Vascular Signaling by Free Radicals

Reactive oxygen species mediate arachidonic acid-induced dilation in porcine coronary microvessels

Christine L. Oltman,1,2 Neal L. Kane,1 Francis J. Miller, Jr.,1,3 Arthur A. Spector,1,4 Neal L. Weintraub,1,2,3 and Kevin C. Dellsperger1,2

Department of Internal Medicine and the Cardiovascular Center,1 University of Iowa, and Department of Veterans Affairs,2 Iowa City, 52246; and Free Radical and Radiation Biology Program3 and Department of Biochemistry,4 University of Iowa College of Medicine, Iowa City, Iowa 52242

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Oltman, Christine L., Neal L. Kane, Francis J. Miller, Jr., Arthur A. Spector, Neal L. Weintraub, and Kevin C. Dellsperger. Reactive oxygen species mediate arachidonic acid-induced dilation in porcine coronary microvessels. Am J Physiol Heart Circ Physiol 285: H2309–H2315, 2003. First published July 17, 2003; 10.1152/ajpheart.00456.2003.—Reactive oxygen species (ROS) are mediators of microvascular dilation. Porcine epicardial coronary arterioles (110 ± 4 μm diameter) were mounted onto pipettes in oxygenated Krebs buffer. Vessels were incubated with vehicle or 1 mM Tiron (a nonselective ROS scavenger), 250 μM polylethylene-glycolated (PEG)-superoxide dismutase (SOD; an O2 scavenger), 250 μM PEG-catalase (a H2O2 scavenger), or the cyclooxygenase (COX) inhibitors indomethacin (10 μM) or diclofenac (10 μM) for 30 min. After endothelin constriction (30–60% of resting diameter), cumulative concentrations of AA (10−10 to 10−5 M) were added and internal diameters measured by video microscopy. AA (10−7 M) produced 37 ± 6% dilation, which was eliminated by the administration of indomethacin (4 ± 7%, P < 0.05) or diclofenac (−8 ± 8%, P < 0.05), as well as by Tiron (−4 ± 5%, P < 0.05), PEG-SOD (−10 ± 6%, P < 0.05), or PEG-catalase (1 ± 4%, P < 0.05). Incubation of small coronary arteries with [3H]AA resulted in the formation of prostaglandins, which was blocked by indomethacin. In separate studies in microvessels, AA induced concentration-dependent increases in fluorescence of the oxidant-sensitive probe dichlorodihydrofluorescein diacetate, which was inhibited by pretreatment with indomethacin or by SOD + catalase. We conclude that in porcine coronary microvessels, COX-derived ROS contribute to AA-induced vasodilation.

cyclooxygenase; coronary microcirculation

The vascular endothelium generates a number of vasoactive agents that are important in the regulation of coronary blood flow. These putative agents may be produced through several enzyme pathways, including nitric oxide synthase (NOS), cyclooxygenase (COX), lipoxygenase (LOX), and the cytochrome P-450 monoxygenase (CYP-450) systems. Responses have been determined to be dependent on vessel size, animal species, and regional circulation. Our group reported that in the dog, arachidonic acid (AA)-induced dilatory responses in the coronary microcirculation are mediated through redundant pathways (15). However, Hein and colleagues (7) demonstrated that in the porcine coronary microcirculation, dilation may be mediated through COX.

Several groups have proposed that reactive oxygen species (ROS) are mediators of microvascular dilation (1, 12, 18). Putative ROS mediators include superoxide anions, hydrogen peroxide, and hydroxyl radicals. Metabolism of AA by COX, LOX, or CYP-P450 may be associated with the production of oxygen-derived free radicals (5, 8). In the microcirculation of the brain, ROS derived through metabolism of AA have been suggested to mediate vasodilatory responses to bradykinin (3).

Mediators of AA-induced relaxation in the coronary microcirculation remain uncertain. Given the interrelationship between metabolic pathways of AA and the production of ROS, we proposed the hypothesis that ROS contribute to AA-induced coronary microvascular dilation. To test this hypothesis, we studied isolated porcine coronary microvessels in a pressurized in vitro system to evaluate the role of ROS using pharmacological techniques. In addition, we coupled our pharmacological approaches with biochemical studies and with fluorescence microtopography to evaluate the source of ROS responsible for the dilatory responses.

MATERIALS AND METHODS

Microvessel procurement. Porcine (male and female) hearts were obtained from a local abattoir. The hearts were quickly harvested and immediately placed in cold (4°C), oxygenated Krebs bicarbonate buffer solution (see details under Isolated microvessels) for dissection.

Isolated microvessels. A standard in vitro pressurized arteriole preparation was used to study coronary microvessels (15, 16). Ventricular microvessels 75–175 μm intraluminal
diameter and ~1 mm in length were carefully removed from the myocardium, cleaned with the aid of a dissecting microscope, and placed in an organ chamber. Each end of the microvessel was cannulated with a glass micropipette and secured with 10-0 ophthalmic suture. The organ chamber was placed on the stage of an inverted microscope. Attached to the microscope were a video camera, a video monitor, and a calibrated video caliper. The organ chamber was connected to a rotary pump that continuously circulated oxygenated Krebs buffer. Krebs-Henseleit solution contained (in mM) 120.0 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 23.0 NaHCO3, 1.2 KH2PO4, 11.0 glucose, and 0.025 EDTA. Solutions were aerated with 20% O2-5% CO2-75% N2 and maintained at 37°C with pH at 7.4. An image of the microvessel was displayed on the video monitor, and intraluminal diameters were measured by manually adjusting the video micrometer. The resolution of the system allowed measurement of very small (1–2 μm) changes in vessel diameter.

Microvessels were allowed to equilibrate for 30 min at a hydrostatic distending pressure of 60 mm Hg under conditions of no flow. KCl (75 mM) was added to the bath to test mobilization and metabolism, and after 30 min, the Krebs buffer, vessel diameter returned to baseline. Endothelin-1 (0.40–1.2 nM) was used to constrict the microvessels to 30–60% of their resting diameter. Cumulative concentration–response relationships were evaluated for AA (10−10 to 10−5 M) 5,8,11,14-eicosatetraynoic acid (ETYA; 10−10 to 10−5 M), PGE2 (10−10 to 10−5 M), or H2O2 (10−7 to 10−2 M) by adding the drug directly to the organ bath. A single dose of SNP (10−4 M) was given at the end of each experiment to determine maximal diameter.

Role of endothelial cells in AA-induced microvascular dilation. Mechanical endothelium denudation was achieved by rubbing the lumen of the vessel with a small wire and confirmed by lack of a dilator response to bradykinin.

Role of COX pathway on AA-induced microvascular dilation. To examine the mechanisms responsible for AA-induced dilation in isolated porcine coronary arterioles, several inhibitors were utilized. Microvessels were incubated with inhibitors specific for the COX pathway for 30 min before being preconstricted with endothelin. Inhibitors included indomethacin (10 μM) or diclofenac (10 μM) to evaluate the role of the COX pathway. AA concentration response studies were then performed.

Role of ROS on AA-induced microvascular dilation. Microvessels were incubated with a nonspecific ROS inhibitor Tiron (1 mM), an O2− scavenger superoxide dismutase (SOD, 250 U/ml), or an H2O2 scavenger catalase (250 U/ml) for 30 min before being preconstricted with endothelin. Polyethylene glycol (PEG) was used as a carrier for SOD and catalase. Separate studies with PEG showed no effects on coronary microvascular dilation to AA (data not shown).

Metabolism of AA by porcine coronary arterioles. Small porcine coronary arteries (~250 μm in diameter) were dissected, placed in test tubes containing 1 ml of Krebs buffer supplemented with 0.1 μM fatty acid-free bovine serum albumin, and maintained in a 5% CO2 incubator (37°C). After 1 h, vehicle or indomethacin (10 μM) was added. One hour later, the Krebs buffer was removed and replaced with fresh buffer containing 1.7 μM [3H]AA along with vehicle or indomethacin. After 1 h, A-23187 (2 μM) was added to induce AA metabolism. After another 30 min, the incubation was terminated by removal of the Krebs buffer.

The radioactivity present in the Krebs buffer after the incubation was measured by liquid scintillation counting. Lipids contained in the medium were extracted twice with 10 ml of ice-cold, water-saturated ethyl acetate. Phase separation was achieved by centrifugation at 600 g for 10 min. The top phases (ethyl acetate) were transferred to new tubes. Fifty-microliter aliquots were removed from the aqueous phases and assayed to determine the amount of radioactivity not extracted. Because of low extraction efficiency, the medium was acidified to pH 4 by using 15 ml of formic acid and reextracted by using two 6-ml aliquots of water-saturated ethyl acetate. The ethyl acetate was evaporated to dryness under N2 and resuspended in 200 ml of CH3CN.

The lipids were separated by reverse-phase HPLC by using a Gilson dual-pump gradient system equipped with model 306 pumps, a model 117 dual wavelength UV detector, a model 231 XL automatic sample injector (Gilson Medical Electronics), and a 5-μm 4.6 × 150-mm Discovery C18 column obtained from Supelco. The elution profile consisted of water adjusted to pH 4.0 with formic acid and an acetonitrile gradient that increased from 30 to 57% over 60 min and then from over 25 min and from 57 to 65% over 25 min, at which time the acetonitrile was taken to 100% and held constant for 15 min. The distribution of radioactivity was measured by combining the column with scintillator solution and passing the eluate through an online flow detector (IN/US Systems) (11, 20, 22).

Detection of ROS. The fluorogenic probe 5-(and 6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to detect intracellular ROS. Small porcine coronary arteries (~250 μm in diameter) were incubated in DCFH (2 μM diluted in Krebs buffer) at 37°C for 15 min, and then AA or vehicle was added for an additional 15 min. Some vessels were pretreated with indomethacin (10 μM) or SOD (250 U/ml) and catalase (250 U/ml) for 15 min before the addition of DCFH. Vessels were washed and placed onto concave microscope slides (Great Scopes; Jamestown, NC) containing Krebs buffer and covered with a coverslip. Images were obtained with a Bio-Rad laser scanning confocal microscope (excitation 488 nm and detection of fluorescence at 525 nm). Images of intact vessels were taken and represent changes in fluorosceint throughout the vessel wall. Addition of AA to DCFH in a cell-free system did not generate fluorescence (model LS50B, Perkin-Elmer Luminescence Spectrometer; Perkin-Elmer Life Sciences, Boston, MA).

Solutions and drugs. Endothelin-1 was purchased from Phoenix Peninsula Laboratories (San Carlos, CA). AA (sodium salt) was purchased from NuChek Prep. [3H]AA was purchased from Perkin-Elmer Life Sciences. DCFH was purchased from Molecular Probes (Eugene, OR). SNP, indomethacin, and other chemical agents were purchased from Sigma Chemical (St. Louis, MO). All solutions and vasoactive agents were freshly prepared on the day of the experiment. SNP was dissolved in Krebs-Henseleit buffer, whereas AA and indomethacin were dissolved in saline.

Statistical analysis. Data are expressed as means ± SE. SigmaStat software (Jandel Scientific) was used for statistical analyses. All concentration-response curves were evaluated for differences using two-way repeated-measures ANOVA, followed by Fisher’s least-significant difference correction for multiple comparisons. Differences with P < 0.05 were considered significant.

RESULTS

AA produced concentration-dependent dilation of porcine coronary microvessels (Fig. 1). The role of endothelial cells in AA-induced microvascular dilation was examined by using denuded vessels. Mechanical endothelium denudation was achieved by denuding the vessel with a small wire and confirmed by the lack of a
dilator response to bradykinin (1 μM, 2.9 ± 1.9% in denuded vessels). AA caused a slight constriction of denuded vessels (1 μM AA, 10.3 ± 10.2%) compared with 49.4 ± 5.4% dilation in intact arterioles (n = 5, *P ≤ 0.05). These findings confirm an obligatory role for the endothelium in AA-induced dilation of porcine coronary microvessels.

We next tested the effects of ETYA (10−10 to 10−5 M), a 20-carbon AA analog that has triple bonds at the 5-, 8-, 11-, and 14-carbons, and therefore is not a substrate for oxygenation via COX, LOX, or CYT-450. Dilation to ETYA was minimal compared with the AA-induced dilation (Fig. 1). This finding suggests that enzymatic metabolism is required for AA-induced dilation of porcine coronary microvessels.

Metabolism of [3H]AA by small porcine coronary arteries. To investigate the metabolism of AA, we initially performed biochemical studies by using ventricular microvessels of the same size used in the functional studies. Despite pooling many microvessels together, we were unable to detect AA metabolites under the incubation conditions described in Materials and Methods presumably because we were not able to obtain a sufficient mass of microvascular tissue. Thus studies were performed using small epicardial porcine coronary arteries (250 μm in diameter). Pooled arteries (70 μg wet wt) were incubated with [3H]AA, after which lipids in the incubation medium were extracted and analyzed by reverse-phase HPLC. Metabolites with retention times consistent with prostaglandins were detected in two independent experiments using vessels obtained from different animals; we did not detect metabolites that comigrated with LOX or CYP-450 products of AA (Fig. 2). Treatment with 10 μM indomethacin abolished the production of prostaglandins.

To determine whether the prostaglandins generated by these vessels are capable of dilating porcine coronary arterioles, we tested responses to PGE2 in endothelin-preconstricted arterioles. As shown in Fig. 2D, PGE2 produced potent concentration-dependent dilation of porcine coronary microvessels.

Role of metabolic pathways on AA-induced microvascular dilation. The role of the COX pathway in AA-induced microvascular dilation was next examined by using indomethacin and diclofenac. AA-induced dilation was eliminated in the presence of either of these COX inhibitors (Fig. 3). These data suggest that AA-induced dilation of porcine coronary microvessels is...
mediated through the COX pathway, in contrast to findings in conduit porcine coronary arteries (9, 21).

**Role of ROS on AA-induced microvascular dilation.**

COX-derived ROS have been suggested to mediate bradykinin-induced dilation of the cerebral microvasculature (3). The role of a nonselective ROS scavenger on AA-induced microvascular dilation was therefore tested. Microvessels were incubated with Tiron (1 mM) for 30 min before being preconstricted with endothelin. Concentration-response studies with AA were then performed. Tiron blocked AA-induced dilation (dilation at 10 μM AA: control: 69 ± 4 vs. Tiron treated: 22 ± 11%, n = 14, P ≤ 0.05) but had no effect on dilation to SNP (data not shown). These data suggest that AA-induced vasodilation in porcine coronary microvessels is mediated by ROS.

To examine the potential role of O₂⁻ and H₂O₂ in AA-induced microvascular dilation, microvessels were incubated with PEG-conjugated SOD (PEG-SOD, 250 U/ml) or PEG-catalase (250 U/ml) for 30 min before being preconstricted with endothelin. Concentration-response studies with AA were then performed. Both PEG-SOD and PEG-catalase attenuated AA-induced dilation (Fig. 4). SNP (10⁻⁴ M) induced 89.4 ± 3.7% and 99.2 ± 2.7% dilation in the SOD and catalase groups, respectively. These data suggest that both O₂⁻ and H₂O₂ play a role in AA-induced vasodilation in porcine coronary microvessels.

Because catalase blocked AA-induced dilation, we examined the capacity of H₂O₂ to dilate coronary microvessels. H₂O₂ completely dilated endothelin-preconstricted microvessels (EC₅₀: 35 ± 9 μM, 102 ± 1% maximal relaxation, n = 5).

The role of ROS in AA metabolism in coronary microvessels was also studied by using the fluorogenic probe DCFH (2 μM). Confocal photomicrographs were taken of intact vessels after 30 min incubation with AA. Increasing concentrations of AA produced increasing fluorescence in coronary microvessels, indicating a concentration-dependent increase in ROS (Fig. 5).

To further study the microvascular generation of ROS by AA, porcine coronary microvessels were evaluated in the presence of AA (10⁻⁶ M) along with indomethacin (10 μM) or the combination of SOD (250 U/ml) + catalase (250 U/ml) for 30 min. Microvascular ROS was then examined by DCFH fluorescence. Treatment with indomethacin alone or with the combination of SOD + catalase attenuated AA-induced fluorescence (Fig. 6).

**DISCUSSION**

Our findings show that in isolated porcine coronary microvessels, AA-induced relaxation is dependent on COX metabolism and the generation of ROS. These findings may have important implications with regard to coronary blood flow regulation, particularly during pathological states such as ischemia, where both AA release and ROS production are prominent (2).

Vascular endothelial cell COX metabolizes AA to prostaglandins and thromboxanes. These compounds can also modulate coronary arterial tone (Fig. 2D). In prior studies by our group, AA-induced relaxation of conduit porcine coronary arteries was not blocked by treatment with 10 μM indomethacin, although the compound effectively blocked prostaglandin I₂ formation (9, 21). However, Hein et al. (7) demonstrated that inhibitors of COX blocked AA-induced dilation of porcine coronary microvessels. Our data confirm these results and suggest that the endothelium is the likely site of COX metabolism, considering the lack of AA-induced dilation in denuded vessels in this study as well as the study by Hein et al. The endothelium was also shown to be required for AA-induced dilation of human coronary arterioles (13). Consistent with this latter study, we also show that ETYA did not produce significant relaxation in porcine coronary arterioles. This confirms that dilation to AA is mediated through enzymatic metabolism of AA.
Besides COX, LOX and CYP-450 monooxygenase enzymes can metabolize AA to produce ROS as well as eicosanoid metabolites capable of inducing vasorelaxation. In particular, the CYP-450 and LOX pathways were suggested to be functionally active in conduit porcine coronary arteries and porcine coronary microvascular endothelial cells, respectively (6, 4, 22). Furthermore, the notion that CYP-450-derived metabolites of AA are putative endothelium-derived hyperpolarizing factors has arisen in part from work performed in porcine conduit coronary arteries. In those prior studies, COX inhibitors failed to block AA-induced vasorelaxation of conduit porcine coronary arteries. However, in the present study, two different chemical inhibitors of COX totally blocked AA-induced dilation of porcine coronary microvessels. Moreover, biochemical studies demonstrated the formation of only COX metabolites of AA in small coronary arteries, consistent with the pharmacological studies in microvessels.

It must be emphasized that the biochemical studies were conducted in small coronary arteries \( \approx 250 \ \mu \text{m} \) in diameter; we cannot exclude the possibility that LOX and/or CYP-450 metabolites could be generated in smaller arterioles. Also, it is possible that other agonist compounds, such as bradykinin or substance P, might stimulate the production of non-COX metabolites of AA. Nevertheless, our functional studies suggest that the dilatory responses to AA in porcine coronary microvessels are mediated entirely by COX metabolism. By inference, neither CYP-450 monooxygenase nor LOX enzymes appear to be necessary for AA-induced dilation of porcine coronary microvessels.

Metabolism of AA via COX is known to generate ROS (8), and ROS generated by COX have been proposed to mediate bradykinin-induced vasodilation in the cerebral microcirculation (3). Several recent studies have demonstrated the importance of endogenously produced H₂O₂ as a mediator of vasodilatory responses in vitro and in vivo (1, 12, 19). In the present study, we found that Tiron, SOD, or catalase attenuated AA-mediated coronary microvascular relaxation. In contrast, the study by Pomposiello et al. (17) did not show an effect of ROS scavengers on AA-induced relaxation in porcine conduit coronary arteries. Because vasomotor responses are related to the size of the vessel studied (14), this suggests that ROS may be of greater importance in the microcirculation than in conduit arteries. Our results with DCHF clearly demonstrate...
that AA induces microvascular ROS production in a dose-dependent manner (Fig. 5). Furthermore, inhibition of COX, which blocked the in vitro dilation to AA and the formation of AA metabolites (Figs. 2 and 3) also blocked the production of ROS (Fig. 6). To our knowledge, no other studies have demonstrated pharmacological inhibition of ROS production using fluorescent microtopography coupled with biochemical and functional responses in the coronary microcirculation. Together, these findings provide strong support for COX-derived ROS as mediators of AA-induced dilation of the coronary microcirculation.

The species of ROS that produce the vasodilation to AA remain(s) to be determined. We have shown that H$_2$O$_2$ produces potent vasodilation in porcine coronary microvessels. This is similar to results observed in other vascular beds (1, 10). In keeping with this notion, we found that treatment with catalase, a scavenger of H$_2$O$_2$, diminished DCHF fluorescence and dilation induced by AA in porcine coronary microvessels. However, if H$_2$O$_2$ were solely responsible for producing the dilation, it is unlikely that the responses could be blocked by treatment with SOD. Thus our findings raise the possibility that both O$_2^-$ and/or other ROS contribute to AA-induced coronary microvascular dilation. Whether there is an interaction between O$_2^-$ and H$_2$O$_2$ and/or other ROS remains to be determined. Also, the mechanism by which ROS-mediated dilation occurs remains to be established. Barlow et al. (1) suggested the mechanism may be related to activation of K$^+$ channels. Likewise, Hein et al. (7) showed that K$^+$ channel inhibitors blocked AA-induced dilation in porcine coronary arterioles. One can speculate that the ROS act ultimately via a K$^+$ channel-mediated response.

SOD or catalase attenuated AA-induced dilation but did not completely block the dilation at the higher concentrations. The combination of an SOD-mimetic [Mn(III)tetrakis(4-benzoic acid) porphyrin chloride, 10 $\mu$M] and PEG-catalase (250 U/mL) did not further inhibit AA-induced dilation (data not shown). These findings suggest that ROS are only partially responsible for the AA-induced dilation, and that another dilatory factor is likely involved. This factor may be PGE$_2$ because it is produced in small porcine coronary arterioles (Fig. 2B) and is a potent dilator of porcine coronary microvessels (Fig. 2D).

We conclude that in porcine coronary microvessels, ROS contribute to AA-induced relaxation through a COX-dependent pathway. These findings suggest that COX-derived ROS may play an important role in the regulation of coronary blood flow in physiological states.

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**DISCLOSURES**

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