Cytochrome P-450 metabolites but not NO, PGI2, and H2O2 contribute to ACh-induced hyperpolarization of pressurized canine coronary microvessels

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THE ENDOTHELIUM PLAYS an important role in regulating the vascular tone by releasing vasodilator substances such as prostacyclin (PGI2), nitric oxide (NO), and endothelium-derived hyperpolarizing factor (EDHF) (37, 50). Part of the endothelium-dependent relaxation is accompanied by endothelium-dependent hyperpolarization of the vascular smooth muscle cells (10) and the contribution of the hyperpolarizing mechanism to endothelium-dependent relaxation increases as the vessel size decreases, whereas the contribution of NO decreases (24, 29, 36, 48).

It has been shown that NO and PGI2, in addition to EDHF, hyperpolarize the membrane potential of vascular smooth muscle cells. NO increases cGMP in vascular smooth muscle cells and the elevation of cGMP induces the activation of large-conductance Ca2+-activated K+ channels (BKCa channels) (1, 38, 53). In some vessels, NO directly activates BKCa channels (3). In guinea pig large coronary and carotid arteries, PGI2 contributes in part to the endothelium-dependent hyperpolarization (43). PGI2 stimulates the synthesis of cAMP (31) and the increase in cAMP activates KATP channels (39). Stretch enhances the hyperpolarizing effect of NO and PGI2 in large guinea pig coronary arteries (43). However, it is unknown whether these factors induce hyperpolarization in coronary microvessels under physiological transmural pressure. Although EDHF has not yet been identified, several candidates have been proposed. In porcine and bovine coronary arteries, epoxyeicosatrienoic acids (EETs), metabolites of arachidonic acid through cytochrome P-450 monooxygenase (CYP450), were suggested to be...
EDHF (5, 18, 23). Several other substances, such as an endogenous cannabinoid and K⁺ released from the endothelium, have been proposed to be EDHF (14, 46). In some arteries, it was reported (9, 15) that the hyperpolarizing response to EDHF might originate in the endothelium and transfer to the smooth muscle through the myoendothelial gap junction. Matoba et al. (34) suggested that H₂O₂ might be EDHF in mouse intravascular pressure to make our experimental conditions more physiological. There are several kinds of membrane potential-sensitive dyes such as merocyanine, cyanine, and oxonol dyes. However, most of them have a low sensitivity to changes in membrane potential and some of them have toxicity to mitochondria (16). In the present study, we chose bis-(1,3-dibutylbarbituric acid/trimethine oxonol [DiBAC₄(3)], which is nontoxic and has a high sensitivity to changes in the membrane potential. In fluorescent dye methods, the photobleaching of excited fluorophore limits the accurate quantitative analysis of the fluorescence intensity under high-energy illumination. Therefore, we used stroboscopic illumination and a synchronized high-speed video system.

METHODS

General preparation. Fifty-three mongrel dogs of either sex (4.0–13.6 kg body wt) were premedicated with intramuscular ketamine (10 mg/kg) and anesthetized with α-chloralose (300 mg/kg iv) and sodium tetraborate (300 mg/kg iv). After the intravenous injection of heparin (200 U/kg), a left thoracotomy was performed in the fifth intercostal space, and the heart was quickly excised from the animal and placed in cold physiological salt solution (PSS). This solution was composed of the following (in mM): 145 NaCl, 5.9 KCl, 2 CaCl₂, 1.2 MgSO₄, 1.2 NaH₂PO₄, 2 pyruvate, 0.02 EDTA, 3 MOPS, and 5 glucose. Cold PSS (1 ml) containing gelatin (36 mg/ml) and glucose. Cold PSS (1 ml) containing gelatin (36 mg/ml) and 5 glucose. Cold PSS (1 ml) containing gelatin (36 mg/ml) and 5 glucose. Cold PSS (1 ml) containing gelatin (36 mg/ml) and 5 glucose.

The dogs were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the experimental and animal care protocol was approved by the Animal Care Committee of the Tohoku University School of Medicine.

Measurements of membrane potential and internal diameter. Changes in membrane potential were evaluated using a membrane potential-sensitive fluorescent dye, DiBAC₄(3), which is an anionic nontoxic dye (16). The membrane potential-dependent partition of DiBAC₄(3) across a cell membrane follows the Nernst equation. When the cell membrane is depolarized, more dye enters the cytosol, and its fluorescence intensity is significantly enhanced by reversibly binding to cytosolic proteins (4). When the cell membrane is hyperpolarized, DiBAC₄(3) detaches from cytosolic proteins, goes outside the cell, and loses fluorescence. Therefore, a decrease in the fluorescence intensity indicates membrane hyperpolarization. To equilibrate the coronary microvessels with DiBAC₄(3), coronary microvessels were perfused with PSS containing 0.25 mM of DiBAC₄(3), and then to 107.5 mM in the dark before the start of the experimental protocols and throughout the experiments. Because the photobleaching of excited fluorophore by high-energy illumination limits accurate quantitative analysis of the fluorescence intensity, the vessels were illuminated with a stroboscope (15 μs/flash, total exposure time 3 ms/s). The maximal wavelength of the illumination light was 495 nm, obtained with a B2 excitation filter (Nikon). The emitted light was passed through a 516-nm filter. Microvascular fluorescent images were observed through a fluorescence microscope enhanced with an image intensifier (model CS100, Hamamatsu Photonics, Hamamatsu, Japan) and recorded at 200 frames/s using a synchronized high-speed video system (model MHS-200, Nac; Tokyo, Japan). To compensate for the small fluctuation of stroboscopic illumination with time, 60 consecutive video frames obtained at 200 frames/s were averaged with a computer (Power Mac G3, Apple Computer) using NIH Image software. Fluorescence intensity and vascular internal diameter were measured on a screen with the use of NIH Image software. The mean value of fluorescence intensity on the averaged image was measured at the center of the vessel. In some experiments, it was also measured at the edge of the vessel.

Experimental protocol 1. To confirm that changes in membrane potential could be detected with the use of DiBAC₄(3), the depolarizing effect of high K⁺ (40 mM) on the fluorescence intensity was assessed in coronary arterial microvessels. After the microvessels were equilibrated with DiBAC₄(3), the K⁺ concentration of PSS was increased from 4 to 40 mM and then returned to 4 mM.

Experimental protocol 2. The correlation between changes in the fluorescence intensity and membrane potential was assessed using an approach similar to that described by He and Curry (22). Eighteen microvessels (211 ± 21 μm internal diameter) from twelve dogs were equilibrated with DiBAC₄(3) and then perfused with the modified PSS containing 1 μM of the cationophore gramicidin, DiBAC₄(3), and 6.6 mM of Na⁺. After 50 min of perfusion with this solution, a microvascular image was acquired and the concentration of Na⁺ was further increased to 107.5 mM. The osmolarity was maintained constant by the addition of N-methyl-d-glucamine. Modified PSS at each concentration of Na⁺ was perfused for 15 min, and the images were acquired at 15 min after the start of each perfusion. Intracellular ion concentrations were assumed to be 140 mM for K⁺ and 10...
mM for Na⁺ (40). The extracellular concentration of K⁺ was 4.7 mM and the other constituents except for Na⁺ were identical to the PSS. When the extracellular concentrations of Na⁺ were 6.6, 29.2, and 107.9 mM, the membrane potentials calculated according to the Goldman equation were −68.9, −39.6, and −7.6 mV, respectively.

**Experimental protocol 3.** To examine the effect of diameter changes on the fluorescence intensity, isolated coronary microvessels (222 ± 24 μm internal diameter; n = 5) were perfused with PSS containing DiBAC₄(3) and 4 mM of K⁺. After the microvessels were equilibrated with DiBAC₄(3), they were constricted with membrane depolarization by high K⁺ (40 mM). EDTA, which chelates metal ions, was then added to the solution to cause full vasodilation while membrane depolarization was maintained. The fluorescence intensity of microvessels constricted by high K⁺ was compared before and after the treatment with EDTA.

**Experimental protocol 4.** To examine whether NO and PGI₂ were involved in the coronary microvascular hyperpolarization by ACh under a physiological transmural pressure, and whether CYP450 metabolites and/or H₂O₂ contributed to the hyperpolarization and vasodilation of coronary microvessels, a total of 32 microvessels from 26 dogs were divided into four groups: the no-inhibitors group (n = 9), the N⁴-nitro-l-arginine (l-NNA)/indomethacin (Indo) group (n = 10), the 17-octadecynoic acid (17-ODYA) group (n = 7), and the catalase group (n = 6). In the no-inhibitors group, after 80 min of perfusion with PSS containing DiBAC₄(3) without any inhibitors, increasing concentrations of ACh (0.1 to 10 μM) were added to the solution for 10 min and then ACh was removed from the solution. In the l-NNA/Indo group, the same procedure was performed as in the no-inhibitors group except for the addition of 100 μM l-NNA (NO synthase inhibitor) and Indo (10 μM, cyclooxygenase inhibitor) to the perfusate. In the 17-ODYA group, the same procedure was performed as in the no-inhibitors group except for the addition of l-NNA, Indo, and 17-ODYA (5 μM; CYP450 monoxygenase inhibitor). In the catalase group, the same procedure was performed as in the no-inhibitors group except for the addition of l-NNA, Indo, and 17-ODYA (5 μM; CYP450 monoxygenase inhibitor). In the catalase group, the same procedure was performed as in the no-inhibitors group except for the addition of l-NNA, Indo, and catalase (1,250 U/ml, an enzyme that dissuates H₂O₂ to form water and O₂). Finally, in each group, 100 μM levcromakalim (K₉ᵥ channel opener) was added to the solution 20 min after the removal of ACh. Inhibitors were added to the PSS for 80 min before ACh application and continued during the experiments. Images were acquired 5 min before and immediately prior to the ACh application and at 2, 5, and 10 min during each concentration of ACh application, 20 min after the removal of ACh, and at 15 min during the levcromakalim application. If the microvessels were not hyperpolarized by the application of levcromakalim and/or their fluorescence intensity did not return to the baseline level after removal of ACh from the solution, those vessels were discarded. In four vessels from three other dogs, the effect of endothelial damage on ACh-induced hyperpolarization was examined. In those vessels, 0.1 ml of air was slowly infused into the lumen through a glass micropipette to selectively damage the endothelium (17, 45), and then the vessels were flushed with PSS and the other side was ligated. After these procedures, the ACh and levcromakalim-induced hyperpolarizations were examined in the no-inhibitors group. In five vessels from three other dogs, a single dose of ACh (1 μM) was perfused for 20 min to obtain the time-response curve by measuring the fluorescence intensity every 2 min during ACh infusion.

In some experiments, the microvessels were kept in minimum essential medium (MEM) containing both penicillin (100 U/ml) and streptomycin (100 μg/ml) for 16–24 h at 3.5°C and then perfused with PSS containing l-NNA and Indo with and without catalase to examine the effect of catalase on the EDHF-mediated vasodilation. Subsequently, ACh or levcromakalim was added to the solution and only the internal diameters were measured.

**Drugs.** DiBAC₄(3) (Sigma) was dissolved in dimethyl sulfoxide (DMSO), and the aliquots of DiBAC₄(3) (0.1 ml, 5 mM) were stored at −20°C until use. An aliquot was freshly dissolved with PSS to 0.25 μM on each experimental day. The final concentration of DMSO was 0.005 vol%. Gramicidin (Sigma) was dissolved in ethanol (5 mM stock solution) and then diluted in PSS on each experimental day. The final concentration of ethanol was 0.02 vol%. 17-ODYA (Sigma) was first dissolved in phosphate buffer and then diluted in PSS. ACh (Sigma) and l-NNA (Sigma) were freshly dissolved with PSS.

**Data analysis.** All values are presented as means ± SE; n indicates the number of vessels. Regression analysis (simple) was performed to assess the correlation between percent changes in fluorescence intensity and the calculated membrane potential. To evaluate the microvascular hyperpolarization induced by ACh or levcromakalim, the percent changes in fluorescence intensity from the baseline were calculated. In each microvessel, the value of the internal diameter in response to 100 μM of levcromakalim was taken for 100% and the changes in internal diameter induced by ACh or levcromakalim from the baseline were calculated. The fluorescence intensity and internal diameter just before the application of ACh or levcromakalim were considered as the baseline. Statistical analysis was performed by Student’s t-test for paired and unpaired observations. When the P < 0.05, the differences were considered to be statistically significant.

**RESULTS**

**Effect of high K⁺ on fluorescence intensity of microvessels.** As shown in Fig. 1, the fluorescence intensity at the edge of the vessel was higher than at the center. The elevation of the K⁺ concentration from 4 to 40 mM increased the fluorescence intensity of the coronary microvessels both at the center and at the edge and decreased the vessel diameter (Fig. 1). Therefore, it was confirmed that the fluorescence intensity of DiBAC₄(3) markedly increased when the membrane potential of microvessels was depolarized.

**Correlation between percent changes in fluorescence intensity and membrane potential.** When the membrane potential was changed from −68.9 to −39.6 mV and to −7.6 mV, the fluorescence intensity significantly increased both at the center and at the edge of the vessel (Fig. 2). As shown in Fig. 2, there was a linear correlation between the percent changes in fluorescence intensity and the calculated membrane potential both at the center (r = 0.774, P < 0.0001) and at the edge (r = 0.747, P < 0.0001).
Effect of diameter change on fluorescence intensity.
The elevation of the K⁺ concentration from 4 to 40 mM significantly increased the fluorescence intensity of the coronary microvessels by 24 ± 4% (P < 0.05) and decreased the vessel diameter by 21 ± 4% (P < 0.05) (n = 5, Fig. 3). When EDTA was added to PSS containing 40 mM of K⁺, the vessel diameter returned to the preconstricted level (2.0 ± 1.0%) but the fluorescence intensity was not affected (24 ± 5%, Fig. 3). These results indicate that changes in the fluorescence intensity are not significantly influenced by the diameter change.

Effect of inhibitors of NO synthase/cyclooxygenase, CYP450 monooxygenases, and H₂O₂ on ACh-induced hyperpolarization. Figure 4 shows a typical change in the fluorescence intensity of the coronary microvascular wall caused by ACh in the presence of L-NNA and Indo. ACh (1 μM) clearly decreased the fluorescence intensity without any change in a reference fluorescence tube indicating EDHF-mediated hyperpolarization. In experimental protocol 4, the internal diameters of microvessels were 172 ± 12 μm (n = 9) in the no-inhibitors group, 182 ± 18 μm (n = 10) in the

Fig. 1. Effect of high K⁺ on fluorescence intensity and diameter of microvessels. A: original image of the fluorescence intensity of coronary arterial microvessels; the fluorescence intensity was measured at the foursided figure. B: changes in fluorescence intensity and vessel diameter with membrane depolarization by high K⁺ (40 mM). The elevation of the K⁺ concentration increased the fluorescence intensity both at the center and at the edge of the vessel and induced vasoconstriction. After the removal of high K⁺, not only the fluorescence intensity but also the vessel diameter returned to the control level.

Fig. 2. Correlation between changes in the fluorescence intensity in response to an elevation in the Na⁺ concentration and membrane potential calculated with the Goldman equation at the center and at the edge of the vessel. Simple regression analysis showed a linear correlation both at the center (r = 0.774, P < 0.0001) and at the edge (r = 0.747, P < 0.0001).

Fig. 3. Effect of diameter change on fluorescence intensity. Line graphs show percent changes in fluorescence intensity and internal diameter by high K⁺ (40 mM). High K⁺ induced an increase in the fluorescence intensity and vasoconstriction. The addition of EDTA to high K⁺ caused vasodilation to the preconstricted level, but did not affect the fluorescence intensity. *P < 0.05, significant difference from 4 mM K⁺. NS, no significant difference.
The L-NNA/Indo group, 224 ± 30 μm (n = 7) in the 17-ODYA group, and 136 ± 28 μm (n = 6) in the catalase group at 60 cmH₂O of intraluminal pressure. There were no significant differences in the internal diameters among the groups. The resting fluorescence intensity just before ACh application was similar among the no-inhibitors, L-NNA/Indo, and 17-ODYA groups, but it was significantly smaller in the catalase group (P < 0.05 vs. other groups) than in the other groups [40.9 ± 3.3 arbitrary units (AU) in the no-inhibitors group, 53.0 ± 4.7 AU in the L-NNA/Indo group, 43.1 ± 3.5 AU in the 17-ODYA group, and 25.0 ± 2.5 AU in the catalase group]. ACh (0.1–10 μM) significantly decreased the fluorescence intensity of the pressurized canine coronary microvessels in a concentration-dependent manner without any inhibitors (−10.7 ± 2.6%, −18.5 ± 3.2%, and −19.0 ± 4.5% at 0.1, 1, and 10 μM of ACh; Fig. 5A and B). After the removal of ACh from PSS, the fluorescence intensity returned to the baseline level (2.8 ± 3.4%, Fig. 5A). When the endothelium

![Fluorescent images of a pressurized coronary microvessel.](image)

**Fig. 4.** Fluorescent images of a pressurized coronary microvessel. The gradations of color were determined according to the fluorescence intensity with the use of NIH Image software. The color bar on the right shows the fluorescence intensity from low (purple) to high (red) values. With membrane hyperpolarization the appearance of a vessel changes to cooler colors. The images show a reference fluorescence tube, which emits constant fluorescence on the left, and a vessel on the right. The colors of the fluorescence of the reference tube were similar among the images. After the application of acetylcholine (ACh; 1 μM) in the presence of N⁶-nitro-L-arginine (L-NNA; 100 μM) and indomethacin (Indo; 10 μM), the color of the vessel changed to a cooler color (middle) and it returned to the control colors (left) after removal of ACh from physiological saline solution (PSS; right). Scale bar represents 100 μm.

![Effect of L-NNA and Indo on ACh (0.1–10 μM)-induced hyperpolarization of pressurized coronary microvessels.](image)

**Fig. 5.** Effect of L-NNA and Indo on ACh (0.1–10 μM)-induced hyperpolarization of pressurized coronary microvessels. A: time course of ACh-induced hyperpolarization without any inhibitors. ACh caused hyperpolarization of the pressurized coronary microvessels in a concentration-dependent manner. B: comparison of ACh-induced hyperpolarization between the no-inhibitors group and the L-NNA/Indo group. In the presence of L-NNA and Indo, ACh also caused hyperpolarization in a concentration-dependent manner. There were no significant differences in the percent changes in fluorescent intensity induced by ACh or levromakalim between the two groups. *P < 0.05, significant difference from baseline.
was selectively damaged with air infusion (124 ± 18 μm internal diameter; n = 4), the ACh-induced hyperpolarization was completely abolished (−1.2 ± 1.5%, −2.1 ± 1.8%, −1.6 ± 1.5% at 0.1, 1, and 10 μM of ACh) but the levcromakalim-induced hyperpolarization was preserved (−14.5 ± 2.5%, P < 0.05 vs. baseline). In the presence of L-NNA and Indo, ACh also decreased the fluorescence intensity of microvessels in a concentration-dependent manner (−6.7 ± 1.5%, −13.2 ± 2.5%, and −17.4 ± 4.0% at 0.1, 1, and 10 μM of ACh; Fig. 5B). There were no significant differences in the changes in fluorescence intensity by ACh between the no-inhibitors and L-NNA/Indo groups. These results indicate that NO and PGI2 did not significantly contribute to the ACh-induced hyperpolarization of the pressurized coronary microvessels. There were also no significant differences in the hyperpolarization induced by levcromakalim (100 μM) between the two groups (Fig. 5B). In the presence of L-NNA and Indo, the addition of 17-ODYA (5 μM) significantly attenuated the ACh (0.1–10 μM)-induced decrease in the fluorescence intensity (−2.6 ± 2.3%, −4.0 ± 3.4%, and −3.4 ± 3.4% at 0.1, 1, and 10 μM of ACh, P < 0.05 at 1 and 10 μM of ACh vs. the L-NNA/Indo group; Fig. 6A). On the other hand, the addition of catalase to L-NNA and Indo did not affect the ACh-induced decrease in the fluorescence intensity of the microvessels (−9.1 ± 2.6%, −13.1 ± 2.4%, and −13.3 ± 3.2% at 0.1, 1, and 10 μM of ACh; Fig. 6B).

There were no significant differences in the hyperpolarization induced by levcromakalim between the L-NNA/Indo and 17-ODYA or catalase groups (Fig. 6A and B). When a single dose of ACh (1 μM) was continuously infused for 20 min into five vessels (123 ± 8 μm internal diameter), minimal fluorescence intensity (−20.7 ± 7.9%) was observed 14.4 ± 2.7 min after the start of ACh, and the minimal fluorescence intensity obtained within 10 min was −18.3 ± 6.7%. There was no significant difference between these two values (P = 0.1992, paired comparison).

Effect of inhibitors of CYP450 monoxygenases and H2O2 on ACh-induced vasodilation. The freshly isolated coronary microvessels were not preconstricted with any drugs and failed to develop spontaneous myogenic tone under 60 cmH2O of transmural pressure in the absence of inhibitors. Therefore, ACh and levcromakalim could not produce vasodilation in the no-inhibitors group (Table 1). In the presence of L-NNA and Indo, the microvessels developed spontaneous myogenic tone in response to 60 cmH2O transmural pressure. When the synthesis of NO and PGI2 was inhibited by the treatment with L-NNA and Indo, ACh (0.1–10 μM) produced significant vasodilation in a concentration-dependent manner. After the removal of ACh, the vessel diameter returned to the baseline level (0.1 ± 0.7%). When microvessels were pretreated with L-NNA, Indo, and 17-ODYA, they could not develop spontaneous myogenic tone. Under this condition, ACh and levcromakalim failed to cause vasodilation. In the presence of L-NNA, Indo, and catalase, the microvessels developed spontaneous myogenic tone and ACh significantly produced vasodilation in a concentration-dependent manner. There were no significant differences in the vasodilation produced by ACh or levcromakalim between the L-NNA/Indo and catalase groups (Table 1).

Fig. 6. Effect of 17-octadecynoic acid (17-ODYA) or catalase on ACh (0.1–10 μM)-induced hyperpolarization of pressurized coronary microvessels. Bar graphs compare the ACh-induced hyperpolarization in the presence or the absence of 17-ODYA (A) or catalase (B). The addition of 17-ODYA to L-NNA and Indo significantly attenuated the ACh-induced hyperpolarization, whereas the hyperpolarizing response to ACh in the presence of catalase was similar to that observed in the absence of catalase. There were no significant differences in the levcromakalim-induced hyperpolarization between the L-NNA/Indo group and the 17-ODYA or catalase groups. *P < 0.05, significant difference from baseline; †P < 0.05, significant difference from L-NNA/Indo group.
HYPERPOLARIZATION OF PRESSURIZED CORONARY MICROVESELS

Table 1. Percent change in internal diameter in response to ACh or levcromakalim

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>ACh (0.1 μM)</th>
<th>ACh (1 μM)</th>
<th>ACh (10 μM)</th>
<th>Levcromakalim (100 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Freshly isolated vessels</td>
<td></td>
<td>ACh (0.1 μM)</td>
<td>ACh (1 μM)</td>
<td>ACh (10 μM)</td>
<td>Levcromakalim (100 μM)</td>
</tr>
<tr>
<td>No inhibitors</td>
<td>9</td>
<td>0.7 ± 1.6</td>
<td>1.5 ± 1.5</td>
<td>2.3 ± 1.8</td>
<td>0.6 ± 1.3</td>
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<tr>
<td>L-NNA + Indo</td>
<td>10</td>
<td>5.1 ± 1.5</td>
<td>5.5 ± 1.5</td>
<td>7.3 ± 2.4</td>
<td>7.9 ± 2.4</td>
</tr>
<tr>
<td>L-NNA + Indo + 17-ODYA</td>
<td>7</td>
<td>0.1 ± 0.3</td>
<td>0.1 ± 0.7</td>
<td>-0.1 ± 0.5</td>
<td>1.9 ± 0.8</td>
</tr>
<tr>
<td>L-NNA + Indo + Catalase</td>
<td>6</td>
<td>4.8 ± 2.0</td>
<td>8.5 ± 3.1</td>
<td>9.2 ± 3.4</td>
<td>9.6 ± 3.1</td>
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Values are means ± SE; n, no. of animals. ACh, acetylcholine; L-NNA, Nω-nitro-L-arginine; Indo, indomethacin; 17-ODYA, 17-octadecynoic acid. *P < 0.05 compared with baseline.

To exclude the possibility that DiBAC₄(3) affected catalase, only the internal diameters of the microvessels, which were kept in MEM for 16–24 h, were measured in the L-NNA/Indo group (138 ± 13 μm internal diameter; n = 7) and in the catalase group (internal diameter, 121 ± 15 μm; n = 6). There were no significant differences in the internal diameters between the two groups. When the microvessels were kept in MEM for 16–24 h, the degree of preconstriction was much greater than that observed in freshly isolated vessels and ACh or levcromakalim produced marked vasodilation in the L-NNA/Indo group. However, catalase did not inhibit the vasodilation produced by ACh or levcromakalim (Table 1, vessels 16–24 h stored in MEM).

DISCUSSION

In the present study, we used a membrane potential-sensitive fluorescent dye to evaluate the changes in the membrane potential of coronary microvessels. With our method, the changes in the membrane potential were directly visualized with sufficient sensitivity and the stable analysis of EDHF-mediated hyperpolarization was possible under a physiological transmural pressure. It is especially important that the photobleaching of excited fluorophore, which limits the accurate quantitative analysis of the fluorescence intensity was eliminated with the use of a strobic illumination. The major findings of this study on pressurized canine coronary microvessels are as follows. First, NO and PGI₂ do not contribute to the ACh-induced hyperpolarization. Second, the metabolites of arachidonic acid through CYP450 are involved in ACh-induced hyperpolarization, whereas H₂O₂ is not.

In some arteries, NO elicits the hyperpolarization of vascular smooth muscle cells by activating BKCa channels or KATP channels through cGMP or by direct activation of BKCa channels (1, 3, 38, 53). The PG₁₂ analogue evokes hyperpolarization by activating KATP channels (13). Moreover, it has been reported that stretch enhances these hyperpolarizing effects in conduit guinea pig coronary arteries (43). However, in the present study, the ACh-induced hyperpolarization in the absence of inhibitors was similar to that observed in the presence of L-NNA and Indo. This suggests that NO and PG₁₂ did not significantly contribute to the ACh-induced hyperpolarization in the coronary microvessels even at the pressurized condition and that the ACh-induced hyperpolarization was mainly mediated by EDHF in these vessels.

The CYP450 metabolites of arachidonic acid have been one of the candidates for EDHF (5, 18, 23). By taking previous reports (5, 18, 23, 35, 41, 52) into consideration concerning coronary arteries in large mammals, such as pigs, bovines, dogs, and humans, the CYP450 metabolites are likely to be involved in the EDHF-mediated responses. In the present study, the addition of 17-ODYA to L-NNA and Indo significantly attenuated the ACh-induced hyperpolarization, indicating that the CYP450 metabolites of arachidonic acid were involved in the EDHF-mediated hyperpolarization of the canine coronary microvessels. It has been reported that coronary arteries including those of dog metabolize arachidonic acid through CYP450 to 14,15-EET, 11,12-EET, 8,9-EET, and 5,6-EET and the corresponding dihydroxyeicosatetraenoic acids (DHETs) (5, 47). EETs dilate vessels by activating BKCa channels in bovine and porcine coronary arteries (5, 23). Furthermore, in canine coronary arteries, not only all four EETs but also DHETs can produce vasodilation and these vasodilator effects are much greater in microvessels (42, 47). Although it is not clear which metabolites of arachidonic acid through CYP450 were involved in the EDHF-mediated hyperpolarization in the present study, the CYP450 metabolites contributed to the EDHF-mediated hyperpolarization.

In the present study, freshly isolated coronary microvessels developed only a small amount of myogenic tone under a physiological transmural pressure without any inhibitors. However, they developed spontaneous myogenic tone in the presence of inhibitors of NO synthase and cyclooxygenase. In these vessels, ACh and levcromakalim produced significant vasodilation. Because a previous study (19) reported that NO but not PG₁₂ attenuated the myogenic tone in pressurized rat coronary arteries, basal release of NO may have attenuated the spontaneous myogenic tone in the present study. In the presence of 17-ODYA in addition to L-NNA and Indo, the microvessels failed to develop myogenic tone. Therefore, it is difficult to discuss the effect of 17-ODYA on EDHF-mediated vasodilation, although the significant effect of 17-ODYA on EDHF-mediated hyperpolarization is clear in the present study. In the presence of L-NNA and Indo, the microvessels were...
significantly constricted in response to the physiological transmural pressure, whereas with the addition of 17-ODYA to those inhibitors they were not. These results may indicate that the CYP450 metabolites of arachidonic acid are involved in the myogenic constriction in response to transmural pressure. In the blood vessel, EETs and DHETs are produced by endothelial cells through CYP2C epoxygenase, whereas smooth muscle cells metabolize arachidonic acid to 20-hydroxyeicosatetraenoic acid (20-HETE) through a CYP4A isozyme, an ω-hydroxylase (6). 20-HETE acts as an intracellular second messenger in smooth muscle cells. It inactivates Ca$^{2+}$-activated K$^+$ channels, depolarizes the cell, increases the intracellular calcium level, and constricts vessels. It is released in response to stretch or an elevation in the transmural pressure and is proposed to mediate the myogenic tone in renal and cerebral arteries (6). Zou et al. (54) have reported that 17-ODYA inhibits the epoxidation and ω-hydroxylation of arachidonic acid with similar potency. Therefore, in our preparation using pressurized canine coronary microvessels, 20-HETE may be produced by the smooth muscle and may mediate the myogenic tone.

Catalase has been reported to inhibit EDHF-mediated relaxation and hyperpolarization in mouse small mesenteric arteries (34). However, in the present study, catalase did not affect the EDHF-mediated hyperpolarization and vasodilation that was simultaneously measured with hyperpolarization. Because the resting fluorescence intensity was significantly smaller in the presence of catalase, there was a possibility that DiBAC$_4$(3) reacted with catalase. Therefore, we evaluated the effect of catalase on the EDHF-mediated vasodilation in the absence of DiBAC$_4$(3). However, even in these experimental conditions, catalase did not inhibit the EDHF-mediated vasodilation. These results indicate that H$_2$O$_2$ is not involved in the EDHF-mediated hyperpolarization and vasodilation in pressurized canine coronary microvessels.

**Critique of method.** In the present study, we used inhibitors such as l-NNA, Indo, 17-ODYA, and catalase. The concentrations of l-NNA (100 μM) and Indo (10 μM) were reported to be sufficient to inhibit endogenous release of NO and PGI$_2$ (34). Furthermore, by using diaminofluorescein-2 diacetate, we confirmed that 100 μM of l-NNA significantly inhibited the ACh (10 μM)-induced NO production in pressurized canine coronary microvessels (30). In rat renal cortical microsomes, it was reported that 5 μM of 17-ODYA inhibited CYP450 epoxygenase activities by >90% (54). In mouse small mesenteric arteries, 1,250 U/ml of catalase were reported to inhibit EDHF-mediated relaxation and hyperpolarization (34). Therefore, the concentration of each inhibitor used in this protocol was considered to be sufficient to inhibit each target.

The observed reduction in fluorescence intensity may reflect the hyperpolarization of endothelium and/or that of vascular smooth muscle. However, the endothelium consists of a single layer of thin endothelial cells, and the vascular wall (~30 μm in thickness in the present case) mostly consists of multiple layers of smooth muscle cells. Also, all observed layers including outer layers at the edges of vessel showed reduced fluorescence intensity. Taken together, the observed ACh-induced decrease in fluorescence intensity most likely reflects hyperpolarization of the vascular smooth muscle cells by EDHF.

In our experiments, 2 mM pyruvate was added to PSS. Pyruvate is a natural oxidant scavenger and abundantly present in mammalian cells. Exogenous pyruvate has been reported to prevent H$_2$O$_2$-induced cell damage and apoptosis (28, 49). Therefore, it might be possible that 2 mM pyruvate in PSS resulted in an underestimation of the role of H$_2$O$_2$ in EDHF-mediated hyperpolarization. However, 3 mM pyruvate can scavenge only 5.9 ± 0.6% and 25.2 ± 4.2% of H$_2$O$_2$ in cells of *Giardia intestinalis* and *Hexamita inflata*, respectively (20). In addition, the estimated hyperpolarization based on the change in fluorescence was approximately −30 mV with 10 μM ACh in the present study. This value is similar to or rather greater than previously reported changes. Taken together, the pyruvate in PSS might reduce the ACh-induced hyperpolarization but the effect would likely be minimal.

In cat coronary arteries, the arterial pressure of microvessels (100–200 μm in internal diameter) was ~50–60% of the aortic pressure (11, 27). When the mean aortic pressure in dogs was ~90 mmHg, the microvascular pressure seemed to be ~45–54 mmHg. Thus we made the intravascular pressure of coronary microvessels 60 cmH$_2$O. We consider “microvessels” as vessels <300 μm in internal diameter, where most of the coronary vascular resistance of arterial microvessels resides (33). Moreover, there are marked physiological and pharmacological differences between arterial microvessels >200 and those <200 μm in inner diameter (25, 26, 32), and the role of EDHF in regulating microvascular tone was greater than that of NO (36). So, in this study, we mainly used vessels <200 μm in inner diameter.

Although the chemical identity of EDHF may be different among species and vascular beds, previous reports (5, 18, 23, 35, 41, 52) concerning coronary arteries in large-sized mammals, including humans, have suggested that CYP450 metabolites of arachidonic acid may be involved in EDHF-mediated responses, and the results of the present study are consistent with those of the previous reports. The present study shows that NO and PGI$_2$ do not contribute to the endothelium-dependent hyperpolarization of pressurized canine coronary microvessels. Therefore, the endothelium-dependent hyperpolarization by ACh was mainly mediated by EDHF in our experimental condition. This hyperpolarization is sensitive to CYP 450 inhibitor but not to catalase. Moreover, catalase does not inhibit the EDHF-mediated vasodilation. These results show that CYP450 metabolites of arachidonic acid but not H$_2$O$_2$ contribute to the EDHF-mediated responses.

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DISCLOSURES

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