H$_2$O$_2$ Is the Transferrable Factor Mediating Flow-Induced Dilation in Human Coronary Arterioles

Yanping Liu, Aaron H. Bubolz, Suelhem Mendoza, David X. Zhang, David D. Gutterman

Rationale: Endothelial derived hydrogen peroxide (H$_2$O$_2$) is a necessary component of the pathway regulating flow-mediated dilation (FMD) in human coronary arterioles (HCAs). However, H$_2$O$_2$ has never been shown to be the endothelium-dependent transferrable hyperpolarization factor (EDHF) in response to shear stress. Objective: We examined the hypothesis that H$_2$O$_2$ serves as the EDHF in HCAs to shear stress.

Methods and Results: Two HCAs were cannulated in series (a donor intact vessel upstream and endothelium-denuded detector vessel downstream). Diameter changes to flow were examined in the absence and presence of polyethylene glycol catalase (PEG-CAT). The open state probability of large conductance Ca$^{2+}$-activated K$^+$ (BK$_{Ca}$) channels in smooth muscle cells downstream from the perfusate from an endothelium-intact arteriole was examined by patch clamping. In some experiments, a cyanogen bromide–activated resin column bound with CAT was used to remove H$_2$O$_2$ from the donor vessel. When flow proceeds from donor to detector, both vessels dilate (donor: 68±7%; detector: 45±11%). With flow in the opposite direction, only the donor vessel dilates. PEG-CAT contacting only the detector vessel blocked FMD in that vessel (6±4%) but not in donor vessel (61±13%). Paxilline inhibited dilation of endothelium-denuded HCAs to H$_2$O$_2$. Effluent from donor vessels elicited K$^+$ channel opening in an iberiotoxin- or PEG-CAT-sensitive fashion in cell-attached patches but had little effect on channel opening on inside-out patches. Vasodilation of detector vessels was diminished when exposed to effluent from CAT-column.

Conclusions: Flow induced endothelial production of H$_2$O$_2$, which acts as the transferrable EDHF activating BK$_{Ca}$ channels on the smooth muscle cells. (Circ Res. 2011;108:566-573.)

Key Words: coronary arterioles • shear stress • reactive oxygen species

As dilation induced by blood flow plays a pivotal role in the physiological control of vascular tone. The mechanism of flow-mediated dilation (FMD) is traditionally thought to involve the synthesis and release of vasodilators including nitric oxide (NO), prostaglandin (PG)I$_2$, and cytochrome P450 metabolites of arachidonic acid. Recent data from our laboratory indicate that FMD occurs in human coronary arterioles (HCAs) from patients with coronary disease via a novel mechanism requiring endothelial production of reactive oxygen species (ROS), specifically hydrogen peroxide (H$_2$O$_2$). Several studies demonstrate that H$_2$O$_2$ elicits non-NO, non–PGI$_2$-induced smooth muscle hyperpolarization and relaxation in mouse mesenteric, human mesenteric, porcine coronary, and human coronary arteries. However, it is unclear whether H$_2$O$_2$ generated in the endothelium truly acts as an endothelium-dependent hyperpolarization factor (EDHF) diffusing to the underlying vascular smooth muscle to elicit dilation, whether it acts locally within the endothelium to release a distinct vasodilating substance, or whether it acts via gap junctions to stimulate smooth muscle relaxation. We hypothesized that H$_2$O$_2$ is indeed released from the endothelium and is the transferrable agent that diffuses to the smooth muscle to elicit hyperpolarization by opening K$^+$ channels, with resultant vasodilation. We used a bioassay system, which combines videomicroscopic, patch clamping, and histofluorescence techniques to test this hypothesis. This unique approach allows for direct determination of the specific role of H$_2$O$_2$ in FMD. We determined that FMD in human coronary arterioles requires endothelial release H$_2$O$_2$. H$_2$O$_2$ is the transferrable substance (EDHF) that activates Ca$^{2+}$-activated K$^+$ channels in the underlying smooth muscle, a process that requires intact intracellular components. This study provides the first direct evidence that H$_2$O$_2$ is the EDHF that mediates FMD in the human coronary microcirculation.

Methods

Videomicroscopic Study

Atrial appendage tissue from human subjects undergoing cardiopulmonary bypass surgery was placed in oxygenated physiological salt
solution (PSS) as described previously. Two HCAs (250 to 300 μm) dissected from the endocardial surface of the atrial appendage were cannulated in tandem and equilibrated in separate chambers with an endothelium intact vessel upstream and an endothelium-denuded vessel downstream. Denudation was performed by injecting 2 mL of air through the lumen before cannulation. Diameters of donor and detector vessels were recorded after development of spontaneous myogenic tone with supplemental endothelin-1 (10⁻¹⁰ to 5×10⁻¹⁰ mol/L) given to achieve 30% to 50% reduction in passive diameter. Flow was produced by changing the heights of the reservoirs connected to the end of donor and detector arteriole in equal and opposite directions to generate a pressure gradient. Intraluminal diameter was measured at pressure gradients of 20 and 100 cm H₂O.

To eliminate the effect of NO and PGI₂, N⁴-nitro-L-arginine methyl ester (L-NAME) (10⁻⁴ mol/L), and indomethacin (10⁻⁵ mol/L) were added to both vessel chambers throughout each experiment. In some studies, polyethylene glycol catalase (PEG-CAT) (500 U/mL) was first applied to the detector chamber 20 minutes before flow. Diameter change to flow was measured in both donor and detector vessels. After washing the chamber, PEG-CAT was applied to the lumen of the donor vessel and FMD was measured again 20-minute later. The calculated percentage dilation to flow was normalized to the maximal dilation in the presence of papaverine (10⁻⁴ mol/L) added at the end of the experiment.

Vasodilation to direct application of H₂O₂ was examined in endothelium denuded HCAs in the absence and presence of papavine (10⁻⁴ mol/L).

In a separate study, a donor vessel was connected to a cyanogen bromide (CNBr)-activated resin column with or without CAT as described below. Effluent collected directly from the donor vessel (bypass column) or diverted through the column was applied to detector vessel incubated with 500 μL of PSS in a separate chamber. Diameter changes of detector vessels were measured in response to addition of effluent in cumulative volumes of 30, 300, and 3000 μL from the donor artery or from column bound with or without CAT.

### Patch-Clamp Studies

HCAs with intact endothelium were cannulated in a vessel chamber with the outflow pipette connected to a 500-μL patch-clamp chamber. The inflow reservoir was set at 100 cm H₂O. Coronary vascular smooth muscle cells acting as detectors, were enzymatically isolated from HCAs derived from the same atrial appendage as the donor HCAs. Unitary K⁺ currents were measured in cell-attached or inside-out membrane patches of vascular smooth muscle cells bathed in PSS as described previously. The effect of vessel effluent on unitary K⁺ currents was evaluated by 2-minute recording intervals, whereas the cells were exposed to either PSS or vessel effluent. In some experiments, 100 nmol/L iberiotoxin (IBTX) was added to the pipette solution to determine the role of large conductance Ca²⁺-activated K⁺ (BKca) channels. In some cases, PEG-CAT was added to the bath solution. The open-state probability (NPo) was determined by the event list analysis using criterion of 50% threshold crossing.

An expanded patch-clamp method is available in the Online Data Supplement at http://circres.ahajournals.org.

### Removing Extracellular H₂O₂ Released From Donor Vessel

We also conducted experiments to determine whether the response to shear at the detector site was the result of donor endothelial. To determine whether H₂O₂ was the substance transferred from the intact vessel and responsible for dilation of the detector vessel, we ran donor vessel effluent through a column containing beads of CNBr activated resin bound with or without CAT (10 mg/mL, CAT-column) to remove extracellular H₂O₂. A column of beads with CAT-free (CF-column) was used as a vehicle control. The effectiveness of CAT-column on removing H₂O₂ was confirmed by measuring H₂O₂ concentration in effluent collected from either column (CAT or CF) using the Amplex Red method (see below) and by detecting H₂O₂ in the detector vessel with fluorescence microscopy.

### Quantitation of Effluent H₂O₂

The presence of H₂O₂ in the effluent from the donor vessel or column (CAT or CF) was determined by quantitative measurement with an Amplex Red H₂O₂ assay kit. Effluent (50 μL) was added to a microplate well containing 100 μmol/L Amplex Red and 0.2 U/mL horseradish peroxidase. The plate was incubated for 30 minutes at room temperature while being protected from exposure to light. Absorbance was measured spectrophotometrically at 560 nm.

### Fluorescence Detection of H₂O₂

Five HCAs were isolated from the same tissue. One was used as a donor connecting to either CAT-column or CF-column. The others were subjected to endothelial denudation (detector vessels) and then incubated for 10 minute with Kreb’s solution, effluent from either the donor vessel or the CAT-column, or the CF-column, respectively. Dichlorodihydrofluorescein (DCFH) (5 μmol/L) was added in each light-protected chamber for 30 minutes to assess fluorescence intensity as described previously.

### Chemicals

All chemicals were purchased from Sigma. Fluorescence dyes and Amplex Red kits were obtained from Molecular Probes.

### Statistics

All data are expressed as means±SEM. Percentage dilation was calculated as the percentage change from the preconstricted diameter to the diameter after agonist or flow (maximal diameter was measured after papaverine; 10⁻⁴ mol/L). Data from vessels or cells exposed to flow or vessel effluent before and after antagonist treatment were compared using a one way ANOVA with repeated measures for dose and condition. The relative fluorescence of cells exposed to bath solution or vessel effluent was compared using a one way ANOVA. All differences were judged to be significant at the level of P<0.05.

### Results

HCAs were obtained from 24 patients with a mean maximal passive internal diameter 277±16 μm. Patient demographics including diagnoses are summarized in Online Table I.

### Presence of a Diffusible Vasodilator in Endothelium of HCAs

The bioassay system was used to confirm the transferable nature of the EDHF responsible for FMD in HCAs. As...
illustrated in Figure 1A, when flow proceeded from donor to detector, both vessels dilated (100 cm H2O: donor: 68±7%; detector: 45±11%, n=5). As anticipated, when flow was reversed (Figure 1B), only the donor (endothelium containing vessel) dilated (100 cm H2O; donor: 40±12%; detector: 11±8%, n=5, P<0.05 versus donor) suggesting a transferable endothelial dilator is essential for FMD in HCAs.

**Effect of Vessel Effluent on Large Conductance BKCa Activity in Human Coronary Smooth Muscle Cells**

To further examine the potential for smooth muscle hyperpolarization by the transferrable dilator agent, a modified bioassay system was used with a donor vessel with effluent superfusing detector coronary vascular smooth muscle cells freshly isolated from the same patient's heart. As illustrated in the sample traces (Figure 4A) and summary data (Figure 4B), the NPo of K+ channels in cell-attached patches was markedly enhanced when coronary smooth muscle cells were superfused with vessel effluent (control versus effluent, 0.0045±0.002 versus 0.11±0.05, n=8, P<0.05 versus control). The augmentation of K+ channel activity was abolished by IBTX, a specific blocker of BKCa channels (0.002±0.001, n=4) indicating the specific involvement of BKCa channels.

**Role of H2O2 in Vessel Effluent-Induced Activation of Ca2+-Activated K+ Channel**

The role of H2O2 in mediating BKCa activity in response to vessel effluent was evaluated by PEG-CAT. Figure 5 shows that in the presence of PEG-CAT, BKCa activity-induced by vessel effluent was attenuated (NPo, without versus with PEG-CAT, 0.11±0.09 versus 0.005±0.004, n=4) consistent with H2O2 as the mediator of smooth muscle hyperpolarization. These results confirm a role for H2O2 in flow-induced activation of BKCa channels.
Effect of Intracellular Components on BK_{Ca} Channel Activity Induced by Vessel Effluent

H_{2}O_{2} can open BK_{Ca} channels by a variety of direct and indirect mechanisms.\textsuperscript{15,16} To determine whether the enhanced BK_{Ca} activity induced by vessel effluent is a direct effect of H_{2}O_{2} or results from activation of other intracellular pathways, we performed inside-out patches where intracellular constituents were eliminated. In contrast to cell-attached patches, the NPo of BK_{Ca} channel activity was reduced when the patches were superfused with vessel effluent, (control versus effluent: 0.26 ± 0.06 versus 0.08 ± 0.04, n = 6, P < 0.05 versus control) (Figure 6A and 6B), suggesting activation of BK_{Ca} channels by vessel effluent requires the presence of intracellular molecules. CAT had no effect on BK_{Ca} channel activities in inside-out patches superfused with vessel effluent.

Effect of Removing Donor Extracellular H_{2}O_{2} on H_{2}O_{2} Production in Detector Vessels

Data presented thus far indicate that a transferrable EDHF mediates FMD in HCAs and that H_{2}O_{2} is critical in this process. To confirm that endothelial H_{2}O_{2} is the EDHF stimulated by shear stress that evokes hyperpolarization and relaxation in smooth muscle cells by a paracrine mechanism; and (2) H_{2}O_{2}-induced...
hyperpolarization and vasodilation result from the opening of BKCa channels in the smooth muscle membrane through an indirect effect requiring intact intracellular signaling.

**Is H2O2 Really an EDHF and Is It Responsible for FMD?**

By definition, EDHF is a transferrable substance that relays a dilator signal from the endothelium to the underlying vascular smooth muscle. This transfer may occur via direct cell to cell communication as with potassium ions traversing gap junctions19 or by a paracrine mechanism involving endothelial production of the chemical which traverses the endothelial cell and acts on adjacent or regional smooth muscle cells (eg, EETs20). H2O2 has been proposed as an EDHF based on the observation that agonist- or flow- induced non-NO-non-PGI2-mediated dilations are partially or completely prevented by catalase in several vascular beds from animals9,10,21 and humans.5,8,22 However, the conclusions derived from these studies are weakened by lack of direct evidence that endothelial H2O2 serves as the paracrine agent. Alternatively some other substance could be released from endothelial cell which traverses the extracellular space, stimulating release of H2O2 in the smooth muscle, or H2O2 could be acting within the endothelium to release a distinct EDHF. A final concern is that by studying intact vessels, it is not possible to eliminate direct communication between endothelium smooth muscle as being necessary for the dilator response involving H2O2. To circumvent these issues, the present study used a novel bioassay system to show that shear-induced endothelial derived H2O2 is the transferable factor responsible for smooth muscle hyperpolarization and vasodilation. This finding is supported by several lines of evidence. Firstly, H2O2 was detected from the effluent of donor vessels during flow. Second, FMD in both detector and donor HCAs was eliminated by application of PEG-CAT into the vessel lumen. Finally, in detector HCAs, both the DCFH fluorescence intensity and dilator response were markedly diminished in vessels exposed to donor effluent in which H2O2 was removed by a resin column coated with CAT compared to vessels incubated with effluent from the CAT-free column. These results provide strong evidence that endothelial H2O2 is indeed the transferrable EDHF responsible for flow-induced vasodilation in HCAs, which is a unique feature of human coronary vascular response to flow, not observed in other species.

**H2O2 Activates BKCa Channels**

It has been reported that exogenous application of H2O2 hyperpolarizes smooth muscle cells in various vascular beds.23–26 However, a variety of K⁺/Ca++ channels contribute to this dilation depending on the species and vascular bed. In rat cerebral and porcine coronary arteries, BKCa channels mediate H2O2-induced dilation because responses are blocked with tetraethylammonium27 or IBTX.28,29 In canine coronary arterioles and rat mesenteric arteries, H2O2-mediated dilation involves activation of K⁺ channels.30 In piglet pial arteries, H2O2 induced dilation was prevented by glibenclamide,25 indicating the contribution of KATP channels. H2O2 has been reported to have a bidirectional effects on BKCa channel activity.15,31,32 In porcine coronary smooth muscle cells H2O2 applied extracellularly increases opening probability.15 In contrast, when H2O2 is applied intracellularly in human embryonic kidney cells coexpressed with BKCa α- and β-subunits13,34 or in human umbilical vein endothelial cells,35 an inhibitory effect was observed. Oxidation of cysteine residue near the Ca²⁺ bowl sensor of BKCa α-subunit has been postulated as one of the mechanisms for the reduction of channel activity by H2O2.31 This differential paracrine and autocrine effects are illustrated in Figure 5.
effect of H₂O₂ on BK<sub>Ca</sub> may be a factor contributing to the divergent results in different preparations. In addition, various concentrations of H₂O₂ were used in different studies ranging from 1 μmol/L to 10 mmol/L. A transition from proliferative to apoptotic signaling occurs across this range of concentration, which may also contribute to the conflicting results. The unique bioassay preparations used in this study provide a physiological (but unmeasured) concentration of H₂O₂ from a donor vessel and obviate many of the problems associated with pharmacological doses.

We found that activation of BK<sub>Ca</sub> channels by H₂O₂ required the presence of intracellular constituents, because BK<sub>Ca</sub> activity was not increased by H₂O₂ in inside-out patches. These results are consistent with the study by Barlow and White,29 where 300 μmol/L H₂O₂ enhanced BK<sub>Ca</sub> channel activity in cell attached, but not in inside-out patches. The mechanisms by which how H₂O₂ activates BK<sub>Ca</sub> channel are complex and not well understood. Multiple signaling pathways have been implicated in H₂O₂-induced responses. The cGMP pathway plays prominently as H₂O₂ can activate guanylate cyclase directly,36,37 or oxidatively modify cysteine residues on PKG1 to produce dimerization and activation of this BK<sub>Ca</sub>-opening kinase.38 Other intermediate signaling pathways have been proposed as mechanisms for BK<sub>Ca</sub> channel opening in response to H₂O₂, including arachidonic acid metabolites,26,39,40 protein kinase C,36 and/or MAPK-ERK activation.11

Study Limitations

Several potential limitations warrant discussion. The amount of flow through each vessel was the same in both directions. However the luminal pressure in the vessel proximal to the higher reservoir may be greater than the luminal pressure in the more downstream vessel. It is possible that this difference in baseline of intraluminal pressure between donor and detector vessels influenced the dilator response to flow. To minimize this limitation, we used larger coronary arterioles (250 to 300 μm) and pipettes with bigger tip sizes. Although a small difference in dilator response to flow was still observed in donor vessels with different direction of flow and a trend of lower FMD in detector vessels, this difference was not statistically significant and did not alter the interpretation of our data.

In patch-clamp studies, the cells were superfused with effluent from a donor vessel containing higher Ca<sup>2+</sup> and lower K<sup>+</sup> than standard bath solutions for single channel recording. Superfusion of cells with effluent may cause 2 confounding influences: (1) reducing channel conductance; and (2) increasing the effect of Ca<sup>2+</sup> on channel open probability. Although this could explain the relatively low single-channel conductance we observed, similar values for conductance of human coronary BK<sub>Ca</sub> channels were observed in this study and previous single channel recordings using standard patch-clamp bath solutions,14 alleviating concerns about alteration of channel conductance by vessel effluent. The enhanced NPo of BK<sub>Ca</sub> channels by effluent in cell-attached patches was inhibited by CAT indicating that H₂O₂ rather than Ca<sup>2+</sup> was the primary effluent-mediating factor.
IBTX applied in single channel recording has to be located in the pipette solution because it is only active on the outside of cell membranes. To obtain control traces, the pipette tip was first dipped into and filled with the regular pipette solution without IBTX and then back filled with pipette solution containing IBTX. This is the only approach that can be used for single channel recording experiments. This is not an ideal preparation, because post-IBTX control traces could be contaminated by IBTX diffusion to the cell surface. However this diffusion takes time, and we observed no difference between pre- and post-IBTX control ion currents.

Although the majority of channel openings triggered by the vessel effluent in patch-clamp recordings were BKCa channels, other channels with different conductance were also observed. Catalase did not alter the activity of these channels (Yanping Liu, unpublished observations, 2005). These channels were activated by vessel effluent but contributed only minimally to the overall current. Thus, other channel types might provide some contribution to the net response to shear, but they are not involved in the prominent catalase-sensitive portion of FMD.

The CAT-column for removing extracellular H2O2 contained CNBr. Despite extensive washing of the beads before use, no toxicity or interference with fluorescence was observed. Vasomotor response was not altered by interposition of naked beads between donor and detector vessels.

We observed a reduction in H2O2 in effluent collected from CF control column with both Amplex red and DCF fluorescence methods. Two possible explanations for this reduction include: (1) CNBr nonspecifically breaks down H2O2; and (2) degradation of H2O2 with time. Further investigation of the effect of CNBr on H2O2 is beyond the scope of this study but it is important to note that: (1) use of naked beads provides an important control for these possibilities, and (2) the remaining H2O2 in the effluent was sufficient to elicit vasodilation similar to the vessel superfused with effluent without running through column. Vasodilation was abolished by effluent collected from CAT-bound column indicating the specificity of CAT column.

Clinical Implications

H2O2 has been proposed as a key mediator of vasodilation to flow in the diseased human heart. This study provides new fundamental information for understanding the dilator pathway involving transfer of H2O2 from endothelial to smooth muscle cells.

Summary

Flow dilates donor and detector vessel dependent on donor endothelial H2O2. H2O2 activates BKCa channel in cell-attached, but not inside-out, patches of human coronary smooth muscle cells. Donor endothelial H2O2 is critical for regulating coronary smooth muscle relaxation. These results suggest that H2O2 induced by shear stress is a true EDHF that activates BKCa channel and induces vasodilation by a mechanism requires intracellular constituents of smooth muscle cells.

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Disclosures

None.

References


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Methods

Patch Clamp Methods
Unitary BK\(_{\text{Ca}}\) currents were obtained in cell-attached or inside-out patches of human coronary smooth muscle cells, which were bathed in the physiological salt solution that was used for perfusion of donor vessel. The cells were subjected to membrane potential of 40 mV. The open state probability was determined by the event list analysis using criterion of 50% threshold crossing. In some experiments, pipette tips were briefly loaded with drug-free pipette solution (in mM: KCl 145, CaCl\(_2\) 1.8, MgCl\(_2\) 1, HEPES 5, pH = 7.4) and then back-filled with pipette solution containing 100 nmol/L iberiotoxin. Unitary currents were recorded immediately for 2 minutes in cell-attached configuration and measured again at the same membrane potential after 5 and 10 minutes to permit drug diffusion to the outside patch surface.

Results

Patient demographics (n=24)

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</tbody>
</table>

Data shown as mean±SE, n indicates the No. of patients studied; M male; F, female; CAD, coronary artery disease; DM, diabetes mellitus; HTN, hypertension; HC, hypercholesterolemia; MI, myocardial infarction; and CHF, congestive heart failure.