Role for Hydrogen Peroxide in Flow-Induced Dilation of Human Coronary Arterioles

Hiroto Miura, John J. Bosnjak, Gang Ning, Takashi Saito, Mamoru Miura, David D. Gutterman

Abstract—Flow-induced dilation (FID) is dependent largely on hyperpolarization of vascular smooth muscle cells (VSMCs) in human coronary arterioles (HCA) from patients with coronary disease. Animal studies show that shear stress induces endothelial generation of hydrogen peroxide (H$_2$O$_2$), which is proposed as an endothelium-derived hyperpolarizing factor (EDHF). We tested the hypothesis that H$_2$O$_2$ contributes to FID in HCA. Arterioles (135±7 μm, n=71) were dissected from human right atrial appendages at the time of cardiac surgery and cannulated with glass micropipettes. Changes in internal diameter and membrane potential of VSMCs to shear stress, H$_2$O$_2$, or to papaverine were recorded with videomicroscopy. In some vessels, endothelial H$_2$O$_2$ generation to shear stress was monitored directly using confocal microscopy with 2',7'-dichlorofluorescin diacetate (DCFH) or using electron microscopy with cerium chloride. Catalase inhibited FID (%max dilation; 66±8 versus 25±7%; P<0.05, n=6), whereas dilation to papaverine was unchanged. Shear stress immediately increased DCFH fluorescence in the endothelial cell layer, whereas treatment with catalase abolished the increase in fluorescence. Electron microscopy with cerium chloride revealed shear stress–induced increase in cerium deposition in intimal area surrounding endothelial cells. Exogenous H$_2$O$_2$ diluted (%max dilation; 97±1%, ED$_{50}$; 3.0±0.7×10$^{-5}$ mol/L) and hyperpolarized HCA. Dilation to H$_2$O$_2$ was reduced by catalase, 40 mmol/L KCl, or charybdotoxin plus apamin, whereas endothelial denudation, deferoxamine, 1H-1,2,4-oxadiazole-[4,3-a]quinoxalin-1-one, or glibenclamide had no effect. These data provide evidence that shear stress induces endothelial release of H$_2$O$_2$ and are consistent with the idea that H$_2$O$_2$ is an EDHF that contributes to FID in HCA from patients with heart disease. The full text of this article is available at http://www.circresaha.org. (Circ Res. 2003;92:e31-e40.)

Key Words: human ▪ coronary microcirculation ▪ flow-induced dilation ▪ hydrogen peroxides

Physiologically, shear stress plays a critical role in the regulation of vascular tonus and vascular homeostasis, contributing to the maintenance of tissue perfusion and vascular integrity. Shear stress–induced release of nitric oxide (NO) from endothelial cells is widely recognized as one of the most important and common mechanisms for shear-induced vasomotion. For example, flow-induced release of NO is responsible for the mediation of flow-induced vasodilation (FID).1–3

Animal studies have reported that the contribution of NO to FID is reduced as oxidative stress increases in the presence of risk factors for cardiovascular disease such as hypercholesterolemia4 and hypertension.5 In humans, in vivo and in vitro studies have demonstrated that relaxant factor(s) other than NO compensate to maintain FID when NO availability is reduced.6,7 We recently reported that FID is mediated largely by endothelium-derived hyperpolarizing factor (EDHF) in human coronary arterioles (HCAs) isolated from patients undergoing cardiac surgery; however, the chemical nature of the specific EDHF remains unknown. Hydrogen peroxide (H$_2$O$_2$) was first proposed as an EDHF by Matoba et al.8 These investigators showed that catalase, a H$_2$O$_2$ scavenger inhibits vasodilation and hyperpolarization to acetylcholine (ACh) in mouse mesenteric arteries.8 Pathological generation of reactive oxygen species (ROS) including H$_2$O$_2$ has been described in diseased vessels under static conditions.9,10 Furthermore, an animal study indicates that the marked generation of endothelium-derived H$_2$O$_2$ contributes to agonist-induced vasodilation in disease states.11

The purpose of this study was to investigate in HCA from patients with heart disease, whether H$_2$O$_2$ is an EDHF, whether it contributes to FID, and whether shear stress elicits endothelial H$_2$O$_2$ generation and release. We also determined the pharmacological characteristics of the vascular response to H$_2$O$_2$, because EDHF is generally recognized to induce membrane hyperpolarization and vasodilation through opening Ca$^{2+}$-activated K$^+$ channels (K$_{Ca}$).5,12,13

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From the Department of Veterans Affairs Medical Center, and Department of Medicine and Cardiovascular Research Center (H.M., J.J.B., D.D.G.), Milwaukee, Wis; Department of Microbiology and Molecular Genetics (G.N.), Medical College of Wisconsin, Milwaukee, Wis; and the 2nd Department of Internal Medicine (T.S., M.M.), Akita University, Akita City, Japan.

Correspondence to Hiroto Miura, Dept of Medicine and Cardiovascular Research Center, Medical College of Wisconsin, 8701 Watertown Plank Rd, Milwaukee, WI 53226. E-mail hmiura@mcw.edu

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Materials and Methods

HCAs were prepared as reported previously. Briefly, HCA were dissected from fresh specimens of right atrial appendage obtained from patients undergoing cardiopulmonary bypass procedures as discarded surgical specimens. Procedures for harvesting tissue samples were in accordance with guidelines established by the local Institutional Review Boards. Demographic data and diagnoses were obtained from hospital records and recorded at the time of surgery.

Videomicroscopy

Videomicroscopy was performed as reported previously. Briefly, isolated HCAs were transferred to a organ chamber containing Krebs solution of the following composition (in mM/mL): NaCl 118, KCl 4.7, CaCl$_2$ 2.5, KH$_2$PO$_4$ 1.2, MgSO$_4$ 1.2, NaHCO$_3$ 20, Na$_2$EDTA 0.026, and glucose 11, pH 7.4, cannulated with glass micropipettes (30 to 50 μm internal diameter, matched for impedance; see next section) and secured. All side branches were tied off with cotton threads. The preparation was transferred to the stage of an inverted microscope (CK2, Olympus) coupled to a CCD camera (VIA-100K, Boeckeler Instruments, Inc). Pharmacological agents other than catalase were added to the oxygenated external bathing solution (37°C).

The vasomotor and endothelial function was confirmed by examining constriction to 50 mM/L KCl and dilation to bradykinin (BK, 10$^{-5}$ mol/L), respectively. Because HCAs develop varying degrees of spontaneous myogenic tone (10% to 60%), Ach (5×10$^{-5}$ to 5×10$^{-7}$ mol/L), a potent and stable vasoconstrictor of HCA, was, if needed, added to adjust tone to a level between 30% to 60% of passive diameter so that all vessels were constricted to a similar level.

Generation of Intraluminal Flow

Both proximal (inflow) and distal (outflow) micropipettes were connected with silicon tubing to a pressure-servo syringe system (Living Systems Inc), which was used to control intraluminal flow without changing intraluminal pressure. The pipettes had similar dimensions and equivalent resistances to flow, as assessed by the changes in perfusion pressure in response to increments of flow by a calibrated flow control peristaltic pump (model FC, Living Systems Inc). Pulse dampers were used to minimize oscillations in flow caused by the pump. Intraluminal flow was monitored with a flowmeter (model 4552, Gilmont Instruments, Inc). Shear stress was calculated by using the flowing formula: 4ρr/πr$^2$, where η is viscosity of the perfusate (0.007 poise at 37°C), Q is perfusates flow (mL/sec), and r is vessel radius (cm).

Vessels were initially pressurized at 60 mm Hg without flow to confirm absence of leaks either at the site of cannulation or via undetected side-branches. Vessel preparations with leaks (flow>0) were discarded (~5% of experiments).

Experimental Protocols of Flow-Induced Vasodilation

Vessel diameter was examined at different flow rates (1, 3, 5, 10, 15, and 20, and 25 μL/min). After determination of the control relationship between vessel internal diameter and flow rate, vascular responses to flow were reexamined in the presence of catalase (1000 U/mL), inactivated catalase, or the combination of catalase and superoxide dismutase (SOD, 200 U/mL), both of which were applied intraluminally and extraluminally. Maximal vasodilator capacity was determined by addition of papaverine (an endothelium-independent dilator, 10$^{-3}$ mol/L). In some experiments, the vasodilation to papaverine (10$^{-3}$ to 10$^{-7}$ mol/L) was examined in the absence or presence of catalase.

H$_2$O$_2$-Induced Vasodilation

To determine the mechanism for vasodilation to H$_2$O$_2$ on HCA, vascular responses to exogenous H$_2$O$_2$ (10$^{-10}$ to 3×10$^{-4}$ mol/L) were examined in the absence or presence of catalase (1000U/mL), inactivated catalase, deferoxamine (10$^{-3}$ mol/L, an inhibitor of the formation of the hydroxyl radical, a ROS derived from H$_2$O$_2$), or 1H-[1,2,4]-oxadiazole-[4,3-a]quinoxalin-1-one (ODQ, 5×10$^{-6}$ mol/L, a selective inhibitor of guanylate cyclase).

H$_2$O$_2$-induced changes in vessel diameter, KCl (40 mM/L) was used to nonspecifically block K$^+$ channels in HCAs. In separate studies, the effects of the combination of charybdotoxin (CTX, 10$^{-7}$ mol/L, a selective block of large and intermediate conductance K$^+$ channels (Kc,)) and apamin (10$^{-6}$ mol/L, a selective blocker of small conductance K$^+$ channels (Ks,)) were also tested. In these protocols, all chemicals were applied extraluminally.

At the end of experiments, maximal passive diameters were obtained by incubating vessels with Ca$^{2+}$-free Krebs solution in the presence of papaverine (10$^{-4}$ mol/L).

Measurement of Vascular Smooth Muscle Membrane Potential

Resting membrane potential (Em) of vascular smooth muscle cells (VSMCs) and changes in Em to endothelin-1 and H$_2$O$_2$ were measured as described previously. Briefly, HCAs were cannulated, pressurized, and suspended in a 20-mL tissue bath. Em was measured by impaling the vessel from the adventitial surface with a glass microelectrode filled with 3 mol/L KCl and connected to a high-impedance biological amplifier (Axoclamp, Axon Instruments).

Detection of Endothelial H$_2$O$_2$ Production With Confocal Microscopy

A closed imaging chamber (model RC-20, Warner Instrument Corp) was used for detection of fluorescence in HCAs using confocal microscopy. HCAs were placed in the chamber and filled with HEPES buffer (pH 7.4 at 37°C). One end of the vessel was cannulated with a micropipette (internal diameter; 30 to 40 μm) inserted to the perfusion inlet of the chamber and secured, and the other end of the vessel was left opened. The chamber was attached onto a heater platform (model PH-5, Warner Instrument Corp) connected to a heater controller chamber system (model TC-344B, Warner Instrument Corp) to keep the system at 37°C and mounted inversely onto the stage of a confocal microscope. After 60-minute equilibration, HCAs were loaded for 30 minutes with a fluorescence probe, 2', 7'-dichlorodihydrofluorescein diacetate (DCFH; 5×10$^{-6}$ mol/L, Molecular Probes), by superfusing HEPES buffer containing DCFH and N$^6$-nitro-l-arginine (10$^{-4}$ mol/L, an NO synthase inhibitor) in the absence or presence of catalase (1000U/mL). After loading the probe, HCAs were perfused to generate shear stress (≈21±2 dyn/cm$^2$) by switching the perfusion from a secondary inlet port to a micropipette cannulating the vessel, and fluorescence images were obtained during 30-minute exposure to shear stress. In some experiments, fluorescence images were obtained without intraluminal flow for 30 minutes as a time control. Fluorescence was excited by 488-nm line of a krypton-argon laser, and emission at 505 to 535 nm was recorded. Images were obtained with a Bio-Rad MRC 600 laser scanning confocal imaging system mounted on a Nikon Optiphot microscope using ×60 or ×100 oil-immersion objective lens. Images were analyzed on a computer with the software program MetaMorph (Universal Imaging Corp).

Detection of Extracellular H$_2$O$_2$ Release With Electron Microscopy

To estimate and localize shear stress-induced H$_2$O$_2$ production, histochemical electron microscopy was performed with cerium chloride, a technique with which allows the specific visualization of H$_2$O$_2$ production in tissues. Briefly, three vessels were isolated from each subject and incubated in 10 mM/L Tris-maleate saline buffer (pH 7.4) containing 5.5 mM/L glucose, 7% sucrose, and 3-amino-1H-1,2,4-triazole (10$^{-5}$ mol/L) for 30 minutes. Two of three vessels were cannulated with a glass pipette and placed in the bath filled with the buffer containing cerium chloride (10$^{-5}$ mol/L). Vessels were exposed to shear stress (≈20 dyn/cm$^2$) by perfusing the
buffer with cerium chloride for 2 minutes in the presence or absence of catalase (1000U/mL). One of three vessels was treated with the buffer containing cerium chloride for 2 minutes without perfusion. Subsequently, vessels were then rinsed with the buffer to wash out unreacted cerium ions, fixed with 2.5% glutaraldehyde in 0.1 mol/L cacodylate buffer, pH 7.4, rinsed and postfixed with 1% OsO4, and then dehydrated and embedded in epoxy resin. Thin cross sections of each vessel were obtained with an UltraCut E microtome. Sections were stained with uranyl acetate and lead citrate and examined with a Hitachi 600 transmission electron microscope at 75 kV.

Materials
CTX was obtained from Research Biochemicals international, DCFH came from Molecular Probes, and all other chemicals were from Sigma Chemical Co. Glutathione peroxidase was prepared in dimethyl sulfoxide and diluted in saline with 1.0 N NaOH, and pH was adjusted with 0.1 N HCl to 7.4. DCFH and ODQ were prepared in dimethyl sulfoxide. All others were dissolved in distilled water. All concentrations represent the final molar concentrations (mol/L) in the organ chambers.

Statistical Analyses
FID and agonist-induced dilation are expressed as a percent, with 100% dilation representing the change from the constricted diameter to the maximal diameter obtained by addition of papaverine (10-4 mol/L). Maximal passive diameters were used as the maximal diameter for papaverine-induced responses. DCFH fluorescence intensity was normalized as a percent change in intensity levels from baseline (100%). Statistical comparisons of maximal percent vasodilation between the treatment groups (factor 2). Interactions were noted between dose (shear) and treatments groups. Corollary dose (shear)-specific contrasts were tested using Bonferroni adjusted t test whenever the interactions were statistically significant. Multivariate analysis was performed, and regression models were made as previously described.13 All procedures were performed using "proc mixed" and "proc reg" programs of SAS for Windows, version 8. Statistical significance was defined as P<0.05. All data are described as mean±SEM. For all data, n indicates the number of patients.

Results
Seventy-one HCAs with a mean maximal passive internal diameter of 135±7 μm (range 67 to 298 μm) were used. Patient demographics including diagnoses are summarized in the Table.

Figure 1 shows the effect of catalase on FID and papaverine responses. An increase in flow produced potent vasodilation, which was attenuated by catalase (Figure 1A; %max dilation, 21±9 versus control 60±9% at 25 μL/min; P<0.05, n=6). Figure 1B shows the relationship between calculated shear stress and FID. FID closely correlated to shear stress. Catalase decreased vasodilation in response to shear stress. In contrast, vasodilation to papaverine was unchanged in the presence of catalase (Figure 1C; %max dilation, 91±4% versus control 91±5%; -log[ED50] 5.2±0.1 versus control 5.8±0.6; P=NS, n=4, respectively). Catalase inactivated by heating10 also had no inhibitory effect on FID (%max dilation, 62±10% versus control 58±8%; P=NS, n=3). The combination of catalase and SOD also reduced FID (%max dilation, 10±6% versus control 50±13% at 25 μL/min; P<0.05, n=4). These results indicate that the inhibitory effect of catalase on FID is unlikely to be nonspecific, suggesting an important role for H2O2 in mediating FID in HCAs.

We evaluated endothelial H2O2 generation in response to shear stress in HCAs using confocal microscopy with DCFH, an H2O2-sensitive fluorescence probe.22,23 Figure 2, left, shows representative images obtained from a vessel with confocal microscopy. In those images, endothelial cells (EC) are recognized as cells positioned parallel to the vessel axis, and VSMCs (SM) are observed as cells oriented perpendicular to the vessel axis. Intraluminal flow produced a marked increase in fluorescence in endothelial cells and minimal increase in VSMCs (Figures 2a through 2d). In vessels treated with catalase, the fluorescence intensities in endothelial cells and VSMCs were unaltered after exposure to intraluminal flow (Figures 2e through 2h). Summary data showing the change in endothelial fluorescence intensity to shear stress is shown in Figure 2, right. After initiating intraluminal flow, the fluorescent intensity in endothelial cells was significantly increased within a minute (138±9%; P<0.05 versus baseline [100%] at 5 minutes). Catalase completely abolished the increase in fluorescence to flow (93±3%; P<0.05 versus baseline [100%] and in the absence of catalase at 5 minutes, respectively). The fluorescence intensity increased only by 9% (P<0.05 versus endothelial cells at 5 minutes). We confirmed that endothelial denudation abolishes shear-induced increase in DCFH fluorescence by obtaining fluores-

### Demographics (n=71)

| Sex (M/F) | 49/22 |
| Age, y | 59±14 |
| Surgical procedure | |
| CABG | 52 |
| Valve replacement | |
| Aortic | 12 |
| Mitral | 13 |
| Tricuspid | 4 |
| Congenital repair | 1 |
| Underlying diseases | |
| CAD | 53 |
| DM | 17 |
| HTN | 38 |
| HC | 23 |
| MI | 17 |
| CHF | 9 |
| (Smoking) | (32) |
| None of the above | 5 |

Data shown as mean±SD. n indicates the No. of patients studied; M, male; F, female; CABG, coronary artery bypass graft; congenital, congenital heart disease; CAD, coronary artery disease; DM, diabetes mellitus; HTN, hypertension; HC, hypercholesterolemia; MI, myocardial infarction; and CHF, congestive heart failure.
Hydroxyl radical via the Haber-Weiss reaction, $H_2O_2^-$

The vascular response to exogenous $H_2O_2$ was

response to shear stress and this $H_2O_2$ might migrate to

endothelial cells of HCA.

Electron microscopy was conducted to detect cerium deposi-

tions, 25,26 in layers of vessels exposed to shear stress. In

vessels without exposure to shear stress, few cerium deposi-

tions were observed in the intima (Figures 3A and 3D). In

contrast, exposure to shear stress markedly increased cerium

depositions in the intimal layer, especially surrounding endo-

thelial cells (Figures 3B and 3E), while treatment with
catalase inhibited the production of cerium depositions (Fig-

ures 3C and 3F). Figure 3H shows whole vascular wall of

vessels exposed to shear stress in the absence of catalase.

Cerium depositions were seen throughout the vascular wall

with the majority of cerium deposits in the intimal layer

compared with the medial layer and most occurring adjacent
to membrane structures. In contrast, few depositions were

observed in the vascular walls of vessels without shear stress
(Figure 3G) or those vessels treated with catalase (Figure 3I).

These findings suggest that endothelial cells release $H_2O_2$ in

response to shear stress and this $H_2O_2$ might migrate to

interact with VSMCs.

To determine the mechanism of $H_2O_2$-mediated vasodi-
lation, the vascular response to exogenous $H_2O_2$ was

examined in HCA. Because $H_2O_2$ can be converted to

hydroxyl radical via the Haber-Weiss reaction, $H_2O_2$-

induced vasodilation may be elicited directly by $H_2O_2$ or

indirectly by hydroxyl radicals.27 The effect of scavenging

$H_2O_2$ by catalase or inhibiting the formation of hydroxyl

radicals by deferoxamine was tested. The presence of
catalase inhibited vasodilation to $H_2O_2$ (Figure 4A; max
dilation, $22\pm3\%$ versus control $97\pm1\%; P<0.05$, $n=4$),

whereas deferoxamine ($\%$ max dilation, $98\pm1\%$ versus

control $99\pm1\%$; $-\log[ED_{50}], 5.0\pm0.3$ versus control

$5.1\pm0.2$; $P=NS$, $n=4$) or inactivated catalase49 (data not

shown) had no effect on vasodilation to $H_2O_2$.

$H_2O_2$ may act on endothelial cells to elicit vasodilation.28

However, endothelial denudation had no effect on vasodila-
tion to $H_2O_2$ (Figure 4B; max dilation, $97\pm2\%$ versus

control $98\pm1\%; -\log[ED_{50}], 4.8\pm0.3$ versus control

$4.8\pm0.3$; $P=NS$, $n=4$). Guanylate cyclase may also mediate

vasodilation to $H_2O_2$.29 Vasodilation to $H_2O_2$ was

unchanged by ODQ, an inhibitor of guanylate cyclase ($\%$ max
dilation, $95\pm2\%$ versus control $97\pm1\%; -\log[ED_{50}], 4.8\pm0.3$

versus control $5.1\pm0.2$; $P=NS$, $n=4$). These results suggest that

$H_2O_2$-induced vasodilation is mediated by endothelium-

independent mechanisms, which do not involve guanylate

cyclase activation or hydroxyl radical formation in HCA.

It has been reported that membrane hyperpolarization

through $K^+$ channel activation contributes largely to vasodi-
lation not only to $H_2O_2$ but also to shear stress.3,8,30 Consistent

with these observations, a high concentration of KCl

(40 mmol/L) reduced $H_2O_2$-induced vasodilation (Figure 4C;

$\%$ max dilation, $64\pm5\%$ versus control $100\pm0\%$; $-\log[ED_{50}], 4.1\pm0.1$

versus control $4.8\pm0.2$; $n=6$, $P<0.05$ for both

comparisons). To directly support the idea that vasodilation to

$H_2O_2$ is dependent largely on membrane hyperpolarization,

changes in Em of VSMCs were measured. As shown in

Figure 4D, $H_2O_2$ produced simultaneous vasodilation and

membrane hyperpolarization in a dose-dependent manner in

HCA. This is consistent with our previous report that FID is

dependent on membrane hyperpolarization via $K^+$ channel

activation in the human coronary microcirculation.3

We next examined which type of $K^+$ channel(s) mediates

vasodilation to moderate doses of $H_2O_2$, because the contribu-
tion of endogenously generated $H_2O_2$ to FID is approxi-

mately 40%. Glibenclamide did not alter $H_2O_2$-induced vaso-
dilation in HCA (Figure 5A; $\%$ max dilation, $35\pm18\%$ versus

control $49\pm15\%$ at $10^{-5}$ mol/L; $P=NS$, $n=6$), whereas the

combination of CTX and apamin significantly decreased the

dilation (Figure 5B; $\%$ max dilation, $3\pm2\%$ versus control

$49\pm14\%$ at $10^{-5}$ mol/L; $P<0.05$, $n=6$). These results suggest that

$K_0$ plays a role in mediating $H_2O_2$-induced dilation of

HCAs.

Discussion

This study is the first to directly examine $H_2O_2$ as a potential

mediator of FID in HCA. The major new findings are 4-fold.
First, H\textsubscript{2}O\textsubscript{2} contributes to FID in HCAs. Second, shear stress elicits endothelial generation and release of H\textsubscript{2}O\textsubscript{2}. Third, H\textsubscript{2}O\textsubscript{2}-induced vasodilation is not endothelium-dependent but is mediated by direct effects on the underlying VSMCs in HCA. Fourth, vasodilation to H\textsubscript{2}O\textsubscript{2} occurs as a result of membrane hyperpolarization consequent to the opening of K\textsubscript{ca} in VSMCs. Taken together, these findings suggest that endothelium-derived H\textsubscript{2}O\textsubscript{2} plays an important role in FID in HCA from patients with heart disease. H\textsubscript{2}O\textsubscript{2} is most likely an EDHF in the human coronary microcirculation.

Flow-Induced Vasodilation
The vasomotor response to shear stress is varied among species and vasculatures. Endothelium-dependent vasodilator responses to shear stress have been demonstrated in a variety of vessels including rat cremaster and gracilis arterioles, and porcine coronary arterioles\textsuperscript{1,2,31} whereas endothelium-independent vasoconstriction is observed in cat and rat cerebral arteries\textsuperscript{32,33} We previously reported that shear stress elicits endothelium-dependent vasodilation in HCA.\textsuperscript{3} In the present study, we show that H\textsubscript{2}O\textsubscript{2} contributes to the FID in HCA.

Shear Stress Elicits Endothelial Production and Release of H\textsubscript{2}O\textsubscript{2}
Numerous in-vivo and in vitro studies have demonstrated that shear stress stimulates the production and release of ROS from endothelial cells, most notably superoxide.\textsuperscript{23,34,35} Human endothelial cells also generate superoxide in response to shear stress.\textsuperscript{35} Superoxide is rapidly dismutated to H\textsubscript{2}O\textsubscript{2}. This may account for the finding by Hsieh et al\textsuperscript{23} of shear stress-induced H\textsubscript{2}O\textsubscript{2} production in cultured human umbilical vein endothelial cells. In contrast to the pathological function of H\textsubscript{2}O\textsubscript{2},\textsuperscript{9,10} a physiological role in vasodilation has been demonstrated. For example, vasorelaxation to the calcium ionophore A23187 in aortas of spontaneously hypertensive rats largely involves H\textsubscript{2}O\textsubscript{2}.\textsuperscript{11} In the present study, we demonstrated that endothelial H\textsubscript{2}O\textsubscript{2} generation is associated with vasodilation to shear stress, a key physiological stimulus for dilation.

Scavenging extracellular H\textsubscript{2}O\textsubscript{2} with catalase could reduce vasodilation to agonists such as BK and ACh in the vasculatures among different species.\textsuperscript{8,36–38} Interestingly, it has been reported that SOD and catalase can reduce vasoconstriction to flow in rat and cat cerebral arteries.\textsuperscript{32,33} Laurindo et al\textsuperscript{34} have reported that shear stress induces endothelial super-
oxide generation with increases in the radical product in plasma of perfused rabbit vessels. These studies support our finding with electron microscopy that endothelial cells release H₂O₂ extracellularly in response to shear stress. The present study is the first demonstration that H₂O₂ is involved in FID, indicating H₂O₂ as an endothelium-derived and transferable vasodilator in FID.

Vasoreactivity to H₂O₂

H₂O₂ induces vasorelaxation in an endothelium-dependent manner in rabbit aorta. However, we found that H₂O₂-induced dilation is endothelium-independent in HCAs, consistent with other studies in porcine coronary arteries.

H₂O₂ may be converted to hydroxyl radicals via Haber-Weiss reaction and this may be necessary for dilation of cat cerebral arteries. However, it is not critical in H₂O₂-induced dilation of porcine coronary arteries or mouse mesenteric arteries. In the present study, deferoxamine had no effect on vasodilation of HCA to H₂O₂.

It has been also reported that H₂O₂-induced vasodilation is mediated by K⁺ATP in porcine coronary arteries, rat cerebral arteries, and mouse mesenteric arteries by K⁺ATP in cat cerebral arterioles, or by soluble guanylate cyclase in bovine pulmonary arteries. Electrophysiological investigations have also revealed H₂O₂-induced direct activation of K⁺ATP in VSMCs isolated from coronary arteries. In the present study, H₂O₂ produced membrane hyperpolarization and vasodilation of HCA, which was sensitive to CTX and apamin but resistant to glibenclamide, deferoxamine, and ODQ.

These findings suggest that in HCAs, H₂O₂ elicits membrane hyperpolarization and vasodilation through the activation of K⁺ATP, hydroxyl radical formation, or soluble guanylate cyclase.
Although FID is mediated largely or in part by NO, prostaglandins, or both in most vessels\textsuperscript{1,2,3} including HCAs isolated from patients without coronary artery disease (CAD),\textsuperscript{3} EDHF contributes largely to FID in HCA from patients with heart disease,\textsuperscript{3} porcine epicardial coronary arteries,\textsuperscript{41} and rat mesenteric arteries.\textsuperscript{42} Animal studies have demonstrated that endothelium-derived H$_2$O$_2$, which is first proposed as an EDHF by Matoba et al\textsuperscript{8} compensates for loss of NO activity and contributes largely to endothelium-dependent vasodilation.\textsuperscript{43} These studies are consistent with the suggestion of the present study that H$_2$O$_2$ acts as an EDHF and maintains FID.

\textbf{H$_2$O$_2$ as an EDHF}

Figure 4. H$_2$O$_2$-induced dilation of HCAs. A, Catalase abolished H$_2$O$_2$-induced dilation of HCAs (*P<0.05, n=4). B, Vasodilation to H$_2$O$_2$ was unaltered by endothelial denudation (P=NS, n=4). C, High concentration of KCl (40 mmol/L) attenuated vasodilation to H$_2$O$_2$. (*P<0.05 vs control, n=6). D, After membrane depolarization and vasoconstriction with endothelin-1 (ET), H$_2$O$_2$ induced a dose-dependent membrane hyperpolarization and vasodilation (*P<0.05 vs ET, n=5).

Figure 5. Effect of K$^+$ channel blockers on H$_2$O$_2$-induced vasodilation in HCA. A, Vasodilation to H$_2$O$_2$ was unchanged in the presence of glibenclamide (10$^{-6}$ mol/L) (P=NS, n=4). B, Combination of CTX (10$^{-7}$ mol/L) and apamin (10$^{-6}$ mol/L) significantly reduced H$_2$O$_2$-induced dilation (†P<0.01, *P<0.05 vs control, n=6).
in HCA isolated from patients with heart disease. The mechanism of dilation to H$_2$O$_2$ involves hyperpolarization and opening of K$_{Ca}$. Therefore, H$_2$O$_2$ is likely an EDHF involved in FID in the human coronary microcirculation.

Potential Limitations of the Study

The local extracellular concentration of H$_2$O$_2$ generated in response to shear stress is not known. In the present study, approximately 10$^{-5}$ mol/L of exogenous H$_2$O$_2$ was required to induce a catalase-sensitive vasodilation that was of similar magnitude to that produced by shear stress (max dilation $\pm$40%). ED$_{50}$ of exogenous H$_2$O$_2$-induced dilation was approximately 3$\times$10$^{-5}$ mol/L in HCAs, whereas other studies reported ED$_{50}$ of 10$^{-5}$ to 10$^{-4}$ mol/L in mouse small mesenteric arteries and 2.5$\times$10$^{-4}$ mol/L in porcine coronary arteries. The different sensitivity of vessels to H$_2$O$_2$ may be dependent on vessel size. Consentino et al reported in aortas from spontaneously hypertensive rats that endogenously generated H$_2$O$_2$ at 10$^{-7}$ to 10$^{-5}$ molar ranges contributes largely to vasorelaxation to A23187. Therefore, the concentration of H$_2$O$_2$ required to induce vasodilation to physiological stimuli such as shear stress, ACh, and BK may be lower than that required for dilation to exogenous H$_2$O$_2$. This could be explained by the interaction of H$_2$O$_2$ with endogenous catalase, by other redox reactions that inactivate H$_2$O$_2$, or convert it to other radical species before diffusing to the active site, or by the additive effect of H$_2$O$_2$ on other vasorelaxant mechanisms.

Although there are several enzymatic sources of ROS in endothelial cells including eNOS, cyclooxygenase, cytochrome P450, NADH oxidase, xanthine oxidase, and sites along the mitochondrial respiratory chain, the source of radicals underlying shear stress–induced ROS production remains unclear. eNOS and cyclooxygenase are activated by shear stress, but activation of these enzymes is not consistent with the finding showing a prominent role of H$_2$O$_2$ in mediating endothelium-dependent relaxation of aorta in diseased animal models. In addition, H$_2$O$_2$ mediates BK-induced vasodilation in mesenteric arteries from patients without CAD. Thus, we speculate that H$_2$O$_2$ may be an important physiological mediator of FID in HCAs, and that the presence of coronary risk factors or CAD may augment the contribution of H$_2$O$_2$ to FID. H$_2$O$_2$ is a relatively stable reactive oxygen intermediate and contributes importantly to inflammation, ischemia/reperfusion injury, and atherosclerosis. It may, therefore have a pathophysiological as well as a normal signaling function. In chronic disease states such as hypercholesterolemia and diabetes mellitus, oxidative stress and H$_2$O$_2$ levels are increased due to the enhanced generation of superoxide from several sources, leading to vascular dysfunction. Endothelial H$_2$O$_2$ production to shear stress may be a double-edged sword in the human coronary microcirculation.

In summary, endothelium-derived H$_2$O$_2$ contributes to FID in isolated HCA from patients with heart disease. Vasodilation to H$_2$O$_2$ is associated with membrane hyperpolarization of VSMCs via K$_{Ca}$ opening, suggesting H$_2$O$_2$ is an EDHF in the human coronary microcirculation. This dilator mechanism may play an important role in maintaining myocardial perfusion when levels are NO are reduced, but it may also lead to the enhanced development of vascular pathology including atherosclerosis.
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Hiroto Miura, John J. Bosnjak, Gang Ning, Takashi Saito, Mamoru Miura and David D. Gutterman

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