate goat secondary antibody conjugated with Alexa Fluor 488 (Invitrogen). Images were immediately taken using an epi-fluorescence microscope (model Eclipse TE-200, Nikon) with a 60x (NA 1.40) oil objective.

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 treatment groups, a two-way repeated measures ANOVA was used. P values of $p < 0.05$ were considered statistically significant.

Results

Role of BK_{Ca} channels in H_2O_2-induced dilation of human coronary arterioles

To examine the mechanism of H_2O_2-induced dilation in HCAs, graded doses of H_2O_2 (10^{-6}–3×10^{-4} mol/L) were applied to endothelium-intact and -denuded HCAs. H_2O_2 induced similar concentration-dependent dilation of both endothelium-intact (Figure 1A; dilation at 10^{-4} mol/L, 84±4%, n=13) and -denuded (Figure 1B; 85±8%, n=4) HCAs, indicating an endothelium-independent smooth muscle mechanism of dilation. The dilation was markedly inhibited by the specific BK_{Ca} channel blocker iberiotoxin (100 nmol/L) in both endothelium-intact (Figure 1A; 3±2%, n=9, $P < 0.05$ versus control) and -denuded (Figure 1B; 19±10%, n=4, $P < 0.05$ versus control) HCAs, implicating BK_{Ca} channels as the smooth muscle end-effectors of H_2O_2-induced relaxation. Consistent with previous data from our laboratory,[6] dilation to H_2O_2 was completely abolished by high K^+ (1±5%, n=7, $P < 0.05$ versus control) as well as by 1,000 U/ml exogenous catalase (10±4%, n=3, $P < 0.05$ versus control), a H_2O_2-metabolizing enzyme, confirming that this relaxation response is elicited through a K^+ channel end-effector and is a H_2O_2-sensitive effect (Figure 1C).

Role of sGC and PKG-Iα in H$_2$O$_2$- and NO-mediated dilation of human coronary arterioles

To further examine the mechanism by which H$_2$O$_2$ induces smooth muscle hyperpolarization through BK$_{Ca}$ channel activation, the role of the sGC-cGMP-PKG pathway in H$_2$O$_2$- and NO-mediated dilation was investigated. As illustrated in Figure 2A, H$_2$O$_2$ 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 H$_2$O$_2$ 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 H$_2$O$_2$ -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) were 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). Further, direct PKG activation by the stable cell-permeable cGMP analogue 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 BK$_{Ca}$ 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.

H$_2$O$_2$-induced protein dimerization of PKG-Iα in human coronary artery smooth muscle cells

Recent evidence suggests that H$_2$O$_2$ directly activates the PKG isoform PKG-Iα but not PKG-Iβ by oxidizing a specific cysteine residue of PKG-Iα, leading to disulfide dimerization of this isoform.[14] To elucidate the role of H$_2$O$_2$ in the dimerization and activation of PKG-Iα in HCAs, immunohistochemistry and Western blotting were used to assess protein expression of this kinase. Immunohistochemical analysis demonstrated expression of PKG-I 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-I exists as a 75 kDa monomer form (Figure 3A, right). Treatment of cultured coronary SMCs with H$_2$O$_2$ induced concentration-dependent dimerization of PKG-I, an effect which was abolished in the presence of the reducing agent β-mercaptoethanol. Given that only PKG-Iα undergoes disulfide dimerization in the presence of H$_2$O$_2$, these results indicate that PKG-I proteins detected in coronary SMCs are mostly the 1α isoform. Immunoblotting studies of cultured human vascular SMCs from multiple vascular beds using PKG-Iα- and PKG-Iβ-specific antibodies also revealed that PKG-Iα is the predominant isoform of PKG-I.[19]

Immunofluorescence was used to 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 H$_2$O$_2$ 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 upon activation by H$_2$O$_2$. Determination of plasma membrane/cytoplasmic ratio of PKG-Iα fluorescence confirmed the plasma membrane localization of this protein in H$_2$O$_2$-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 BK_{Ca} channels in coronary smooth muscle cells

BK_{Ca} channel expression is known to be modulated by disease,[20,21] but its expression in HCAs from patients with CAD is not clear. Therefore, the expression of BK_{Ca} channels in coronary SMCs of patients with and without CAD was examined. Expression of BK_{Ca} α-subunit was detected at both transcript (mRNA) (Figure 4A) and protein (Figure 4B) levels from patients with and without CAD. The average protein expression level of BK_{Ca} α-subunits showed no significant difference between patients with CAD and those without, although in 1–2 samples (out of 8) from CAD patients the level of BK_{Ca} channel expression was much higher than average. The expression of BK_{Ca} channels on the plasma membrane was further verified via immunofluorescence, which demonstrated BK_{Ca} channel immunoreactivity (in green) in smooth muscle cells of patients with CAD (Figure 4C, middle) or without CAD (Figure 4C, upper). Cell nuclei were stained in blue. Analysis of plasma membrane/cytoplasmic ratio of BK_{Ca} fluorescence showed a similar plasma membrane distribution of this protein in smooth muscle cells from patients with and without CAD (1.25±0.03 and 1.23±0.03, respectively; P>0.05). To examine the functional status of BK_{Ca} channels, single-channel activity and conductance were measured at different patch potentials using 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 BK_{Ca} 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^+ (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 BK_{Ca} single-channel currents recorded from conduit coronary SMCs.[22]

Effect of H_2O_2 on BK_{Ca} channel currents in isolated human coronary artery smooth muscle cells

To investigate the activation of BK_{Ca} channel currents by exogenously applied H_2O_2, BK_{Ca} 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 H_2O_2 to the bath induced activation of BK_{Ca} single-channel currents (NPo, 0.0050±0.0017 before versus 0.0495±0.0147 after H_2O_2; 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 H_2O_2) (Figure 5A), indicating that H_2O_2 activates smooth muscle BK_{Ca} channels. The activation of BK_{Ca} channels by H_2O_2 was concentration-dependent in the concentration range of 10 to 100 µmol/L (data not shown). Compared to cell-attached patches, the effect of H_2O_2 on BK_{Ca} 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 H_2O_2 activation of BK_{Ca} channels requires an intracellular second messenger signaling pathway. To determine whether PKG participates as an intracellular signaling molecule in the H_2O_2-induced BK_{Ca} activation, the effects of H_2O_2 on BK_{Ca} single-channel currents were measured 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 BK_{Ca} single-channel activity elicited by H_2O_2 (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 BK_{Ca} channels in human coronary arteriolar relaxation to flow

To verify the functional significance of the proposed signaling pathway, the roles of guanylate cyclase, PKG, and BK_{Ca} in FMD was examined. H_2O_2 has been demonstrated to be a key mediator of FMD in HCAs.[4,6] Pretreatment with the sGC inhibitor ODQ had no effect on FMD (dilation at 100-cm H_2O 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; $P<0.05$), suggesting the involvement of downstream PKG in the relaxation response to increased flow. Furthermore, flow-induced dilation was significantly impaired in the presence of iberiotoxin (30±5% versus 67±4% of control; n=6, respectively; $P<0.05$), implicating a role for BK_{Ca} 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 H_2O_2-mediated smooth muscle hyperpolarization and dilation in the human coronary microcirculation. The major new findings are threefold. First, exogenous H_2O_2-induced dilation of HCAs results from the opening of smooth muscle BK_{Ca} channels. Second, the opening of BK_{Ca} channels requires intracellular signaling through H_2O_2-induced dimerization and translocation of PKG-Iα. Third, shear stress, which releases endogenous endothelium-derived H_2O_2, dilates HCAs by activating a similar signaling pathway as exogenous H_2O_2. In sum, these data indicate that H_2O_2-induced protein dimerization and activation of PKG-Iα and subsequent opening of BK_{Ca} channels serves as an important mechanism responsible for H_2O_2-mediated dilation of HCAs. The findings of this study provide further support for the proposed role of H_2O_2 as a diffusible EDHF in the human coronary microvessels[4] and may apply to other coronary stimuli that induce a H_2O_2-mediated dilation, such as metabolic coronary dilation.[23]

PKG in H_2O_2-induced dilation

The role of cGMP as a second messenger for NO-induced vasodilation has been well established.[24] 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 (type Iα and Iβ) in SMCs, followed by the initiation of a variety of cellular processes leading to smooth muscle relaxation.[25] 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 H_2O_2-mediated dilation in HCAs, indicating that H_2O_2 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.[6]

The PKG-Iα and -Iβ are two 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 H_2O_2 through disulfide dimerization.[14] The activation of different targets within the cGMP-PKG pathway by H_2O_2 and NO is further supported by the findings that H_2O_2-mediated dilation was abolished by high K^+, whereas NO-mediated dilation was only partially inhibited by high K^+ (data not shown). It is generally agreed that PKG-I mediates smooth muscle relaxation through both Ca^{2+}-dependent (e.g., K^+ channel activation) and Ca^{2+}-independent (e.g., changes in the activity of myosin light chain phosphatase) mechanisms, but two PKG-I isoforms may differ in their specific mechanisms of relaxation.[25] Therefore,

it is possible that the selective activation of PKG-Iα but not PKG-Iβ contributes, at least partially, to H_2O_2-induced high K^+-sensitive dilation. It is also interesting to note that H_2O_2 activates PKG-Iα through a marked (>10-fold) increase in the affinity (indicated by a decrease in K_m) for substrate, whereas cGMP activates PKG-Iα primarily by increasing its maximum velocity (V_{max}) without changes in the K_m for substrate.[14] Thus, an increase in PKG-Iα affinity for selected substrates (e.g., K^+ channels) may serve as another potential mechanism underlying high K^+-sensitive dilation to H_2O_2. The (patho)physiological significance of the specific signaling initiated by H_2O_2 versus NO in vascular smooth muscle remains to be clarified.

It has been well demonstrated that H_2O_2 relaxes bovine pulmonary and coronary arteries primarily through sGC activation.[26–28] The mechanism by which H_2O_2 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 H_2O_2 by this enzyme.[26] Pretreatment of the reaction mixture with the catalase inhibitor 3-amino-1,2,4-triazole (3-AT) reduces the stimulatory effect of catalase in the presence of H_2O_2 on sGC activity, further suggesting the role of catalase in H_2O_2-induced activation of sGC.[26] Despite the stimulatory effect on sGC by H_2O_2, H_2O_2-induced relaxation is resistant to the sGC inhibitor ODQ in both pulmonary and coronary arteries.[27] Therefore, the possible contribution of sGC in H_2O_2-induced dilation of human coronary arterioles can not be completely excluded. However, the effectiveness of ODQ as an inhibitor of sGC activation by H_2O_2 may depend on vascular beds studied; for example, ODQ inhibits a portion of H_2O_2-induced dilation in porcine coronary arteries.[9] In addition, 3-AT does not affect H_2O_2-induced dilation in HCAs (authors' unpublished observations), indicating that catalase or its derived compound I is not involved in the vasodilatory responses of H_2O_2.

K^+ channels in H_2O_2-induced dilation

There is substantial evidence that H_2O_2 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 H_2O_2-induced hyperpolarization or dilation, and the signaling cascade leading to K^+ channel activation remains largely unexplored. Activation of ATP-sensitive K^+ (K_{ATP}) channels has been implicated in H_2O_2-mediated dilation of porcine and cat cerebral arteries.[29,30] A number of studies indicate that BK_{Ca} channels play an important role in H_2O_2-induced dilation of porcine coronary arteries and arterioles,[7–9] whereas other studies show that voltage-gated K^+ (Kv) channels but not BK_{Ca} channels contribute to the dilatory effect of H_2O_2 in canine and rat coronary arterioles.[31,32] By measuring both isolated vascular reactivity and single K^+-channel activity, we demonstrate that H_2O_2-induced dilation of human coronary arterioles depends on increased opening of BK_{Ca} channels. In particular, our patch-clamp studies showed that H_2O_2 activated BK_{Ca} 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 H_2O_2 on BK_{Ca} activity was markedly reduced by the PKG-Iα inhibitor DT-2. Taken together, these data provided strong evidence that H_2O_2-induced BK_{Ca} channel opening requires intracellular signaling, i.e. the activation of PKG-Iα in smooth muscle cells.

Several recent studies indicate that BK_{Ca} 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.[33–35] Specifically, phosphorylation of S^{1151} by PKC during receptor agonist stimulation renders the channel responsive to PKG but prevents its activation by PKA, whereas PKC phosphorylation of both S^{1151} and S^{695} renders the channel insensitive to PKG and PKA. PKG sensitivity can be rescued by protein phosphatase 1, a BK_{Ca}-associated protein that is

constitutively active against phosphorylation of S^{695}. The balance between protein kinases and protein phosphatases may vary in different smooth muscle preparations, contributing to the sensitivity of BK_{Ca} channels and potentially other K^+ channels to PKG and activators such as H_2O_2.

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 BK_{Ca} channel.[20] For example, an upregulation of BK_{Ca} 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 BK_{Ca}-mediated dilation with an unexpected increase in the expression of BK_{Ca} 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 BK_{Ca} α-subunits between patients with CAD and those without, and more importantly, the BK_{Ca} channel is functional and mediates the dilation of coronary arterioles to H_2O_2 and flow in disease. Thus, our data further underscore the complexity of BK_{Ca} channel regulation by cardiovascular pathologies.[20]

It remains to be determined whether flow- and H_2O_2-induced activation of BK_{Ca} 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, H_2O_2-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 H_2O_2-induced dilation in the human versus animals may simply reflect species-specific differences, there is evidence that Kv channels may be importantly involved in H_2O_2-induced dilation in relatively healthy subjects without preexisting CAD or many other cardiovascular disorders (author's unpublished observations). Because functional BK_{Ca} channels are present in subjects both with and without CAD and risk factors, the activation of BK_{Ca} channel-mediated dilation in CAD may result from the modulation of signaling pathways (e.g. protein phosphorylation) rather than from the change of the number of functional BK_{Ca} channels.

Interestingly, when applied to the cytosolic side of an excised membrane patch in human embryonic kidney (HEK)-293 cells overexpressing the BK_{Ca} α-subunit, H_2O_2 inhibits BK_{Ca} channel currents by oxidizing the cysteine residues on the α- subunit.[36–38] In the present study, H_2O_2 had no inhibitory effect on BK_{Ca} 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 over-expression systems or the H_2O_2 concentrations (50 µmol/L used in the current study, and < 1 mmol/L H_2O_2 used in others).[36–38] In HCAs, the dilation induced by the highest concentration of H_2O_2 (300 µmol/L) was blocked by high K^+ but not affected by the BK_{Ca} 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, BK_{Ca} channels exhibit smaller single-channel conductance (e.g., 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 BK_{Ca} blocker paxilline and the conductance of these currents increases to approximately 240–250 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 BK_{Ca} channels.

Study limitations

Exogenous H_2O_2 has been widely used to study the role of H_2O_2 in cell signaling.[2] In the present study, EC_{50} (the concentration of drug that produces a 50% maximal response) of exogenous H_2O_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,[7–11,26–32] are higher than those reported for endogenously generated H_2O_2 ($<10^{-6}$ mol/L) by physiological stimuli such as shear stress[4] and A23187.[39] This could be explained by the fact that the intracellular H_2O_2 may only reach 1–15% of the exogenously applied concentration,[2] probably due to endogenous anti-oxidant systems that protect cells from ROS. It is also possible that H_2O_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_2O_2 induced PKG-Iα dimerization and translocation to the plasma membrane in coronary SMCs. The dimerization results in the activation of PKG-Iα associated with a decrease in the enzyme's K_m value.[14] It is presumed, though not directly tested, that H_2O_2-induced dimerization of PKG-Iα contributes to the opening of BK_{Ca} channels and smooth muscle dilation in HCAs. Because there are no specific pharmacological agents available to inhibit the dimerization of PKG-Iα by H_2O_2, testing this hypothesis would require genetic manipulation of cysteine residues of PKG-Iα,[14] an approach not feasible for isolated human blood vessels. Similarly, we did not directly assess PKG-Iα-mediated protein phosphorylation of BK_{Ca} channels, the result of which could provide further support for the activation of BK_{Ca} by dimerized PKG-Iα. Since specific antibodies for PKG-targeted residue in BK_{Ca} (serine 1072) are not available, the results obtained with a general anti-phosphoserine antibody may be difficult to interpret because other protein kinases (such as PKC) also phosphorylate serine residues of the BK_{Ca} channel.

Clinical implications

Flow-induced dilation of coronary arterioles from patients with CAD is mediated by a unique mechanism requiring the release of H_2O_2 from endothelial cells and subsequent smooth muscle hyperpolarization.[4–6] In the absence of CAD or its risk factors, other traditional mediators (i.e., NO and PGI_2) may play a more prominent role in dilation of coronary arterioles.[6] In the present study, we provide evidence that the dimerization of PKG-Iα and subsequent activation of BK_{Ca} channels are two key intracellular signaling events responsible for H_2O_2-induced smooth muscle hyperpolarization and relaxation, thus revealing a fundamental signaling mechanism responsible for the role of H_2O_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.[23] By serving as a potent vasodilator in the coronary microcirculation, H_2O_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.[23]

Novelty and Significance

What Is Known?

- Hydrogen peroxide (H_2O_2) is a relatively stable reactive oxygen species (ROS) that serves as a signaling molecule in diverse physiological and pathophysiological responses.

- H_2O_2 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.

- H_2O_2 dilates blood vessels through the activation of smooth muscle K^+ channels, including the large-conductance Ca^{2+}-activated K^+ (BK_{Ca}) channel.

What New Information Does This Article Contribute?

- Exogenous H_2O_2 opens arterial smooth muscle BK_{Ca} channels resulting in dilation of human coronary arterioles.

- The mechanism by which H_2O_2 opens BK_{Ca} channels involves dimerization and translocation of protein kinase G (PKG)-Iα.

- A similar signaling pathway, involving BK_{Ca} activation via PKG-Iα, mediates flow-induced and H_2O_2-dependent dilation of human coronary arterioles.

Accumulating evidence suggests that H_2O_2 serves as an important signaling molecule in the vascular system, although it is traditionally viewed as a harmful by-product of cell metabolism. Endothelial H_2O_2, 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 H_2O_2 relaxes coronary arterioles remain unclear. In this study, we found that exogenous H_2O_2 dilates human coronary arterioles by opening smooth muscle BK_{Ca} channels through dimerization and translocation of PKG-Iα. Flow or shear stress, which releases endothelium-derived H_2O_2, dilates coronary arterioles by activating a signaling pathway similar to that induced by exogenous H_2O_2. Our findings provide further support for the proposed role of H_2O_2 as a diffusible endothelium-derived hyperpolarizing factor in both animals and humans. and have implications for antioxidant treatment strategies in patients with coronary disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Non-standard abbreviations and acronyms

cAMP	adenosine 3',5'-cyclic monophosphate
3-AT	3-amino-1,2,4-triazole
cGMP	guanosine 3',5'-cyclic monophosphate
BK_{Ca} channel	large conductance Ca^{2+}-activated K^+ channel
CAD	coronary artery disease
EC	endothelial cell
EDHF	endothelium-derived hyperpolarizing factor
FMD	flow-mediated dilation
HCA	human coronary arteriole
HCASMC	human coronary artery smooth muscle cell
H_2O_2	hydrogen peroxide
IbTX	iberiotoxin

K_{ATP} **channel**	ATP-sensitive K^+ channel
K_{Ca} **channel**	Ca^{2+}-activated K^+ channel
Kv channel	voltage-gated K^+ channel
L-NAME	N^G-nitro-L-arginine methyl ester
NPo	open state probability
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PKA	protein kinase A or cAMP-dependent protein kinase
PKC	protein kinase C
PKG	protein kinase G or cGMP-dependent protein kinase
PSS	physiological salt solution
ROS	reactive oxygen species
sGC	soluble guanylate cyclase
SMC	smooth muscle cell

Acknowledgments

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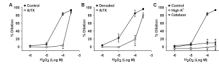
Figure 1. Role of BK$_{Ca}$ channels in H$_2$O$_2$-mediated dilation of human coronary arterioles
H$_2$O$_2$ induced similar dose-dependent dilation of both endothelium-intact (**A**) and -denuded (**B**) arterioles. These relaxations were inhibited by 100 nmol/L iberiotoxin (IbTX), a BK$_{Ca}$ channel blocker, and eliminated by high K$^+$ (80 mmol/L) and exogenous catalase (1,000 U/ml) (**C**). n=3–13 vessels/each group; *P<0.05 vs. control.
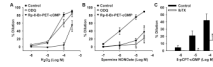
Figure 2. Role of guanylate cyclase and PKG in H$_2$O$_2$- and NO-mediated relaxation of human coronary arterioles

A: H$_2$O$_2$-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 that were inhibited by both Rp-8-Br-PET-cGMP and ODQ. **C**: The stable cGMP analogue 8-pCPT-cGMP dose-dependently dilated coronary arterioles, a response inhibited by the BK$_{Ca}$ channel blocker iberiotoxin (100 nmol/L). n=4–15 vessels/each group; *$P<0.05$ vs. control.
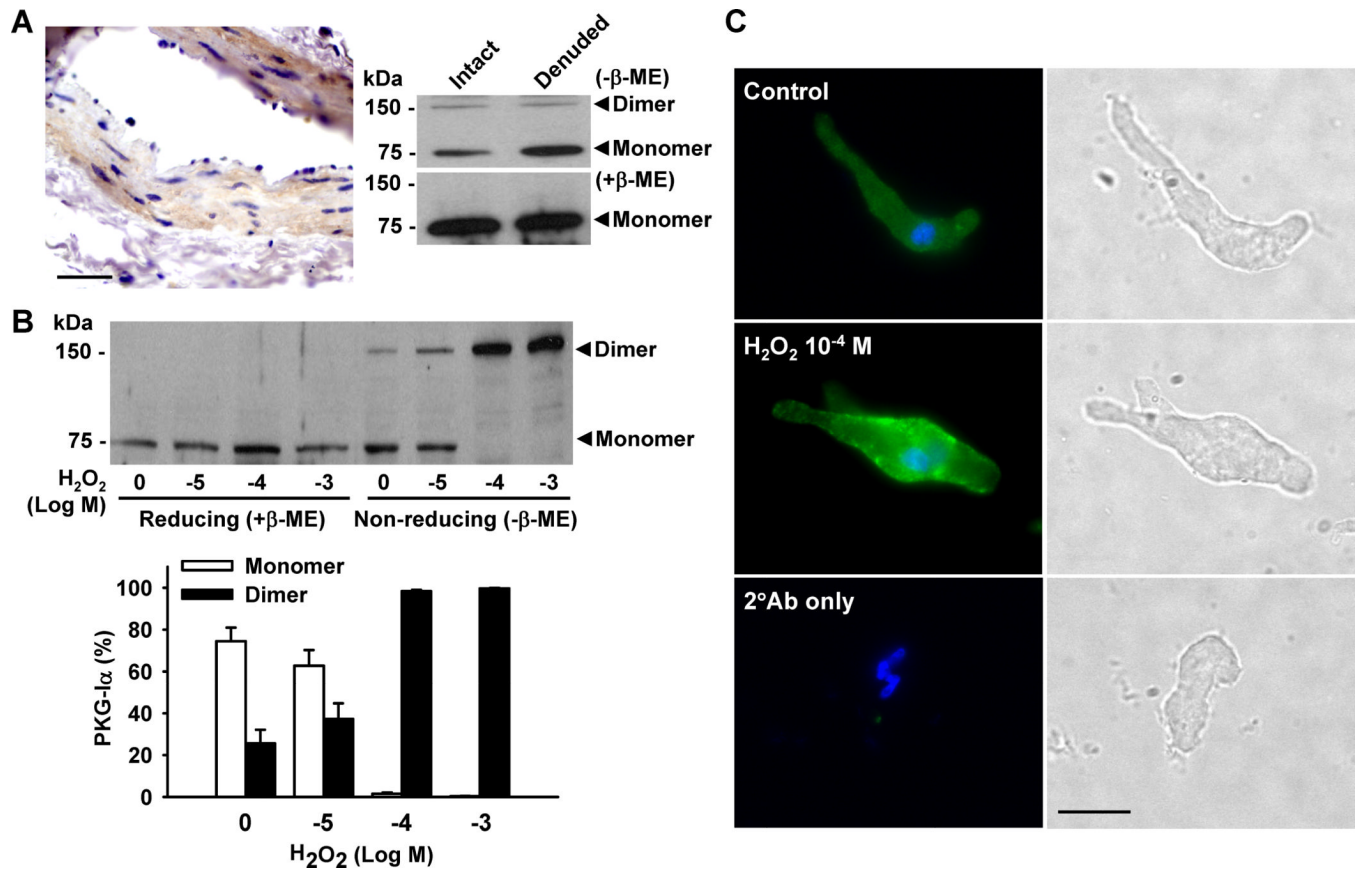
Figure 3. H$_2$O$_2$-induced protein dimerization of PKG-I in human coronary artery smooth muscle cells

A: The protein expression of PKG-I in HCAs was detected by immunohistochemical analysis (left panel; scale bar = 50 µm) and Western blot (right panel). Under basal conditions, PKG-I was primarily in monomeric form. **B**: Treatment of HCASMCs with H$_2$O$_2$ induced concentration-dependent dimerization of PKG-I 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-I under control conditions (upper). H$_2$O$_2$ (100 µmol/L) induced punctate expression of PKG-I along the plasma membrane (middle). Scale bar = 20 µm. Data are representative of 3 independent experiments with 5–10 cells/group/experiment.
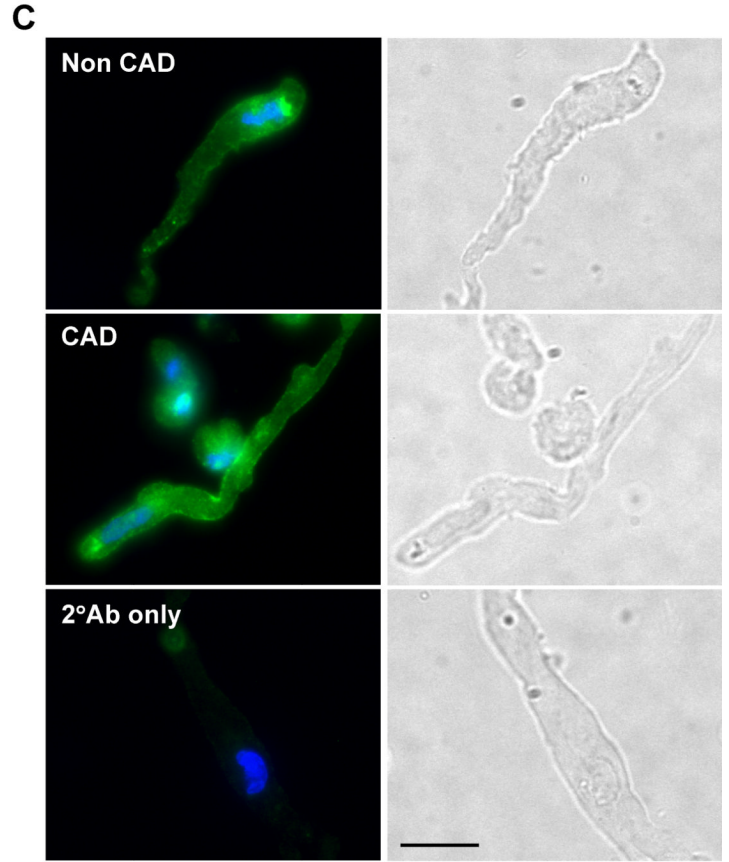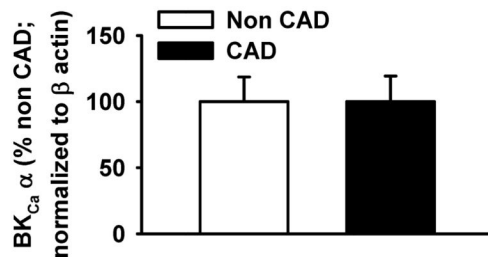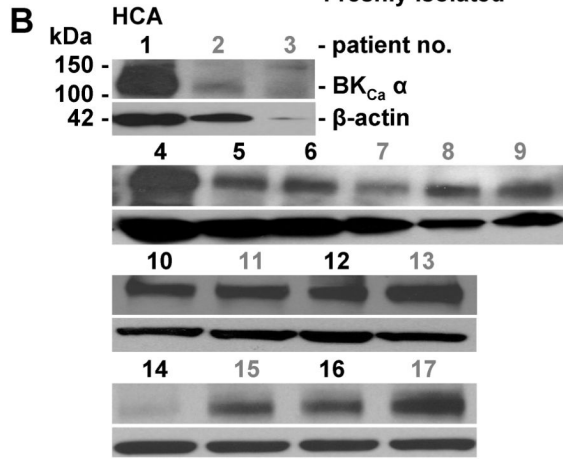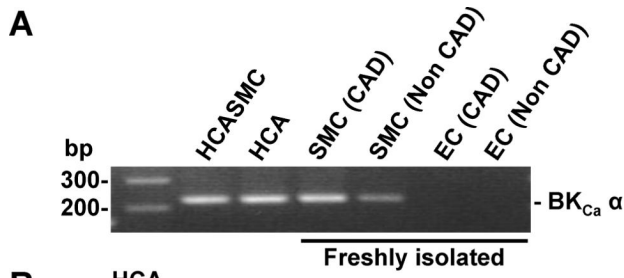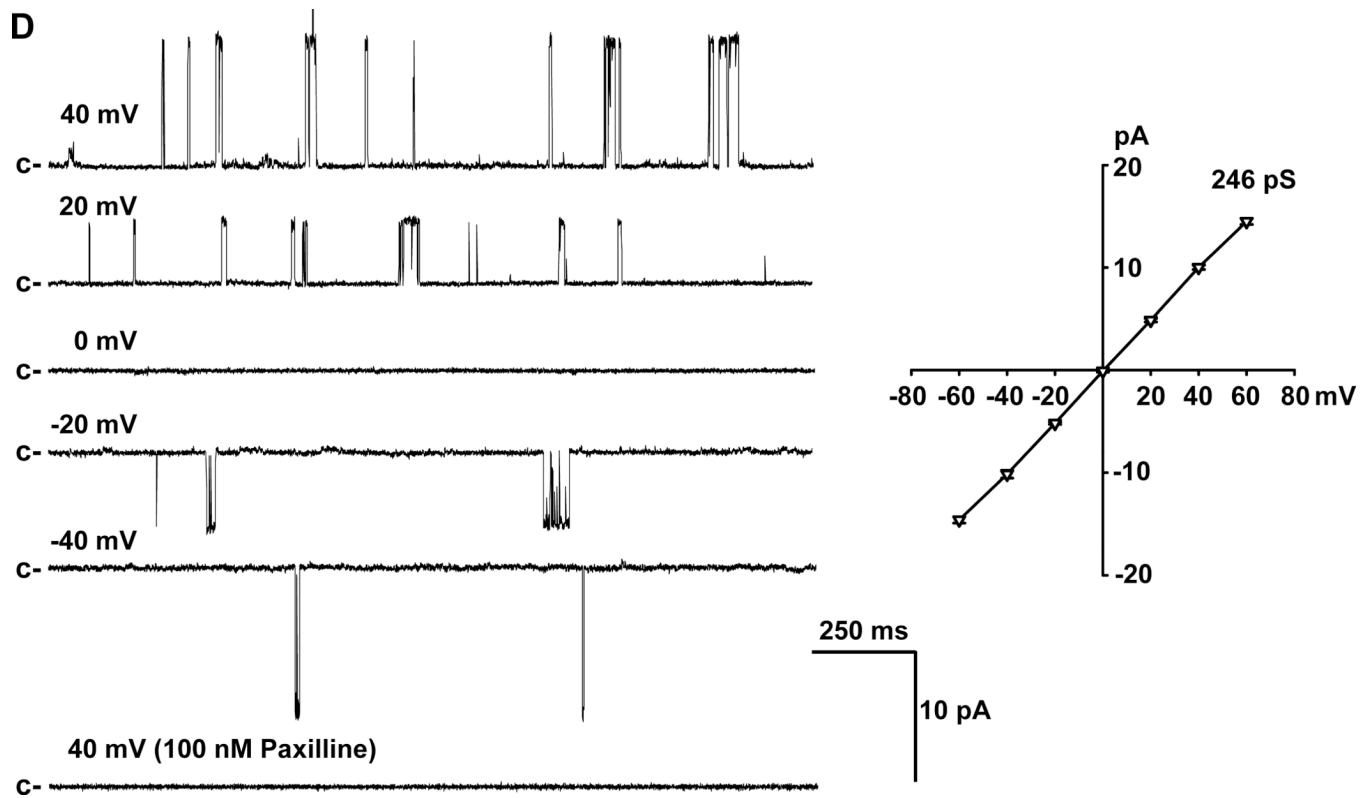
Figure

Figure 4. Expression of BK$_{Ca}$ channels in coronary smooth muscle cells
A: Expression of BK$_{Ca}$ channel α-subunits was detected at mRNA level in freshly isolated SMCs from patients with and without CAD, as indicated by a representative image of RT-PCR analysis. **B**: BK$_{Ca}$ α-subunit protein was expressed in coronary arterioles from patients with (patient no. in black) and without CAD (patient no. in gray). Lower, summarized data; n=8 and 9 for CAD and non CAD, respectively. **C**: Presence of BK$_{Ca}$ 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 5–10 cells/ group/ experiment. **D**: In inside-out patches of freshly isolated SMCs from human coronary arterioles, an increase in membrane potential enhanced BK$_{Ca}$ channel open probability (left) that was abolished by 100 nmol/L paxilline, a specific BK$_{Ca}$ 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). c, closed state. n=4 patches.
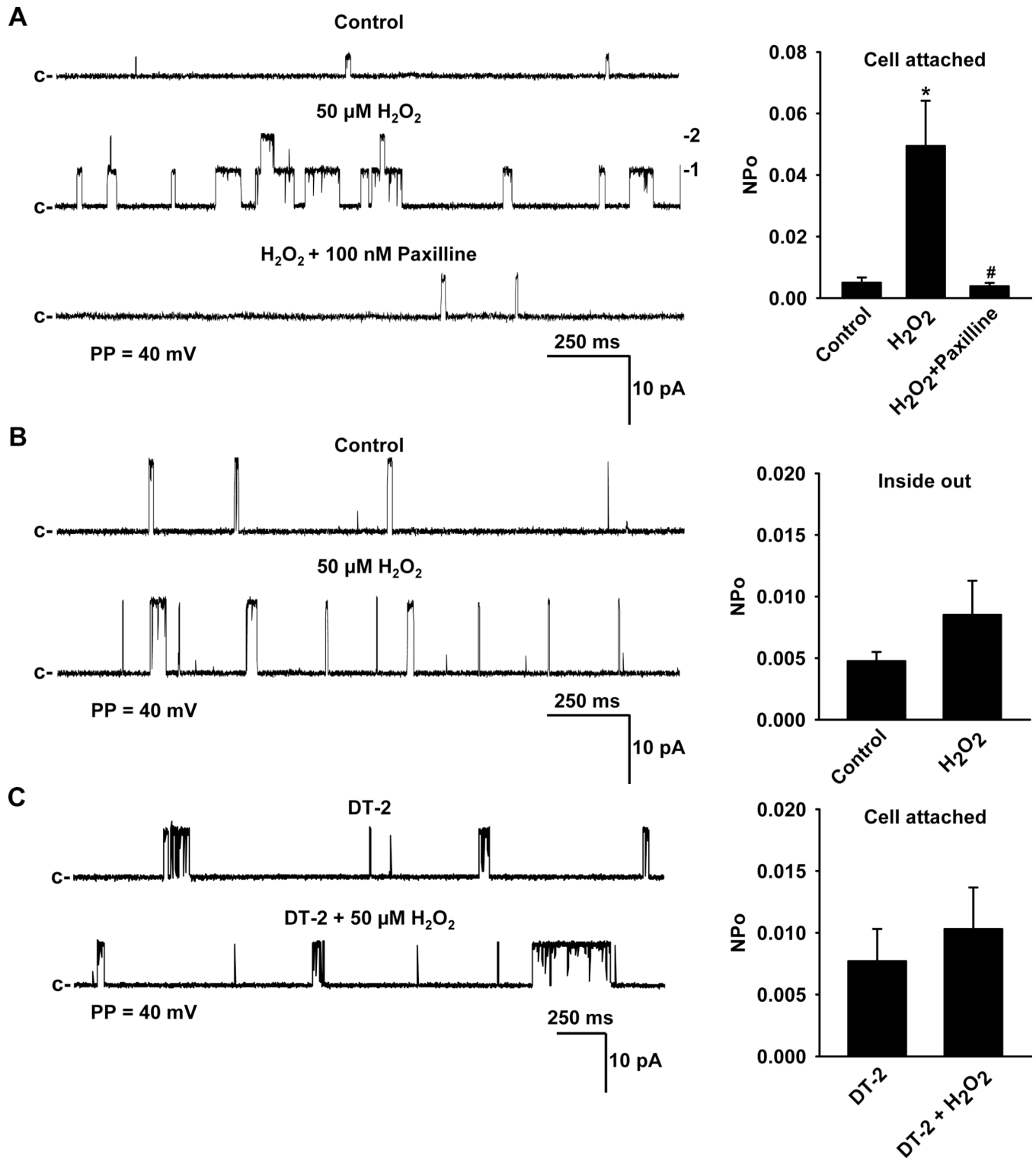
Figure 5. Effect of H_2O_2 on BK_{Ca} channel currents in freshly isolated coronary arteriolar smooth muscle cells
Using cell-attached patches (**A**), H_2O_2 (50 µmol/L) increased BK_{Ca} single-channel currents in a paxilline (100 nmol/L)-sensitive fashion. However, H_2O_2-induced BK_{Ca} activation was

greatly diminished in single-channel recordings from inside-out patches which lack intracellular constituents (**B**). H_2O_2-activated BK_{Ca} single-channel currents recorded from cell-attached patches were reduced in the presence of 10 µmol/L DT-2, a PKG-Iα inhibitor (**C**). c, closed state; PP, patch potential. n=6–12 patches/each group; *$P<0.05$ vs. control, [#]$P<0.05$ vs. H_2O_2.
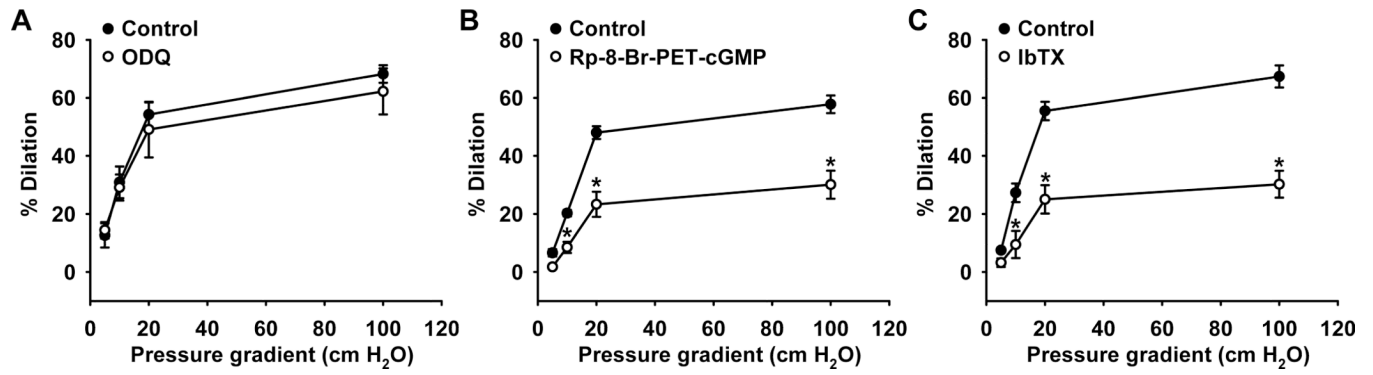
Figure 6. Role of guanylate cyclase, PKG, and BK$_{Ca}$ channels in human coronary arteriolar relaxation to flow
Fluid flow induced dilation of human coronary arterioles was attenuated by the PKG inhibitor Rp-8-Br-PET-cGMP (**B**) and by the BK$_{Ca}$ channel blocker iberiotoxin (**C**). The guanylate cyclase inhibitor ODQ, had no effect (**A**). n=3-6 vessels/each group; *$P<0.05$ vs. control.