Electron Spin Resonance Detection of Hydrogen Peroxide as an Endothelium-Derived Hyperpolarizing Factor in Porcine Coronary Microvessels

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Objective—Endothelium-derived hyperpolarizing factor (EDHF) plays an important role in modulating vascular tone, especially in microvessels, although its nature has yet to be elucidated. This study was designed to examine whether hydrogen peroxide (H$_2$O$_2$) is an EDHF in porcine coronary microvessels with use of an electron spin resonance (ESR) method to directly detect H$_2$O$_2$ production from the endothelium.

Methods and Results—Isometric tension and membrane-potential recordings demonstrated that bradykinin and substance P caused EDHF-mediated relaxations and hyperpolarizations of porcine coronary microvessels in the presence of indomethacin and N$^o$-nitro-L-arginine. The contribution of H$_2$O$_2$ to the EDHF-mediated responses was demonstrated by the inhibitory effect of catalase and by the relaxing and hyperpolarizing effects of exogenous H$_2$O$_2$. Endothelial production of H$_2$O$_2$ was quantified in bradykinin- or substance P–stimulated intact blood vessels by ESR spectroscopy. Tiron, a superoxide scavenger that facilitates H$_2$O$_2$ formation, enhanced bradykinin-induced production of H$_2$O$_2$, as well as the EDHF-mediated relaxations and hyperpolarizations. By contrast, cytochrome P-450 inhibitors (sulfaphenazole or 17-octadecynoic acid) or a gap junction inhibitor (18 glycyrrhetinic acid) failed to inhibit the EDHF-mediated relaxations. Involvement of endothelium-derived K$^+$ was not evident in experiments with ouabain plus Ba$^{2+}$ or exogenous K$^+$.

Conclusion—These results provide ESR evidence that H$_2$O$_2$ is an EDHF in porcine coronary microvessels. (Arterioscler Thromb Vasc Biol. 2003;23:1224-1230.)

Key Words: endothelium • endothelium-derived hyperpolarizing factor • hydrogen peroxide • membrane potential

The endothelium plays an important role in maintaining vascular homeostasis by synthesizing and releasing several vasodilating factors, including prostacyclin, nitric oxide (NO), and the still-unidentified endothelium-derived hyperpolarizing factor (EDHF). Since the first reports of the existence of EDHF, several candidates for EDHF have been proposed, including cytochrome P-450 metabolites, endothelium-derived K$^+$, and gap-junctional electrical communication between endothelial cells and smooth muscle cells. We and others have recently demonstrated that endothelium-derived hydrogen peroxide (H$_2$O$_2$) is an EDHF of mouse and human mesenteric arteries, piglet pial arterioles, and human and canine coronary microvessels (in flow-induced and autoregulatory relaxation, respectively). However, it remains to be determined whether H$_2$O$_2$ is also an EDHF in agonist-induced relaxation of the coronary microvessels, in which EDHF plays an important role in modulating vascular tone. Moreover, endothelial production of H$_2$O$_2$/EDHF remains to be demonstrated by methods other than conventional tension or membrane-potential recordings. The present study was thus designed to examine whether H$_2$O$_2$ plays a role as an EDHF in porcine coronary microvessels by using an electron spin resonance (ESR) method to directly detect endothelial production of H$_2$O$_2$.

Methods
This study was reviewed by the Committee on Ethics in Animal Experiments of the Kyushu University and was carried out according to the Guidelines for Animal Experiments of the Kyushu University and of the Japanese Government.

Animals and Tissue Preparation
We used a total of 36 domestic male pigs (Nihon Crea, Tokyo, Japan; 2 to 3 months old and weighing 25 to 30 kg). Animals were humanely killed with a lethal dose of pentobarbital sodium, and then the right ventricular free wall was carefully removed. Epicardial right coronary arteries and distal coronary microvessels (250 to 300 µm in diameter) were excised from the right ventricular free wall and
carefully cleaned of adherent perivascular connective tissue under a microscope. The microvessels were cut into 1.2-mm rings for organ chamber experiments.

Organ Chamber Experiments
Experiments were performed in 37°C Krebs’ solution bubbled with 95% O₂ and 5% CO₂. Isometric tension was recorded in isolated coronary microvessels and epicardial arteries. 95% O₂ and 5% CO₂. In some experiments, the vascular endothelium was removed and then ESR measurements were performed with an X-band EPR spectrometer (JES-RE-1X, Jeol Ltd). In this system, H₂O₂ was detected as a specific waveform of a stable nitro oxide radical, and relative signal intensity was determined in each experiment with a manganese marker as a control. H₂O₂ concentration was determined from the relative ESR signal–H₂O₂ concentration relation obtained from preliminary experiments. Response to bradykinin was determined as the fold increase in H₂O₂ signal from baseline signal intensity under control conditions.

Drugs and Solution
The ionic composition of the Krebs’ solution (mmol/L) was as follows: Na⁺ 144, K⁺ 5.9, Mg2+ 1.2, Ca²⁺ 2.5, HPO₄⁻ 1.2, HCO₃⁻ 24, Cl⁻ 129.7, and glucose 5.5. Bradykinin, indomethacin, l-NNNA, catalase (No. C-40), PEG-SOD, SNP, 18α-AG, 17-ODYA, apamin, charybdotoxin, TBA, BaCl₂, and ouabain were obtained from Sigma Chemical Co. Catalase from another supplier (No. 106836, Roche Diagnostics Co) was also used. 1-Hydroxy-2,2,5,5-tetramethyl-3-imidazoline-3-oxide was obtained from Acros Organics, and p-acetamidophenol and horseradish peroxidase were obtained from Wako Pure Chemical Industries. Levcromakalim was a gift from SmithKline Beecham Pharmaceuticals (Philadelphia, Pa). Indomethacin was dissolved in 10⁻² mol/L Na₂CO₃, 18α-AG and 17-ODYA were dissolved in dimethyl sulfoxide. Sulfaphenazole was dissolved in dimethyl sulfoxide or ethanol, and in both cases, identical results were obtained. Levcromakalim was dissolved in 90% ethanol, and all other drugs were dissolved in distilled water. The solvents used did not affect the mechanical or electrical responses at their final bath concentrations.

Statistical Analysis
Data are shown as mean ± SEM. Dose–response curves were analyzed by 2-way ANOVA, followed by Scheffe’s post hoc test for multiple comparisons. Other values were analyzed by paired Student’s t test or 1-way ANOVA, according to propriety. P < 0.05 was considered statistically significant.

Results
EDHF Responses of Porcine Coronary Microvessels
Bradykinin (10⁻¹⁰ to 10⁻⁷ mol/L) elicited concentration-dependent relaxations of porcine coronary microvessels, which were almost resistant to indomethacin or indomethacin plus l-NNNA, but were highly sensitive to the combined treatment with indomethacin, l-NNNA, and KCl (n = 5, Figure 1A). In the presence of KCl alone, bradykinin still elicited significant relaxations, which were partially sensitive to additional treatment with indomethacin, whereas the remaining relaxations could be attributed to NO (n = 6, Figure 1B). Relaxations resistant to indomethacin and l-NNNA were abolished by charybdotoxin plus apamin, Kᵣ channel inhibitors (maximal relaxation 84 ± 3% vs 13 ± 7% in the absence and presence of charybdotoxin/apamin, respectively; n = 6, P < 0.05), suggesting that those relaxations are mediated by EDHF. Bradykinin elicited concentration-dependent membrane hyperpolarizations of vascular smooth muscle cells in the presence of indomethacin and l-NNNA (Figure 1C). These results suggest a primary role for EDHF in bradykinin-induced relaxations of porcine coronary microvessels, with a small contribution from NO and vasodilator prostaglandins. Substance P is another vasodilating agonist in porcine coronary arteries. 18,19 Substance P (10⁻¹² to 10⁻⁹ mol/L) also elicited indomethacin- and l-NNNA–resistant relaxations, which were significantly inhibited by additional treatment with KCl (maximal relaxation 36 ± 12% and 14 ± 7% in the absence and presence of KCl, respectively; n = 6, P < 0.05), suggesting the involvement of EDHF in these relaxations.

EDHF in Coronary Microvessels
Inhibitory Effects of Catalase on Vascular Responses

To examine the role of H₂O₂ in EDHF-mediated relaxations, we first examined the inhibitory effect of catalase, which selectively dismutates H₂O₂ into oxygen and water.⁹,¹⁰ Catalase (Sigma No. C-40 used throughout this study) markedly inhibited the EDHF-mediated relaxations elicited by bradykinin (n = 11005, P < 0.05; Figure 2A). Catalase from another supplier (Roche) also significantly inhibited the EDHF-mediated relaxations (maximal relaxation 83% vs 64% in the absence and presence of catalase, respectively; n = 6, P < 0.05), although the extent of inhibition was smaller than that of Sigma catalase. We found that catalase requires a 2-hour incubation period to exert a significant inhibitory effect on relaxation (data not shown). By contrast, catalase did not affect NO-mediated relaxations in the presence of indomethacin and KCl in response to bradykinin, excluding its inhibitory effect on NO-mediated responses (n = 6; Figure 2A). Catalase also significantly inhibited bradykinin (10⁻⁷ mol/L)-induced hyperpolarizations (n = 5; Figure 2B left). To further clarify the specificity of catalase, aminotriazole was simultaneously applied, which inhibits the peroxide-binding site of catalase and thus, enzymatically inactivates the enzyme.⁹ When inactivated with aminotriazole, catalase lost its inhibitory effect on EDHF-mediated hyperpolarizations (n = 3; Figure 2B middle). Furthermore, catalase did not affect hyperpolarizations to levcromakalim, a direct K⁺ channel opener (n = 3; Figure 2B right). Catalase also significantly inhibited EDHF-mediated relaxations in response to substance P in the presence of indomethacin and L-NNA (maximal relaxation 36±12% vs 10±6% in the absence and presence of catalase, respectively; n = 6, P < 0.05).

Effect of Exogenous H₂O₂

We then examined whether exogenously applied H₂O₂ could mimic the EDHF-mediated relaxations and hyperpolarizations of coronary microvessels without the endothelium. H₂O₂ (10⁻⁸ to 10⁻⁴ mol/L) caused concentration-dependent relaxations that were inhibited by either the K⁺ channel inhibitor TBA or high extracellular K⁺ (n = 4 to 6; Figure 3A). H₂O₂ (10⁻³ to 10⁻⁴ mol/L) also elicited significant hyperpolarizations of blood vessels without the endothelium (n = 7; Figure 3B).

ESR Detection of Endothelial H₂O₂ Production

It is mandatory to directly detect endothelial production of H₂O₂ to conclude that H₂O₂ is an EDHF. Because direct measurement of H₂O₂ is difficult, especially in living tissues, we performed ESR spectrometry of H₂O₂ by using p-acetamidophenol, 1-hydroxy-2,2,5,5-tetramethyl-3-imidazoline-3-oxide, and horseradish peroxidase to form stable nitroxide radicals as an H₂O₂ indicator.¹⁷ To increase the amount of H₂O₂, epicardial coronary arteries as well as microvessels were used in the ESR experiments. H₂O₂ production was detected as an ESR signal of nitroxide radicals in perfusates of porcine coronary arteries. Bradykinin elicited an
increase in H$_2$O$_2$ production, which was highly sensitive to catalase (500 U/mL) (Figure 4A). Bradykinin significantly increased H$_2$O$_2$ production (n=5, P<0.05; Figure 4B). The mean concentration of H$_2$O$_2$ in the perfusate of the arteries on stimulation with bradykinin was 1.5±0.2×10$^{-6}$ mol/L (n=5). Removal of the endothelium abolished the H$_2$O$_2$ signal, confirming that H$_2$O$_2$ production in response to bradykinin is endothelium dependent (n=5; Figure 4B). An increase in the substance P–induced H$_2$O$_2$ signal was noted but insignificant (1.2±0.7-fold increase from baseline, n=5).

**Effect of Tiron and PEG-SOD on H$_2$O$_2$ Production and Vascular Responses**

To characterize endothelial H$_2$O$_2$ production, Tiron (10$^{-3}$ mol/L, a cell-permeable SOD mimetic) and PEG-SOD were applied to porcine coronary microvessels. Because Tiron and SOD react with superoxide anions to form H$_2$O$_2$, they are expected to enhance agonist-induced H$_2$O$_2$ production from the endothelium if H$_2$O$_2$ is converted from superoxide. Indeed, Tiron significantly enhanced bradykinin-induced H$_2$O$_2$ production from porcine coronary arteries (n=5, P<0.05; Figure 4B). By contrast, PEG-SOD (1000 U/mL) failed to enhance H$_2$O$_2$ production from porcine coronary arteries (n=5; data not shown). Pretreatment with Tiron also significantly enhanced the bradykinin-induced, EDHF-mediated relaxations (n=5, P<0.05; Figure 5A) and hyperpolarizations (n=6, P<0.05; Figure 5B) in the presence of indomethacin and l-NNA. However, Tiron did not affect endothelium-independent relaxations to either SNP (10$^{-10}$ to $10^{-6}$ mol/L; maximal relaxation 97±1% vs 96±2% in the absence and presence, respectively, of Tiron; n=6) or levcromakalim (10$^{-8}$ to 10$^{-5}$ mol/L; maximal relaxation 60±13% vs 50±16% in the absence and presence of Tiron, respectively; n=4). By contrast, pretreatment with PEG-SOD (1000 U/mL) failed to enhance EDHF-mediated relaxations to bradykinin in the presence of indomethacin and l-NNA (maximal relaxation 85±4% vs 81±3% in the absence and presence of PEG-SOD, respectively; n=4).

**Role of Cytochrome P-450 Epoxygenases, K$^+$, and Gap Junctions in EDHF-Mediated Responses**

It has been reported that cytochrome P-450 epoxygenases are involved in the synthesis of EDHF in epicardial porcine coronary arteries, because EDHF-mediated responses were inhibited by sulfaphenazole, an inhibitor of cytochrome P-450 epoxygenase. In the present study, we used sulfaphenazole, an inhibitor of cytochrome P-450 epoxygenase.
Coronary Microvessels

observations that K⁺ releases from the endothelium is an EDHF in rat hepatic arteries. This notion was based on the observations that K⁺ hyperpolarized and relaxed vascular smooth muscle by activating Na/K pumps and inward rectifier potassium channels (Kir channels) and that Ba²⁺ plus ouabain, inhibitors of Kir channels and Na/K pumps respectively, inhibited the EDHF-mediated responses. In the present study, although Ba²⁺ (3×10⁻³ mol/L) plus ouabain (10⁻³ mol/L) shifted the concentration-response curve to bradykinin to the right in the presence of indomethacin and L-NNa in porcine coronary microvessels, the combination failed to affect maximal vasodilator responses (n=4; Figure 6B). Exogenously applied K⁺ (5 to 20 mmol/L) caused only contractions in the arteries studied (n=3; data not shown). To examine the role of gap junctions in the EDHF-mediated responses, 18α-GA (10⁻⁴ mol/L) failed to inhibit the EDHF-mediated relaxations of porcine coronary microvessels (n=5; Figure 6C).

Discussion

H₂O₂ as a Primary EDHF in Porcine Coronary Microvessels

In porcine coronary microvessels, bradykinin elicited relaxations attributable to prostaglandins, NO, and EDHF. The contribution of EDHF to bradykinin-induced relaxation is dominant compared with that of NO or prostacyclin. As a primary relaxing factor in response to bradykinin, EDHF appears to compensate for the relaxations after inhibition by NO and prostaglandins. By contrast, the extent of EDHF-mediated relaxation in response to substance P is relatively small. In the present study, catalase markedly inhibited the bradykinin-induced, EDHF-mediated relaxations and hyperpolarizations of porcine coronary microvessels. Although previous reports failed to find an inhibitory effect of catalase on EDHF-mediated responses in porcine coronary arteries, recent investigations successfully utilized catalase to inhibit EDHF-mediated vascular responses. The different results with catalase may be caused by the difference in vessel size and/or experimental conditions, including the catalase preparations. Indeed, in the present study, a longer (≈2 hours) incubation period was required to observe the inhibitory effect of catalase. Similarly, myeloperoxidase, another large molecule, also needs 2 to 3 hours to accumulate in the subendothelial space by way of transcytosis. Thus, it is not surprising that exogenous catalase, a large molecule, also needs a longer incubation period to penetrate into the myoendothelial space. The difference in the effect of different catalase preparations can also be caused by the different efficiencies of penetration into the myoendothelial space. In the present study, the specificity of catalase was confirmed by the following findings: (1) catalase did not affect endogenous NO-mediated relaxations; (2) enzymatically inactivated catalase lost its inhibitory effect on EDHF-mediated hyperpolarizations; (3) catalase did not affect levromakalim-induced, direct hyperpolarizations of vascular smooth muscle; and (4) catalases from different suppliers significantly inhibited EDHF-mediated responses. Exogenously applied H₂O₂ mimicked the EDHF-mediated relaxations and hyperpolarizations of blood vessels without the endothelium. H₂O₂-induced relaxations were sensitive to a Kir channel inhibitor or high extracellular K⁺, suggesting that those relaxations are mainly caused by hyperpolarization of the smooth muscle through activation of Kir channels. These functional data also indicate that H₂O₂ plays a primary role as an EDHF in porcine coronary microvessels.

ESR Detection of Endothelial H₂O₂ Production

It is important to detect a reasonable amount of H₂O₂ released from the endothelium. In the present study, ESR measurement demonstrated that endothelial cells of porcine coronary microvessels produce a significant amount of H₂O₂ on stimulation by bradykinin. There was a difference between the H₂O₂ concentration detected in the perfusate from blood vessels and that required to cause vascular relaxations/hyperpolarizations. It is conceivable that the concentration of endogenous H₂O₂ detected in the perfusate is lower than that in myoendothelial space where EDHF works, whereas the concentration of exogenous H₂O₂ required to cause vascular relaxations may be higher than that in the myoendothelial space because exogenous H₂O₂ rapidly reacts with endogenous peroxidases. Endothelial cells have a capacity to produce superoxide anions from several intracellular sources,
including endothelial NO synthase, cyclooxygenase, lipoxygenase, cytochrome P-450 enzymes, and NAD(P)H oxidases. In the present study, H$_2$O$_2$ formation can be attributed to superoxide-producing enzymes, because Tiron facilitated H$_2$O$_2$ production as well as EDHF-mediated vascular responses, although PEG-SOD affected neither H$_2$O$_2$ production nor EDHF-mediated relaxations. The different effect of Tiron and PEG-SOD might be caused by differences in cell permeability, although long-term treatment with PEG-SOD has been reported to augment endothelium-dependent vascular responses in a previous study. Among the possible sources of superoxide anions, we have demonstrated that endothelial NO synthase is a major source of H$_2$O$_2$ in mouse mesenteric arteries. However, the source of superoxide and H$_2$O$_2$ in porcine coronary arteries remains to be determined in a future study.

In the present study, the extent of EDHF-mediated relaxations to substance P was smaller than that to bradykinin. In order to test whether the EDHF-mediated relaxations to substance P is smaller than that to bradykinin, further studies are needed to clarify whether endothelial production of H$_2$O$_2$ as an EDHF can be generalized to various vascular beds and stimuli, including endothelial agonists and physiological stimuli.

**Other Candidates for EDHF**

Epoxycosatrienonic acids, major products of cytochrome P-450 epoxygenase, have been proposed to be EDHFs in some vascular beds, including epicardial porcine coronary arteries. In the present study, we concluded that cytochrome P-450 metabolism may not be involved in the EDHF-mediated responses of porcine coronary microvessels because sulfaphenazole, a specific inhibitor of cytochrome P-450 epoxygenase, did not affect the EDHF-mediated relaxations or hyperpolarizations. The difference in vessel size may account for the different conclusion between a previous report and the present study. 17-ODYA, another inhibitor of cytochrome P-450, also failed to inhibit the EDHF-mediated relaxations or hyperpolarizations, a consistent finding with a previous report that studied epicardial porcine coronary arteries.

K$^+$ released from the endothelium has also been suggested to be an EDHF in epicardial porcine coronary arteries. We attempted to inhibit Na/K pumps and K$_\text{ir}$ channels, proposed targets of endothelium-derived K$^+$, by using ouabain plus Ba$^{2+}$. Although the combination of ouabain plus Ba$^{2+}$ shifted the dose-response curve to bradykinin to the right, the combination did not significantly affect the maximal responses to bradykinin. Ouabain-induced membrane depolarization may decrease bradykinin-induced Ca$^{2+}$ influx into the endothelium, which is essential for the production of both NO and EDHF. We have observed that ouabain (10$^{-3}$ mol/L) inhibits NO production in epicardial porcine coronary microvessels (authors’ unpublished observations). Thus, the specificity of ouabain plus Ba$^{2+}$ is questionable in the present preparations. Furthermore, exogenously applied K$^+$ elicited only contractions and depolarizations of the smooth muscle under the same experimental condition where bradykinin-induced, EDHF-mediated relaxations were consistently observed. Thus, involvement of K$^+$ was not evident in porcine coronary microvessels in the present study.

It has also been proposed that intercellular electrical communication through gap junctions could contribute to endothelium-dependent hyperpolarizations/relaxations of epicardial porcine coronary arteries. In the present study, 18α-GA failed to inhibit the EDHF-mediated relaxations in porcine coronary microvessels. Thus, gap junctions may not be required to activate the H$_2$O$_2$-hyperpolarization pathway in porcine coronary microvessels.

**Mechanism of H$_2$O$_2$-Induced Hyperpolarization and Relaxation**

In the present study, EDHF-mediated responses were inhibited by charybdotoxin plus apamin or TBA, K$_\text{ir}$ channel inhibitors. Recently, it has been suggested that the combination of charybdotoxin plus apamin inhibits the endothelium from synthesizing EDHF. If so, it is unknown at the present time which mechanism is involved in the EDHF-mediated hyperpolarization at the smooth muscle level. In the present study, H$_2$O$_2$ elicited hyperpolarizations and relaxations of vascular smooth muscle that were sensitive to a K$_\text{ir}$ channel inhibitor or high extracellular K$^+$. It is conceivable that H$_2$O$_2$ activates K$_\text{ir}$ channels in smooth muscle to cause hyperpolarizations, as reported previously. Indeed, H$_2$O$_2$ is known to activate K$_\text{ir}$ channels by direct modulation of the channels, by a cGMP-dependent mechanism, or by a lipoxygenase-mediated mechanism in porcine coronary arteries. The precise mechanism by which H$_2$O$_2$ activates K$_\text{ir}$ channels remains to be clarified in future studies.

In summary, the present study provides direct evidence for endothelial production of H$_2$O$_2$ with use of an ESR method. Further studies are needed to clarify the physiological and pathological significance of the novel role of endothelium-derived H$_2$O$_2$ as an EDHF.

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