Supplemental Material

H_2O_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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Short title: Zhang et al. PKG and BK_{Ca} in H_2O_2-induced dilation

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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% CO_2 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 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 5.5 glucose, and 10 HEPES (pH 7.4). Coronary arterioles ($\approx$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 $CaCl_2$, 1.2 $MgSO_4$, 20 $NaHCO_3$, 1.2 KH_2PO_4, 0.026 Na_2EDTA, and 11 glucose. Krebs-PSS in the myograph chamber and buffer reservoir was gassed continuously with a mixture of 21% O_2-5% CO_2-74% N_2 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 H_2O_2 (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 Ca^{2+}-activated K^+ (BK_{Ca}) channels; catalase (1000 U/ml), a H_2O_2 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 H_2O_2-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 N^G-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 ($\approx$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% O_2, 5% CO_2, and 74% N_2. The internal diameter of arterioles was measured with video system composed of an inverted microscope (Olympus CK2; magnification $\times$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 H_2O) 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-Ca^{2+} dissociation solution consisting of (in mM) 145 NaCl, 4.0 KCl, 0.05 $CaCl_2$, 1.0 $MgSO_4$, 10 glucose, and 10 HEPES, with 0.1% BSA (pH 7.4). The solution was carefully removed, 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 $CaCl_2$, 1.0 $MgCl_2$, 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 CaCl$_2$, 1.0 MgCl$_2$, 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 CaCl$_2$, 1.0 MgCl$_2$, 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 [Ca^{2+}] 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 H$_2$O$_2$ (10–100 μM) in the absence or presence of paxilline (100 nM, a specific and cell-permeable inhibitor of BK$_{Ca}$ channels). Paxilline instead of iberiotoxin was used in these studies because iberiotoxin binds to the extracellular site of BK$_{Ca}$ 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 H$_2$O$_2$-induced BK$_{Ca}$ 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 H$_2$O$_2$ 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 N$_2$. 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 BK$_{Ca}$ 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

difluoride 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 H$_2$O$_2$ 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% H$_2$O$_2$ 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 NH$_4$Cl, 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 BK$_{Ca}$, 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 H$_2$O, 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 Na_2CO_3).

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

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Supplemental Results

Online Table I. Patient demographics (n=77)

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

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

	Tension, mN				
	Basal (-inhibitor)	Basal (+inhibitor)	ET-1	Papaverine (100 µM)	n
H$_2$O$_2$-induced dilation					
Control	1.2±0.2		8.8±1.2	ND	13
IbTX		1.9±0.4	10.2±1.9	ND	9
Catalase		0.9±0.2	13.1±5.1	ND	3
Control (Denuded)	0.9±0.1		7.0±1.0	0.8±0.3	4
IbTX (Denuded)		0.9±0.1	7.1±0.9	1.3±0.2	5
Control	1.3±0.4		5.8±0.8	1.4±0.4	15
ODQ		1.7±0.7	6.1±0.9	1.9±0.7	9
Rp-8-Br-PET-cGMP		1.3±0.5	5.7±1.0	1.7±0.5	6
Spermine NONOate-induced dilation					
Control	0.5±0.1		3.9±0.5	0.5±0.1	11
ODQ		1.2±0.6	4.7±0.7	1.4±0.6	4
Rp-8-Br-PET-cGMP		0.8±0.3	5.3±0.9	1.1±0.3	4
8-pCPT-cGMP-induced dilation					
Control	1.0±0.2		6.2±1.0	0.9±0.2	7
IbTX		0.7±0.2	6.8±1.7	0.9±0.4	5

	Internal diameter, µm				
	Passive	Active (-inhibitor)	Active (+inhibitor)	ET-1	n
Flow-mediated dilation					
Control	128±22	126±22		59±9	3
ODQ	128±22		128±21	58±8	3
Control	138±15	135±16		75±11	5
Rp-8-Br-PET-cGMP	138±15		125±9	64±4	5
Control	129±22	128±22		75±15	6
IbTX	129±22		129±22	74±15	6

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