Cytochrome P-450 pathway in acetylcholine-induced canine coronary microvascular vasodilation in vivo

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Widmann, Mark D., Neal L. Weintraub, Jonathan L. Fudge, Leonard A. Brooks, and Kevin C. Dellsperger. Cytochrome P-450 pathway in acetylcholine-induced canine coronary microvascular vasodilation in vivo. Am. J. Physiol. 274 (Heart Circ. Physiol. 43): H283–H289, 1998.—In the canine coronary microcirculation, acetylcholine (ACh)-induced vasodilation of large (∼100 µm) epicardial arterioles (LgA), but not small (∼100 µm) epicardial arterioles (SmA), is blocked by nitric oxide (NO) synthase inhibitors in vivo. We hypothesized that the ACh-induced vasodilation of SmA is mediated by a cytochrome P-450 metabolite of arachidonic acid (AA). Epicardial coronary microvascular diameters in dogs were measured at baseline and after treatment with topically applied ACh (1, 10, and 100 µM), AA (1, 5, and 10 µM), or sodium nitroprusside (SNP; 10–100 µM). Coronary microvascular diameters of AA were compared among control dogs (group NO); dogs pretreated with L-NNA alone (group OC); dogs pretreated with L-NNA plus clotrimazole (CIO; 1.6 µM topically) or 17-octadecynoic acid (ODYA; 2 µM topically), cytochrome P-450 monooxygenase inhibitors (groups NC and NY, respectively); dogs pretreated with CIO alone (group OC); and dogs pretreated with L-NNA plus CIO with AA as the agonist (group AA). ACh-induced vasodilation of LgA was abolished by L-NNA alone, whereas in SmA, L-NNA was without effect. CIO alone did not inhibit ACh-induced dilation in either SmA or LgA. However, the combinations of L-NNA plus either CIO or ODYA abolished ACh- and AA-induced dilation of SmA (100 µM ACh: NC, 3 ± 5%; NY, 8 ± 2%; 10 µM AA: 6 ± 3%) but did not affect responses to SNP. These results suggest that the ACh-induced vasodilation of SmA is mediated in part by cytochrome P-450 metabolites of AA and provide the first evidence that the cytochrome P-450 pathway contributes to the regulation of coronary resistance vessels in vivo.

arachidonic acid; arginine analog; endothelium-derived hyperpolarizing factor; clotrimazole; intravital microscopy

ENDOTHELIAL CONTROL of vasomotor dilation is mediated by several autacoid factors including nitric oxide (NO), prostaglandin (PG) I2, and endothelium-derived hyperpolarizing factor (EDHF) (5, 10, 17). The contribution of these mediators to endothelium-dependent responses is variable, depending on the species, vascular bed, and vessel size. In the coronary circulation, the majority of coronary vascular resistance resides in microvessels <150 µm in diameter (4). In an earlier study from our laboratory (14), we demonstrated that vasodilation of large (∼120 µm) epicardial arterioles, but not small (∼120 µm) epicardial arterioles, to acetylcholine (ACh) was blocked by NO synthase (NOS) inhibitors in vivo. Other studies (14, 18) in the canine coronary microcirculation demonstrated that ACh-induced relaxation is not affected by cyclooxygenase inhibition with indomethacin. Taken together, these observations suggest that ACh-induced vasodilation of small epicardial arterioles is not mediated solely by NO or PGI2. To our knowledge, the role of EDHF in mediating coronary microvascular dilatation to ACh has not been thoroughly investigated.

Recently, epoxyeicosatrienoic acids, cytochrome P-450 metabolites of arachidonic acid (AA), have been identified as EDHF and have been suggested to contribute to methacholine-induced vasodilation in bovine epicardial coronary arteries (3). In the coronary circulation of the rat, EDHF-mediated vasodilation has been reported (9) to be blocked by inhibitors of cytochrome P-450. However, these results are not uniform among different species and vascular beds (6, 16). Thus we hypothesized that ACh-induced vasodilation of small arterioles in the canine coronary microcirculation is mediated in part by cytochrome P-450 monooxygenase metabolites of AA.

To address this hypothesis, we determined the effects of inhibitors of cytochrome P-450 enzymes on ACh-induced microvascular responses in the canine beating left ventricle in the presence of cyclooxygenase and NOS inhibitors.

METHODS

General Preparation

Mongrel dogs (n = 83; 4–8 kg body wt) of either sex were sedated with ketamine (20 mg/kg sc) and acepromazine (0.2 mg/kg sc) and anesthetized with α-chloralose (60 mg/kg iv), urethan (150 mg/kg iv), and sodium borate (25 mg/kg iv). Additional doses of anesthetic were administered throughout the experiment to maintain an adequate level of anesthesia. A catheter (PE-150) was inserted into the external jugular vein for administration of drugs and fluids. Another catheter (PE-205) was inserted into the internal carotid artery for monitoring of arterial pressure and measurement of arterial blood gases. Auffed endotracheal tube was inserted into the trachea. To minimize respiration-induced cardiac motion, dogs were ventilated with a high-frequency jet ventilator synchronized to the cardiac cycle as previously described (4, 7, 15). The ventilator settings were adjusted to maintain physiological blood gases and pH at all times. Positive end-expiratory pressure (3–5 cmH2O) was applied to prevent atelectasis. To inhibit cyclooxygenase, indomethacin (5 mg/kg iv) was administered and allowed to circulate for 20 min before thoracotomy (13).

A left thoracotomy was performed in the fifth intercostal space, the fourth and fifth ribs were resected, and a left lower lobectomy was performed to create an adequate thoracic window. The pericardium was incised to suspend the heart in a cradle. A catheter (PE-150) was inserted into the left atrial appendage for administration of fluorescein-labeled dextran. A 5-Fr catheter (Millar Instruments, Houston, TX) was placed in the left ventricle via the left atrial appendage for
recording of left ventricular pressure and the first derivative of pressure with respect to time (dP/dt). Snare were placed around the descending thoracic aorta and the inferior vena cava to control arterial pressure. The epicardial surface was kept moist by suffusion of Krebs solution (containing (in mM) 118.3 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 25 NaHCO3, 1.2 KH2PO4, pH 7.4, at 37°C and gassed with 95% O2-5% CO2-75% N2) at 2 ml/min. Body temperature was maintained (37 ± 1°C) with a servo-controlled thermal blanket. Arterial blood gases were maintained within the physiological range throughout the protocols (pH: 7.39 ± 0.02; PCO2: 34 ± 2 mmHg; PO2: 95 ± 10 mmHg).

Microscope and Video System

Measurements of coronary microvessels were obtained using intravital microscopy (Zeiss), with epi-illumination of the cardiac surface by a computer-controlled strobe (Chadwick Helmuth, Almonte, CA) as described previously (4, 7, 15). The strobe was triggered by causing the left ventricular dp/dt signal to flash once per cardiac cycle in late diastole. Fluorescein isothiocyanate dextran (molecular weight 487,000; Sigma Chemical, St. Louis, MO) was injected into the left atrium to illuminate the internal microvascular diameter and to differentiate arterioles from venules by sequence of illumination. A Zeiss Neufluora (x≥6.3, NA = 0.02) objective, when coupled with a x6.3 relay lens, measured microvascular diameters with 2.5-µm resolution. Images were transmitted to a video camera (General Electronic, Owensboro, KY) via a x10.0 or x6.3 relay lens. Digital data images were selected and stored in a computer (IBM 486). Images were later recalled on a high-resolution monitor, and the microvascular diameters were measured using a digitizing tablet and a computer to calculate the vessel diameter in microns. All vessel measurements represent the mean of up to three images at each experimental condition.

Drugs

ACh, N-nitro-L-arginine (L-NNA), clotrimazole, sodium nitroprusside (SNP), AA, and indomethacin were purchased from Sigma Chemical. The 17-octadecynoic acid (ODYA) was purchased from Biomol (Plymouth Meeting, PA). ACh, AA, and SNP were dissolved in Krebs-Henseleit buffer, whereas L-NNA and ODYA were dissolved in saline. These drugs were added to the epicardial perfusate at 10 times their final concentration using a Harvard infusion pump at a rate of 0.2 ml/min. Clotrimazole was initially dissolved in 100% ethanol to provide an initial stock solution of 10⁻² M and was subsequently dissolved into the Krebs perfusate to provide a final concentration of 1.6 µM. Vehicle studies demonstrated that this concentration of ethanol did not affect ACh-induced vasodilation.

Protocols

After the general surgical preparation, at least 30 min were allowed for stabilization of monitored variables. The microvascular field of study was identified and coronary arterioles were verified by injection of fluorescein-labeled dextran. Baseline measurements of hemodynamics, hematocrit, microvascular diameters, and arterial blood gases were performed.

General protocol. A similar protocol sequence was performed in each group receiving ACh. During the surgical preparation, all animals were pretreated with indomethacin (5 mg/kg iv). Baseline images were obtained 30 min after the epicardium was superfused with the appropriate inhibitors (or their vehicles) but before ACh was administered. After this 30-min period, ACh (1, 10, and 100 µM topically) was given along with continuous suffusion of the inhibitors or their vehicles, and coronary microvascular diameters were measured 1, 5, and 10 min following the initiation of the topical suffusion. The time course of ACh-induced vasodilatation was examined, because vasodilation attributed to EDHF has been reported to be transient (1, 2). A Ch was administered to establish that vessels were capable of relaxing. Large vessels (>100 µm) dilating <20% or small vessels (<100 µm) dilating <40% in response to 100 µM SNP were excluded from analysis. The length and bore of the tubing were not changed throughout the duration of these studies. Hemodynamics were continuously monitored and were unaffected by the topical suffusion of ACh or SNP.

After the protocols were completed, the dogs were killed with an overdose of anesthetic followed by saturated potassium chloride (10 ml).

The following protocols used combinations of inhibitors of NOS (L-NNA) and cytochrome P-450 (clotrimazole and ODYA) as shown in Table 1.

Protocol 1. Group OO (18 dogs) served as the control group. Neither L-NNA nor an inhibitor of cytochrome P-450 was administered. The percentage change from baseline in response to topically applied ACh (1, 10, and 100 µM) was determined at 1, 5, and 10 min for each dose. Measurements were made in both small (n = 11 dogs) and large (n = 7 dogs) arterioles.

Protocol 2. In group NO (24 dogs), to evaluate the response of the coronary microcirculation to L-NNA (70 µM topically), coronary microvascular diameter was measured during ACh infusions at 1, 5, and 10 min for each dose. Measurements were made in both small (n = 17 dogs) and large (n = 7 dogs) arterioles. One dog from each vessel size (total = 2) was excluded from analysis due to inadequate SNP responses.

Protocol 3. In group OC (15 dogs), to evaluate the effect of clotrimazole (1.6 µM topically) alone on coronary microvascular diameter, coronary microvascular diameters were measured during increasing concentrations of ACh in the presence of clotrimazole. Measurements were made in both small (n = 9 dogs) and large (n = 6 dogs) arterioles.

Protocol 4. Because our earlier study (14) demonstrated that L-NNA alone is not sufficient to completely inhibit small coronary arterial responses to ACh, in group NC (15 dogs)

Table 1. Presence and absence of inhibitors of nitric oxide synthase and cytochrome P-450 in protocol groups

<table>
<thead>
<tr>
<th>Group</th>
<th>NOs</th>
<th>Cytochrome P-450</th>
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<tbody>
<tr>
<td>OO</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>NO</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>OC</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>NC</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>NY</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+ and −, Presence and absence, respectively, of inhibitors of nitric oxide synthase (NOs) and cytochrome P-450.
we tested the hypothesis that the combination of clotrimazole and L-NNA will inhibit ACh-induced small coronary microvascular dilatation. Coronary microvascular diameters were measured in response to ACh in the presence of both L-NNA (70 µM topically) and clotrimazole (1.6 µM topically) 1, 5, and 10 min after each dose. Measurements were made in both small (n = 7 dogs) and large (n = 8 dogs) arterioles.

Protocol 5. In group NY (6 dogs), to further investigate the role of cytochrome P-450 in mediating ACh-induced coronary microvascular dilatation, we determined the effects of treatment with a chemically distinct inhibitor of cytochrome P-450 enzyme, ODYA. ACh responses were measured in the presence of both L-NNA (70 µM topically) and ODYA (2.0 µM topically). Measurements were made only in small arterioles. One dog was excluded from analysis due to inadequate SNP responses.

Protocol 6. In group AA (5 dogs), to test the hypothesis that AA-induced dilation is mediated in part by cytochrome P-450, we determined the microvascular response to ACh (1, 5, and 10 µM topically) in the absence of inhibitors and in the presence of indomethacin and L-NNA. We subsequently investigated whether the indomethacin- and L-NNA-resistant vasodilation is inhibited by clotrimazole. After baseline measurements were made in the presence of indomethacin, SNP (10 µM) was given. Fifteen minutes after SNP was discontinued, L-NNA was given for 30 min. Coronary microvascular measurements and hemodynamics were again measured, and then AA was administered in increasing doses. Diameters were measured at 1, 5, and 10 min as with ACh. After administration of the high dose of AA and a 30-min recovery period, repeat baseline measurements were made. Clotrimazole was then administered for 30 min, and repeat measurements were made. AA was then applied as described earlier. After a 15-min period, SNP was administered while measurements of diameter and hemodynamics were made. Preliminary studies with AA in other animals demonstrated that repeated doses of AA in this time course provide identical results.

Statistics

Data are displayed as means ± SE. For studies with ACh, one-way analysis of variance (ANOVA) with repeated measures was used to evaluate the changes in hemodynamic variables and microvascular diameters during each protocol. Blood gas data within groups were analyzed by Student’s t-tests. Baseline diameters for each treatment group were compared with baseline diameters from the control group using an unpaired Student’s t-test and the Bonferroni correction. ACh dose-response curves among treated and control groups were compared using two-factor repeated-measures ANOVA. For the protocol with AA, paired Student’s t-test with Bonferroni correction was used to compare responses and hemodynamics before and after clotrimazole. A P < 0.05 was considered statistically significant.

RESULTS

Hemodynamics, Hematocrit, and Blood Gases

Baseline heart rate [group OO: 165 ± 5; group NO: 157 ± 5; group OC: 150 ± 7; group NC: 165 ± 5; group NY: 154 ± 10; and group AA: 155 ± 7 beats/min; P = not significant (NS)], mean arterial blood pressure (group OO: 87 ± 3; group NO: 93 ± 2; group OC: 88 ± 3; group NC: 88 ± 5; group NY: 86 ± 5; and group AA: 86 ± 6 mmHg; P = NS), and blood gas measurements were similar among all groups. There were no significant changes in these parameters during the course of each protocol. Hematocrit was different only between groups OO and OC; however, all dogs had hematocrits >30% (group OO: 36.9 ± 1.5; group NO: 32.4 ± 1.1; group OC: 31.2 ± 0.9; group NC: 32.8 ± 1.4; group NY: 35.3 ± 2.0; and group AA: 36.2 ± 0.7%; group OO vs. group OC, P < 0.05; other groups, P = NS).

Baseline Coronary Microvascular Diameters and Responses to SNP

Because previous studies (14) have indicated that the vasomotor responses to ACh differ in small versus large arterioles, we analyzed the small (<100 µm) and large (>100 µm) arteriolar diameters separately. Baseline control diameters in small arterioles were not different among the various groups (Table 2). Further, the percentage increase in diameter in response to SNP did not differ among the groups (100 µM SNP; group OO: 78 ± 9; group NO: 76 ± 9; group OC: 66 ± 9; group NC: 83 ± 10; and group NY: 63 ± 12%; P = NS). In the large arterioles there was more variability among the baseline diameters, and only groups OO and NO were different (P < 0.05) (Table 2). As in the small arterioles, the responses to SNP in the large arterioles were not different among the groups (10 µM SNP; group OO: 50 ± 7; group NO: 39 ± 8; group OC: 48 ± 7; and group NC: 43 ± 8%; P = NS).

In dogs that were pretreated with indomethacin, small coronary microvascular diameters were not significantly different before and after administration of L-NNA (79 ± 16 µm before; 82 ± 14 µm after; P = NS), clotrimazole (61 ± 9 µm before; 57 ± 7 µm after; P = NS), or ODYA (not shown).

Coronary Microvascular Responses to ACh Alone

The changes in diameters of the small and large coronary arterioles in response to ACh for 10 min are displayed in Figs. 1 and 2. In the control protocol (group OO), ACh induced a dose-dependent vasodilation in all sizes of coronary arterioles. Individual vessel responses after ACh (100 µM) administration for 10 min are shown in Fig. 3A.

Table 2. Baseline average coronary microvascular diameters for both small and large coronary arterioles

<table>
<thead>
<tr>
<th>Group</th>
<th>Inhibitors</th>
<th>Small Arterioles</th>
<th>Large Arterioles</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Diameter, µm</td>
<td>Diameter, µm</td>
</tr>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>n</td>
</tr>
<tr>
<td>OO</td>
<td>-/-</td>
<td>66 ± 4</td>
<td>121 ± 4</td>
</tr>
<tr>
<td>NO</td>
<td>+/-</td>
<td>69 ± 4</td>
<td>138 ± 5*</td>
</tr>
<tr>
<td>OC</td>
<td>-/+</td>
<td>68 ± 5</td>
<td>133 ± 9</td>
</tr>
<tr>
<td>NC</td>
<td>+/-</td>
<td>61 ± 5</td>
<td>139 ± 11</td>
</tr>
<tr>
<td>NY</td>
<td>+/-</td>
<td>60 ± 4</td>
<td>ND</td>
</tr>
<tr>
<td>AA</td>
<td>+/-/-</td>
<td>73 ± 7</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE for n dogs and vessels. Small arterioles had diameters <100 µm, and large arterioles had diameters >100 µm. ND, experiments were not done. *P < 0.05 vs. group OO.
ated ACh responses. However, the combination of both L-NNA and clotrimazole (group NC) abolished ACh-induced vasodilation (Fig. 1). The individual vessel responses during treatment with indomethacin (group OO; Fig. 3A), L-NNA (group NO; Fig. 3B), or clotrimazole (group OC; Fig. 3C) show that the magnitude of vasodilation was inversely related to baseline vessel diameter. Individual vessel responses during treatment with both L-NNA and clotrimazole (group NC; Fig. 3D) did not demonstrate a significant dependency on baseline vessel size.

In separate experiments, we investigated the effects of L-NNA and ODYA (2.0 µM; group NY) on ACh-induced vasodilation in small arterioles. Consistent with the results obtained with the combination of L-NNA and clotrimazole, this combination of inhibitors also abolished ACh-induced vasodilation (Fig. 1).

Large Coronary Arteriolar Responses to ACh at 10 min

In the large arterioles, L-NNA alone (group NO) abolished ACh-induced vasodilation (Fig. 2), whereas clotrimazole alone (group OC) had no significant effect. The results obtained with the combination of L-NNA plus clotrimazole (group NC) did not differ from those obtained with L-NNA alone (P = 0.07; group NO). Individual responses to ACh (100 µM) at 10 min are shown in Fig. 3.

Small Coronary Arteriolar Responses to AA at 10 min

In small arterioles there was dilation to AA in the presence of indomethacin and L-NNA (Table 3).
Table 3. Coronary microvascular responses to arachidonic acid

<table>
<thead>
<tr>
<th>Drug</th>
<th>Change in Diameter From Baseline, %</th>
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<tbody>
<tr>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>AA (1 µM)</td>
<td>11 ± 2</td>
</tr>
<tr>
<td>AA (5 µM)</td>
<td>20 ± 4</td>
</tr>
<tr>
<td>AA (10 µM)</td>
<td>28 ± 5</td>
</tr>
<tr>
<td>SNP (10 µM)</td>
<td>50 ± 8</td>
</tr>
</tbody>
</table>

Values are means ± SE. Indo, indomethacin; L-NNA, Nω-nitro-L-arginine; AA, arachidonic acid; SNP, sodium nitroprusside. *P < 0.05 vs. Indol-NNA and control.

The addition of clotrimazole, the AA-induced vasodilation was substantially impaired (Table 3). SNP (10 µM) responses were normal and unchanged before (51 ± 6%) and after (53 ± 7%) the AA protocol.

Time Course of Coronary Microvascular Responses

The time-response curves for the highest dose of ACh (100 µM) for both small and large arterioles are displayed in Table 4. In the control (group OO), ACh induced a time-dependent vasodilation in all coronary arterioles. Coronary microvascular diameter was greatest at the earliest time measured (1 min), with a progressive decrease in diameter by 10 min. In small arterioles, the presence of either L-NNA (group NO) or clotrimazole (group OC) alone did not significantly alter the ACh time-response curve. The combination of L-NNA and an inhibitor of cytochrome P-450 (groups NC or NY) attenuated the ACh-induced responses at all times observed (Table 4). In large arterioles, L-NNA alone (group NO) or the combination of L-NNA plus clotrimazole (group NC) resulted in inhibition of ACh-induced responses at all time points (Table 4). Similar time-response curves were also noted for ACh at 1 and 10 µM in both small and large arterioles (data not shown). In contrast, during the highest dose of AA (10 µM), there was no significant effect of time on microvascular responses during indomethacin and L-NNA administration. The addition of clotrimazole resulted in inhibition of the ACh-induced dilation at all time points.

Table 4. Time course of coronary microvascular responses to 100 µM ACh

<table>
<thead>
<tr>
<th>Group</th>
<th>NOSP-P-450</th>
<th>Vessel Size</th>
<th>1 min</th>
<th>5 min</th>
<th>10 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>OO</td>
<td>−/−</td>
<td>Small</td>
<td>51 ± 7</td>
<td>50 ± 10</td>
<td>43 ± 5</td>
</tr>
<tr>
<td>OC</td>
<td>−/+</td>
<td>Small</td>
<td>49 ± 10</td>
<td>47 ± 8</td>
<td>39 ± 5</td>
</tr>
<tr>
<td>NO</td>
<td>++</td>
<td>Small</td>
<td>59 ± 5</td>
<td>44 ± 7</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>NC</td>
<td>++</td>
<td>Small</td>
<td>59 ± 5</td>
<td>44 ± 7</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>NY</td>
<td>++</td>
<td>Small</td>
<td>59 ± 5</td>
<td>44 ± 7</td>
<td>40 ± 4</td>
</tr>
<tr>
<td>OO</td>
<td>−/−</td>
<td>Large</td>
<td>41 ± 5</td>
<td>40 ± 5</td>
<td>38 ± 7</td>
</tr>
<tr>
<td>OC</td>
<td>−/+</td>
<td>Large</td>
<td>42 ± 5</td>
<td>40 ± 5</td>
<td>37 ± 7</td>
</tr>
<tr>
<td>NO</td>
<td>++</td>
<td>Large</td>
<td>43 ± 5</td>
<td>40 ± 5</td>
<td>37 ± 7</td>
</tr>
<tr>
<td>NC</td>
<td>++</td>
<td>Large</td>
<td>44 ± 5</td>
<td>40 ± 5</td>
<td>37 ± 7</td>
</tr>
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</table>

Values are means ± SE and represent %change in diameter. *P < 0.05 vs. group OO for respective vessel size; †P = 0.07 vs. group OO for large vessels.

Discussion

There were four principal findings in our study. First, in the presence of cyclooxygenase inhibition, ACh-induced vasodilation was abolished in large coronary arteries (>100 µm) by L-NNA, an inhibitor of NOS. Second, ACh-induced dilation in small coronary arteries (<100 µm) was not inhibited by the administration of either L-NNA or clotrimazole, an inhibitor of cytochrome P-450 epoxygenase. Third, ACh-induced dilation in small arterioles was abolished by the combination of L-NNA and clotrimazole or a different cytochrome P-450 inhibitor, ODYA. Fourth, AA-induced dilation in small arterioles is resistant to inhibition with indomethacin and L-NNA but is abolished by the addition of clotrimazole, an inhibitor of cytochrome P-450.

The observation that L-NNA abolished ACh-induced vasodilation of large coronary arterioles is consistent with a previous report from our group (14). In that study, the specificity of the arginine analogs was demonstrated by showing reversibility of the inhibitory effects with L-arginine. Further, as shown in this study, no differences to SNP responses were observed following treatment with L-NNA. These observations suggest that L-NNA selectively inhibited the ACh-induced production of NO by the coronary microvessels.

In contrast to the results obtained with L-NNA in large coronary arterioles, L-NNA failed to attenuate ACh-induced vasodilation of small coronary arterioles. A previous study from our laboratory using the same preparation (14), L-NNA partially attenuated ACh-induced dilation of small arterioles. The apparent discrepancy between our present results and those previously published is explained by the differences in the baseline (control) diameters in the small arteriolar groups (Table 2, group NO) and the methods of calculating the change in diameter. In the previous study (14), vessels <120 µm in diameter were included in the small vessel group, resulting in a mean diameter of 83 ± 6 µm. In our present study, only vessels <100 µm were included in the small vessel group, resulting in a mean diameter of 69 ± 4 µm. When the current data set was reanalyzed using the same size parameters and calculation methods as in the Komaru et al. (14) study (with vessels <120 µm in diameter classified as small arterioles), partial attenuation of ACh-induced vasodilation was also observed with L-NNA. Thus the results obtained with L-NNA in the present study are consistent with those obtained in the previous study. Collectively, these data support that the ability of L-NNA to inhibit ACh-induced vasodilation is highly dependent on the baseline arteriolar diameter. The fact that inhibition of ACh-induced vasodilation was nearly complete at 10 min in large arterioles suggests that our dose of L-NNA was adequate and, consequently, that ACh-induced vasodilation of small arterioles occurred, at least in part, through a mechanism distinct from L-arginine-derived NO.

Because all animals were pretreated with indomethacin, it is unlikely that a significant portion of ACh-induced vasodilation was due to PGI2-induced vasodila-
tion. Several investigations (14, 18) have demonstrated that cyclooxygenase inhibition failed to inhibit ACh-induced arteriolar vasodilation.

We investigated the hypothesis that the L-NNA-resistant vasodilation of small coronary arterioles is mediated by a pathway that includes cytochrome P-450 metabolism. This hypothesis was formulated on the basis of previous reports that inhibitors of cytochrome P-450 enzyme activity blocked agonist-induced relaxations of bovine and porcine epicardial coronary arteries (3, 12) and bradykinin-induced, NO-independent vasodilation in the isolated, perfused rat heart (2, 9). We observed that the L-NNA-resistant, ACh-induced vasodilation of small coronary arterioles was abolished by either clotrimazole (1.6 µM) or ODYA (2 µM), chemically dissimilar inhibitors of cytochrome P-450 enzymes (Fig. 1). These inhibitor compounds have been reported to possess nonspecific effects when given at twofold or higher concentrations than those used in our study in other vascular beds (8, 19). The concentrations used in our study have not been associated with nonspecific effects in the coronary circulation (9, 12).

We found that clotrimazole alone had no effect on ACh-induced vasodilation in large or small coronary arterioles and that the combination of L-NNA plus clotrimazole or ODYA did not inhibit responses to SNP. Moreover, we gave the inhibitor compounds by topical rather than systemic or intra coronary administration to avoid the possibility of confounding hemodynamic effects or alterations in myocardial metabolism. Thus our studies provide strong support for the hypothesis that the L-NNA-resistant, ACh-induced vasodilation of small coronary arterioles is mediated by a pathway that includes cytochrome P-450 metabolism.

Although the L-NNA-resistant, ACh-induced vasodilation in small arterioles was completely abolished by the addition of clotrimazole or ODYA, clotrimazole alone (absence of L-NNA) did not affect ACh-induced responses. Thus multiple redundant dilator mechanisms are possibly present. Inhibition of the NOS pathway alone or in combination with cyclooxygenase blockade (14) (Fig. 1) did not inhibit small coronary arteriolar microvascular dilatation in response to ACh. Likewise, the combination of cytochrome P-450 inhibition with cyclooxygenase blockade also did not reduce ACh-induced coronary microvascular dilatation. When all three pathways are inhibited (NOS, cyclooxygenase, and cytochrome P-450), complete inhibition of ACh-induced vasodilation occurred (Fig. 1). These multiple redundant pathways are present in the small coronary arterioles, but in large arterioles inhibition of NOS alone (14) or together with cyclooxygenase (Fig. 2) is sufficient.

Our data are consistent with the hypothesis that ACh-induced vasodilation responses in the small arterioles may, at least in part, be mediated by endothelium-derived, cytochrome P-450 metabolites of AA. This is further supported by our results with inhibition of AA-induced vasodilation. Epoxycosatrienoic acids are cytochrome P-450 monooxygenase metabolites of AA that are released by endothelial open calcium-activated potassium channels and produce hyperpolarization in vascular smooth muscle cells, resulting in arterial vasodilation (3, 11, 12). Although we did not measure vascular smooth muscle cell membrane potential, it is likely that endothelium-dependent hyperpolarization accompanied ACh-induced vasodilation and was inhibited by the combination of NOS and cytochrome P-450 monooxygenase blockade.

The time-course experiments raise the possibility that ACh induces vasodilation in both small and large arterioles through mechanisms that are insensitive to the inhibitors tested in this study. Whereas the combination of NOS and cytochrome P-450 enzyme blockade returned the vessel diameters nearly to baseline by 10 min, significant dilator responses were observed at 1 min in all groups (Table 4). It is unlikely that this observation was due to inadequate exposure to blocking agents, because these were applied for at least 30 min before ACh exposure and this finding was consistent for each dose of ACh (1, 10, and 100 µM). Because ACh was administered topically, alterations in hemodynamics in response to administration of ACh were not observed and are an unlikely cause of this observation. The possibility of rapid tachyphylaxis to ACh could be considered but is unlikely, because all three doses of ACh produced vasodilation at 1 min. Further experiments using other agonists that cause endothelium-derived relaxation are required to further determine the mechanism of this observation.

In summary, our study suggests that ACh-induced vasodilation of small coronary arterioles is mediated in part by a cytochrome P-450 metabolite of AA. Moreover, these data provide the first evidence that the cytochrome P-450 pathway contributes to the regulation of coronary resistance vessels in vivo.

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