Synthesis of Lipoxygenase and Epoxigenase Products of Arachidonic Acid by Normal and Stenosed Canine Coronary Arteries

Mark Rosolowsky, J.R. Falck, James T. Willerson, and William B. Campbell

Coronary vascular injury promotes blood cell–vessel wall interactions that influence arachidonic acid metabolism and coronary blood flow patterns. Since lipoxygenase and cytochrome P-450 epoxigenase metabolites of arachidonic acid are synthesized by vascular and inflammatory cells and have a variety of important biological actions, we investigated the metabolism of arachidonic acid by these pathways in normal and stenosed, endothelially injured canine coronary arteries. We found and confirmed by gas chromatography/mass spectrometry that primarily 12- and 15-hydroxyeicosatetraenoic acids (HETEs) are synthesized by both coronary artery segments. Lesser amounts of 11-, 9-, 8-, and 5-HETEs are also produced. 15-Ketoepoxicosatetraenoic acid is also synthesized. The synthesis of 14C-HETEs is fivefold to 10-fold greater by the stenosed than the normal coronary artery. Specific radioimmunoassays indicated that the stenosed coronary artery synthesized 93±14 and 1,102±154 ng/g of tissue of 15- and 12-HETE, respectively, while the normal coronary artery produced 17±3 and 162±68 ng/g of tissue of 15- and 12-HETE, respectively. Products comigrating with 14,15-; 11,12-; 8,9-; and 5,6-epoxyeicosatrienoic acids (EETs) and the corresponding dihydroxyeicosatrienoic acids (DHETs) were detected predominantly in stenosed coronary arteries by high-pressure liquid chromatography. The structures of the EETs were confirmed by GC/MS. The EETs and prostaglandin I2 produced endothelium-independent, concentration-related relaxations of dog coronary artery rings. These data indicate that normal and stenotic coronary arteries metabolize arachidonic acid to HETEs, DHETs, and EETs along with prostaglandins; however, the synthesis of these metabolites is greater in the stenosed, endothelially injured vessel. The EETs may be synthesized during the development of cyclic flow variations and counteract the vasoconstrictor effects of thromboxane A2. (Circulation Research 1990;66:608–621)

Metabolites of arachidonic acid are important mediators of inflammation, platelet aggregation, and vascular tone. The balance between platelet thromboxane A2 (TXA2) and vascular prostacyclin (PGI2) production has been proposed to regulate vessel patency in vascular disease.1–3 To examine this association, we used a canine model of concentric coronary artery stenosis. This model is characterized by cyclic flow variations that are spontaneous decreases in coronary blood flow followed by sudden returns of flow to normal. This laboratory has demonstrated that stenosed left anterior descending (LAD) canine coronary arteries synthesized less PGI2 than normal coronary arteries. In contrast, TXB2, a metabolite of TXA2, was present in these stenosed and damaged vessels but was absent from normal coronary arteries.4 In stenotic vessels, the endothelial layer was missing, platelets adhered to the lumen, and leukocytes infiltrated into the media of the vessel. These histological changes appeared to account for the alterations in TXA2 and PGI2 synthesis. In 70% of the animals, a thromboxane-synthetase inhibitor or thromboxane-receptor blocker reduced spontaneous declines in coronary blood flow induced by the stenosis, indicating that TXA2 plays a role in the cyclic flow patterns in this model.5

There is increasing evidence that lipoxygenase or cytochrome P-450 epoxigenase products of arachidonic acid are released from vascular and inflamma-
tory cells and exhibit properties that may influence thrombus development and vascular tone. For example, hydroperoxyeicosatetraenoic acids (HPETEs) were shown to contract or relax blood vessels in a number of species. These effects may be due to inhibition of PG12 synthase or additional mechanisms, such as release of intracellular calcium. The hydroxyeicosatetraenoic acids (HETEs), metabolites of HPETEs, promote leukocyte and smooth muscle cell migration. Leukotriene (LT) B4 and 5-HETE, metabolites of the 5-lipoxygenase pathway, were found to stimulate granule release and induce chemotaxis of neutrophils. The epoxyeicosatrienoic acids (EETs) are products of the cytochrome P-450 epoxygenase pathway. Several of these compounds have been reported to inhibit Na+,K+-ATPase, mobilize calcium, dilate microvessels, and inhibit platelet aggregation. Therefore, these lipoxygenases and cytochrome P-450 epoxygenase products have the potential to influence the development of occlusive thrombi in vascular disease.

In the present study, we investigated the metabolism of arachidonic acid in normal canine coronary arteries. Emphasis was placed on identifying metabolites of the lipoxygenase and cytochrome P-450 epoxygenase pathways and determining the influence of these metabolites on coronary vascular tone and platelet aggregation in vitro. Additionally, a comparison of arachidonic acid metabolism was made between normal and stenotic vessels.

Materials and Methods

Tissue Incubations

Mongrel dogs (20–30 kg) were anesthetized with sodium pentobarbital (30 mg/kg i.v.) before being placed on a respirator and ventilated with room air. Catheters were inserted into the carotid artery to monitor blood pressure and into the femoral vein to administer supplemental anesthetic. A thoracotomy was performed at the fifth intercostal space, and the heart was suspended in a pericardial cradle. In 42 dogs, a segment of the LAD coronary artery was exposed by dissection, and a Doppler flow probe was placed around the vessel. Control hemodynamic measurements (heart rate, arterial blood pressure, and mean and phasic coronary blood flow) were obtained before a hard plastic cylindrical constrictor was placed distal to the flow probe. Gentle pinching of the coronary artery with rubber-clad vascular forceps and application of the constrictor were associated with endothelial injury, platelet adherence, and leukocyte infiltration as shown by light and electron microscopy. This manipulation induced regular patterns of spontaneous declines in coronary blood flow that were partly due to platelet aggregates formed at the site of stenosis and distal vasoconstriction. Tapping the area distal to the constrictor dislodged the platelet thrombus and restored coronary blood flow to baseline without affecting the flow probe or constrictor. These cyclic blood flow patterns continued for 3 hours before the heart was excised during a nadir in coronary blood flow and placed in 0.9% sodium chloride solution. The damaged LAD segment that was circumscribed by the constrictor and the left circumflex coronary artery were dissected and cleared of connective tissue and fat. No attempt was made to remove the adherent platelet thrombus from the LAD segment. The vessels were placed in 10 mM HEPES buffer containing 150 mM NaCl, 5 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5.5 mM glucose and were sectioned into small rectangles (approximately 15 mm2) with a razor blade. This procedure did not disrupt the endothelium as indicated by light microscopy. The stenosed LAD or normal left circumflex coronary artery was then immersed in 1 ml fresh HEPES buffer and incubated with 14C-arachidonic acid (1 μCi, 1 Ci/mmol) and 50 μM unlabeled arachidonic acid for 5 minutes at 37°C before the calcium ionophore A23187 (20 μM) was added. The incubation proceeded for another 10 minutes before the tissue was removed, blotted dry, and weighed. The incubation buffer was removed and stored frozen (−40°C) until analyzed. To obtain a radioactive profile of arachidonic acid metabolism, equivalent weights of stenosed LAD and normal left circumflex coronary arteries of one dog were incubated. This was repeated with similar results three times with tissue from separate dogs. In contrast, to obtain adequate mass for gas chromatography/mass spectrometry (GC/MS) analysis, the incubation media of the stenosed LAD or the entire normal left circumflex coronary artery from six dogs were pooled. This resulted in approximately 12 times more normal vessel than stenosed coronary artery being incubated (1.0 vs. 0.08 g). This experiment was repeated three times (18 additional dogs) with similar results. In a separate group of four dogs without cyclic flow variations, normal left circumflex coronary arteries were dissected and pretreated with a cyclooxygenase inhibitor (indomethacin, 10 μM), a combined cyclooxygenase/lipoxygenase inhibitor (BW755c, 50 μM), or a cytochrome P-450 inhibitor (metyrapone, 50 μM) for 10 minutes in 1 ml of 10 mM HEPES buffer before incubation with 14C-arachidonic acid and A23187 at 37°C. Parallel incubations were carried out under identical conditions, but in the absence of coronary tissue, to evaluate the possibility of nonenzymatic metabolism of arachidonic acid.

Extraction and Chromatographic Methods

Incubation media was acidified to pH 3.0 with glacial acetic acid, treated with ethanol to a final concentration of 15%, and extracted over 6 ml octadecylsilylextraction columns (Analytichem, Harbor City, California), as previously described. The eluate was dried under a stream of nitrogen and then dissolved in acetonitrile and chromatographed on a Unimetrics C-18 column (5 μm, 4.6×250 mm) using a liquid chromatograph (model 431, Beckman Instruments, Fullerton, California). In solvent program 1, solvent A was distilled water, and solvent B contained acetonitrile/glacial acetic acid (999:1). A linear gradient from 50% solvent B in solvent A to...
100% solvent B over 40 minutes was used at a flow rate of 1 ml/min. A portion of the sample was chromatographed. The effluent was collected in fractions (five per minute) and mixed with scintillation fluid, and the radioactivity was measured by liquid scintillation spectrometry to obtain a profile of radioactive metabolites. The remainder of the sample was immediately chromatographed under the same conditions, and the radioactive peaks comigrating with the prostaglandins (PGs) (fractions 10–50), diHETE/dihydroxyeicosatrienoic acid (DHET) (fractions 50–105), HETE (fractions 105–130), and EET (fractions 130–155) standards were collected. These fractions were then extracted with 2 vol cyclohexane/ethyl acetate (1:1). The combined organic extracts were dried under a stream of nitrogen. Solvent program 2 was used to rechromatograph the PG fraction. Solvent A contained 0.025 M phosphoric acid in water and solvent B was 100% acetonitrile. An isotropic elution was used with 31% solvent B in solvent A at a flow rate of 1 ml/min. Prostaglandin standards were injected with the sample, and the absorbance was monitored at 192 nm. Column eluate was collected in 0.5 ml fractions, and radioactivity was measured in a Beckman liquid scintillation counter. The other fractions were then rechromatographed on the appropriate normal phase high-pressure liquid chromatography (HPLC) system on an Ultrasphere-Si silica column (5 μm, 4.6×250 mm, Altex), using a Beckman 421 chromatograph with a diode array ultraviolet (UV) detector (model 1040A, Hewlett-Packard, Palo Alto, California). The diHETE/DHET fraction was resolved with a mobile phase of 1% to 5% isopropanol in hexane/glacial acetic acid (999:1) over 50 minutes at a flow rate of 3 ml/min (solvent program 3). The HETE fraction was resolved with a gradient from 0.5% to 1.5% isopropanol in hexane/glacial acetic acid (999:1) over 40 minutes at 3 ml/min (solvent program 4). The EET fraction was resolved using an isotropic mobile phase of hexane/isopropanol/glacial acetic acid (995:4:1) at a flow rate of 2 ml/min (solvent program 5). Fractions of the column eluate were collected every 0.2 minutes for all normal phase HPLC methods, and the radioactivity was determined in aliquots of these fractions. For solvent programs 3–5, UV absorbance spectra from 200 to 350 nm were obtained every 6 seconds from the column eluate.

In a separate group of four animals that underwent cyclic flow variations, incubations were performed to examine whether LTs were synthesized by stenosed and normal coronary arteries. The incubation buffer from these experiments was extracted over octadecylsilyl extraction columns that were washed sequentially with methanol, water, and 0.1% EDTA before sample addition. The LTs were eluted with 10 ml methanol, and the solvent was removed under a stream of nitrogen at room temperature. The extract was dissolved in methanol and chromatographed on a Nucleosil C18 column (5 μm, 4.6×250 mm, Phenomenex, Torrance, California). For this solvent program (program 6), solvent A contained water/acetic acid (999:1) adjusted to pH 5.6 with ammonium hydroxide. Solvent B was 100% acetonitrile. A linear gradient from 30% solvent B in solvent A to 100% solvent B over 45 minutes was used at a flow rate of 1 ml/min. Ultraviolet absorbance was monitored at 280 nm.

Gas Chromatography/Mass Spectrometry

The radioactive peaks from the HETE fraction were collected, and the solvent was removed under a stream of nitrogen. These metabolites were esterified with ethereal diazomethane at 4°C for 20 minutes. The ether was evaporated under a stream of nitrogen, and the residue silylated with bis(trimethylsilyl)-trifluoroacetamide (BSTFA) (Supelco, Bellefonte, Pennsylvania) in acetonitrile for 18 hours at 40°C. The material comigrating with 15-ketoeicosatetraenoic acid (15-KETE) was incubated for 12 hours at room temperature with 2% methoxyamine hydrochloride (Pierce Chemical, Rockford, Illinois) in pyridine to form the methoxime. After removal of the pyridine under a stream of nitrogen, the product was dissolved in ether and washed three times with water. The methyl ester was formed as described above. These samples were subjected to GC/MS analysis with a Finnigan 4500 Series gas chromatograph/mass spectrometer (San Jose, California) using a 6-foot glass column packed with 3% SP 2100-DOH on Supelcoport (Supelco). The carrier gas was nitrogen at a flow rate of 20 ml/min, and the column temperature was 210°C. Electron impact spectrometry was performed at an ionization energy of 70 eV. Fragmentation patterns and elution times were compared with similarly derivatized HETE and 15-KETE standards. The radioactive metabolites of the EET fraction were dissolved in acetonitrile containing pentafluorobenzyl bromide (Pierce Chemical) and N,N-disopropylethylamine (10:1:5 vol/vol/vol) for 20 minutes at room temperature. The solvents were removed under a stream of nitrogen. The residue was filtered through a column of silicic acid using methylene chloride. The EET pentafluorobenzyl esters were analyzed using a 14 meter DB-5 column (J&W Scientific, Georgetown, Texas) with a linear gradient from 190°C to 340°C at 8°C/min. Negative ion chemical ionization mass spectroscopy was performed with methane as the reagent gas.

Radioimmunoassays

To quantitate endogenous production of 12-HETE and 15-HETE, segments of normal circumflex or stenosed LAD coronary arteries of 10 dogs were dissected free of myocardium, cleaned, sectioned, and incubated with 20 μM A23187 in 1 ml HEPES buffer at 37°C for 30 minutes. The media was decanted and frozen in a methanol/dry ice bath. The vessels were then blotted dry and weighed. The arachidonic acid metabolites in the unextracted samples were quantitated by specific radioimmuno-
Specific antibodies were raised in rabbits against 12-HETE-bovine serum albumin or 15-HETE-thyroglobulin complex. The 12-HETE antiserum (kindly provided by Dr. L. Levine, Brandeis University, Waltham, Massachusetts) was sensitive to 5 pg of 12-HETE and produced 50% displacement of [3H]-12-HETE with 30 pg. It cross-reacted 100% with 12-HETE, 1% with 5-HETE, 5% with LTb4, 0.001% with arachidonic acid, and less than 0.001% with other HETEs, DHETs, PGs, or diHETEs. The 15-HETE antibody was sensitive to 5 pg 15-HETE and had a 50% displacement of [3H]-15-HETE with 30 pg. This antibody cross-reacted 100% with 15-HETE, 0.2% with 8,15-diHETE, 0.15% with 5,15-diHETE, 0.02% with 5-HETE, and less than 0.001% with other HETEs, DHETs, PGs, or diHETEs.

**Biological Activity of Metabolites**

Platelet aggregation was measured by the turbidometric method of Born. Canine blood was collected in a polypropylene tube containing 3.2% sodium citrate (9:1 vol/vol). The blood was centrifuged at 100g for 10 minutes, and the platelet-rich plasma was removed. The blood was then centrifuged for 10 minutes at 1,500g to obtain platelet-poor plasma. Aggregation studies were performed with a dual channel Sienco aggregometer (Morison, Colorado) balanced against platelet-rich and platelet-poor plasma for 0 and 100% light transmission, respectively. Metabolites were added to the cuvettes in ethanol, and the vehicle was evaporated under a stream of nitrogen before platelet-rich plasma (0.4 ml) was added. Platelet aggregation of platelet-rich plasma was induced by collagen. The amount of collagen given to aggregate platelets ranged between 1 and 3 μg/0.4 ml and was chosen to yield approximately 60% light transmission.

For vascular reactivity studies, 14 dogs were killed with sodium pentobarbital (50 mg/kg), and the left circumflex coronary artery was dissected free and cleaned of adhering fat and connective tissue. The vessel was cut into 3-4-mm long rings, and care was taken not to disturb the endothelium. The rings were suspended on a pair of stainless steel hooks in a 15-ml water-jacketed organ chamber. One hook was anchored to a steel rod, and the other was attached to a force transducer (model FT-03C, Grass Instruments, Quincy, Massachusetts). The transducer was connected to a rack and pinion device that served to adjust the length and basal tension of the ring. Tension was recorded on a Grass Model 7D polygraph. The endothelium was removed from some rings by gently rolling the intimal surface over a pair of forceps. The organ chamber was filled with a Krebs' bicarbonate solution (pH 7.4) of the following millimolar composition: NaCl 119, KCl 5, NaHCO3 24, KH2PO4 1.2, MgSO4 1.2, glucose 11, EDTA 0.02, and CaCl2 3.2. The solution was bubbled with 95% O2-5% CO2 and maintained at 37°C. The vessels were challenged with repeated exposures to 20 mM KCl and progressive increases in basal tension.

To determine the optimal resting tension. After the vessels were equilibrated for 1 hour, the effect of various arachidonic acid metabolites was determined on basal tension or after contraction induced by the thromboxane-mimetic U46619 (20-60 nM) or porcine endothelin (5 nM). In some experiments, vessels were treated with indomethacin (10-5 M) or propranolol (5×10-5 M) or the endothelium was removed before addition of the metabolites. All metabolites except prostacyclin were given in ethanol and compared with vehicle controls. The final concentration of ethanol in the tissue bath never exceeded 0.1%. PGI2 was dissolved in ethanol/NaHCO3 (9/1 vol/vol), pH 9.0.

Statistical analysis was performed by an analysis of variance to determine significant differences among groups followed by an unpaired t test to determine differences between groups. A value of p<0.05 was considered statistically significant.

**Materials**

12- or 15-HPETE, 12- or 15-HETE, and 15-KETE were purchased from Biomol, Plymouth Meeting, Pennsylvania, and the purity was determined by HPLC. Prostaglandins and U46619 were purchased from Cayman Chemicals, Ann Arbor, Michigan, and the LTs were provided by Drs. J. Rokash and A. Ford-Hutchinson at Merck-Frosst, Ltd, Pointe-Claire, Canada. The epoxyeicosatrienoic acids and dihydroxyeicosatrienoic acids were synthesized by the method of Corey et al. [12-]Arachidonic acid (1 Ci/mmol), [3H]-12-HETE (225 Ci/mmol), and [3H]-15-HETE (183 Ci/mmol) were purchased from New England Nuclear, Boston, Massachusetts. Arachidonic acid, indomethacin, propranolol and the calcium ionophore, A23187, were purchased from Sigma Chemical, St. Louis, Missouri. All organic solvents were HPLC grade and purchased from Burdick and Jackson, Muskegon, Michigan. Collagen for aggregation was from Hormon-Chemie, Munich, FRG, and endothelin was from Peptides International, Louisville, Kentucky.

**Results**

During the course of the 3-hour experiment there was an average of 10 episodes of cyclic blood flow patterns per hour for each dog. Heart rates and aortic blood pressures remained stable throughout the duration of the experiment.

**Identification of Arachidonic Acid Metabolites**

The radioactive profile of a typical incubation of equivalent weights of stenosed LAD and normal left circumflex coronary arteries is shown in Figure 1. In the normal left circumflex, radioactive peaks were observed that comigrated with the PG standards in fractions 10–50 (2–10 minutes), the HETE standards in fractions 105–130 (21–26 minutes), and the EET standards in fractions 130–155 (26–31 minutes). Arachidonic acid eluted in fractions 170–190 (34–38 minutes). When incubations were conducted under
the same conditions but in the absence of vessels, radioactive peaks were detected in fractions 160–170 and in fractions 170–190. Thus, the peak in fractions 160–170 would appear to represent an autooxidation product. The profile of metabolites produced by the stenosed LAD differed from the normal vessel. Also, the extent of metabolism of 14C-arachidonic acid was greater than the stenosed than the normal vessels. The stenosed vessel synthesized radioactive metabolites comigrating with PGs, HETEs, and EETs as did the normal vessel; however, the stenosed vessel additionally synthesized 14C-metabolites comigrating with the diHETEs/DHETs (fractions 50–105).

To obtain an adequate mass of metabolites for GC/MS analysis, the stenosed LAD and the entire normal left circumflex coronary artery of six dogs were incubated as described in “Materials and Methods.” The media of each incubation was pooled and extracted. The metabolites were first separated by reverse-phase HPLC as in Figure 1. The radioactive peaks comigrating with the PGs, diHETEs/DHETs, and EETs were collected for further analysis by HPLC. These pooled incubations were repeated three times and similar results were obtained.

Figure 2 shows the further purification of the metabolites found in the PG fraction from stenosed and normal dog coronary arteries. This separation used reverse-phase HPLC and solvent program 2. The major PG metabolite synthesized by the normal coronary artery was 6-keto-PGF1α. In contrast, the major metabolites produced by stenosed and endothelially injured coronary arteries included TXB2, PGF2α, PGE2, and PGD2. No 6-keto-PGF1α synthesis was detected. These findings are in agreement with our previous results.4

The HETE fraction of each sample was rechromatographed on normal phase HPLC (solvent program 4). The radioactivity and ultraviolet absorbance at 235 nm of the column eluate are shown in Figure 3. Seven major radioactive peaks were detected. Peak 1 had a UV absorbance maximum at 278 nm and comigrated with 15-KETE. When peak 1 was treated with sodium borohydride for 15 minutes at room temperature, the UV absorbance maximum shifted to 235 nm, and the product comigrated with 15-
Radioactivity in acetic hexne/glacial the of 235 at 3.

FIGURE 3. Separation of the 14C-metabolites of the hydroxyeicosatetraenoic acid (HETE) fraction (fractions 105–130, Figure 1) of the normal circumflex and stenosed left anterior descending coronary arteries. The fractions were chromatographed on an Ultrasphere-Si silica gel column using solvent program 4. A linear gradient was used from 0.5% to 1.5% isopropanol in hexane/glacial acetic acid (999:1) over 40 minutes at a flow rate of 3 ml/min. Fractions were collected every 0.2 minutes. Radioactivity in the eluate is plotted in the upper panel and ultraviolet absorbance at 235 nm in the lower panel. A 235, absorbance at 235 nm.

HETE on normal phase HPLC (solvent system 4). These data suggest that peak 1 is 15-KETE which is reduced to 15-HETE by sodium borohydride. Analysis of the derivatized material by GC/MS gave fragments at m/z 91 (M-270), 106 (M-255), and 180 (M-181) and a chromatographic peak that had the same elution time as the methoxime, methyl ester of authentic 15-KETE. Peaks 2–7 demonstrated UV absorbance at 234 or 236 nm, indicating the presence of a conjugated diene structure that is characteristic of the HETEs. Peaks 2–7 were collected, derivatized to form the methyl ester and trimethylsilyl ether, and analyzed by GC/MS. A summary of the fragmentation pattern of each peak is shown in Table 1. The

<table>
<thead>
<tr>
<th>Peak</th>
<th>Ultraviolet absorbance maximum (nm)</th>
<th>M*</th>
<th>Fragment ions (m/z)</th>
<th>Compound</th>
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</thead>
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<tr>
<td>1</td>
<td>278</td>
<td>406</td>
<td>91(100), 106(3), 180(3)</td>
<td>15-KETE*</td>
</tr>
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<td>2</td>
<td>236</td>
<td>406</td>
<td>295(40), 316(9), 406(1)</td>
<td>12-HETE</td>
</tr>
<tr>
<td>3</td>
<td>236</td>
<td>406</td>
<td>225(39), 316(27), 335(9), 406(2)</td>
<td>15-HETE</td>
</tr>
<tr>
<td>4</td>
<td>236</td>
<td>406</td>
<td>225(76), 316(8), 406(1)</td>
<td>11-HETE</td>
</tr>
<tr>
<td>5</td>
<td>234</td>
<td>406</td>
<td>255(60), 316(7), 406(1)</td>
<td>9-HETE</td>
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<tr>
<td>6</td>
<td>234</td>
<td>406</td>
<td>265(38), 316(8), 406(1)</td>
<td>8-HETE</td>
</tr>
<tr>
<td>7</td>
<td>234</td>
<td>406</td>
<td>203(8), 255(6), 316(4), 305(3), 406(1)</td>
<td>5-HETE</td>
</tr>
</tbody>
</table>

Values in parentheses represent the relative abundance of the ions. The peak number corresponds to the radiolabeled peaks found in Figure 3.

*Peak 1 comigrated with 15-ketoecosatetraenoic acid (KETE) and had an ultraviolet absorbance maximum of 278 nm. Following treatment with sodium borohydride, the ultraviolet absorbance maximum was 235 nm and the modified compound comigrated with 15-hydroxyeicosatetraenoic acid (HETE). Thus, Peak 1 would appear to be 15-KETE.
mass spectra of peaks 2–7 contained an M⁺ ion at 406, which is consistent with a methyl ester–trimethylsilyl ether of a HETE. Peak 2 gave major ions at m/z 295 [M-11, loss of CH₂-C(H₂)=CH-(CH₂)₂-CH₃] and 316 [M-90, loss of (CH₃)₂SiOH]. The latter ion was present in the mass spectra of peaks 2–7. This mass spectrum and the GC elution time were identical to the methyl ester–trimethylsilyl ether of synthetic 12-HETE. Peak 3 gave major ions at m/z 225 [M-181, loss of CH₂-CH=CH-CH₂-CH=CH-(CH₂)₂-COOCH₃], 335 [M-71, loss of (CH₃)₂CH=CH-(CH₂)₂COOCH₃], and 316. This was characteristic of 15-HETE and identical to the derivatized standard for 15-HETE. Peak 4 gave a mass spectrum identical to the methyl ester–trimethylsilyl ether of 11-HETE with major ions of 225 and 316. Peaks 5 and 6 had mass spectra that were similar to the methyl ester–trimethylsilyl ethers of 9-HETE and 8-HETE, respectively. Major ions were observed at m/z 255 (M-151) and 265 (M-141) for peaks 5 and 6, respectively. Peak 7 had major ions at m/z 203 [loss of CH=CH-CH=CH₂-CH=CH-(CH₂)₂-CH₃], 255 [loss of CH₂-CH=CH-CH₂-CH=CH-(CH₂)₂-CH₃], and 305 [loss of (CH₂)₂-COOCH₃]. The mass spectrum and GC elution time were identical to the derivatized 5-HETE standard.

While each HETE was produced by both stenosed LAD and normal left circumflex coronary arteries, the amounts of HETEs were greater in the stenosed vessel. Table 2 compares ¹⁴C-HETE production obtained by incubating 0.08 g of stenosed LAD and 1.06 g of normal left circumflex with ¹⁴C-arachidonic acid and 20 µM A23187. The stenosed vessel synthesized fivefold to 10-fold more ¹⁴C-HETE per gram of tissue than normal coronary artery. Twenty-fold more ¹⁴C-15-KETE was produced by the stenosed LAD than by the normal left circumflex coronary artery.

To examine endogenous production of 12- and 15-HETE by stenosed LAD and normal left circumflex vessels, vessel segments were incubated and the media were assayed by specific radioimmunoassays. Both the normal and stenotic vessels synthesized 12- and 15-HETE; however, there was approximately five times more 15-HETE and seven times more 12-HETE synthesized by the stenosed LAD than the normal left circumflex (Table 3). The release of 12-HETE exceeded 15-HETE in both the normal and stenosed vessel when release of endogenous arachidonic acid was stimulated by A23187. In contrast, when exogenous ¹⁴C-arachidonic acid was added, the synthesis of ¹⁴C-15-HETE was greater than that of ¹⁴C-12-HETE. Thus, the source of the arachidonic acid may have influenced the relative amounts of 12- and 15-HETE produced.

The metabolites comigrating with the EET standards (fraction 130–155, see Figure 1) were further purified by normal phase HPLC (solvent program 5, Figure 4). Six radioactive peaks were observed in the fraction from the normal coronary artery and seven peaks in the fraction from the stenosed vessel. Peaks 1–5 failed to absorb in the range 200–350 nm. Peak 6 had a UV absorbance maximum of 278 nm. The radioactivity profile from the stenosed vessel differed in several other respects from the normal vessel. In the stenosed vessel, peak 1 was larger than in the normal vessel, and peak 5 was absent. Two additional peaks were detected in the stenosed vessel and migrated between peaks 1 and 4. Peaks 2–5 comigrated with 14,15-EET; 11,12-EET; 8,9-EET; and 5,6-EET standards, respectively. The radioactive peaks were converted to their pentafluorobenzyl esters and analyzed by negative ion chemical ionization mass spectrometry. Peak 1 contained contaminating material and failed to give a clear mass spectrum. The mass spectra for peaks 2–5 are shown in Figure 5. Each metabolite exhibited a major ion at m/z 319 (M⁺-1), which is characteristic of an EET. We were unable to obtain a molecular ion for peak 6. It may contain other functional groups which prevented it from eluting from the column or it may not contain a carboxyl. Based on a molecular weight of 320, the absence of UV absorbance and comigration with known standards on HPLC and GC, peaks 2–5 were assigned as 14,15-EET; 11,12-EET; 8,9-EET; and 5,6-EET, respectively. Normal phase HPLC resolution of radiolabeled metabolites of the diHETE-DHET fraction (fraction 50–105, Figure 1) of the stenosed coronary artery is shown in Figure 6. Five major radioactive peaks were observed. Four of these peaks comigrated with standards for 14,15-DHET; 11,12-DHET; 8,9-DHET; and 5,6-DHET. These

### Table 2. Synthesis of ¹⁴C-HETE by Normal and Stenosed Canine Coronary Arteries

<table>
<thead>
<tr>
<th></th>
<th>Normal circumflex (cpm/g)</th>
<th>Stenosed LAD (cpm/g)</th>
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<tr>
<td>15-KETE</td>
<td>2,376</td>
<td>50,550</td>
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<td>5-HETE</td>
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</tbody>
</table>

LAD, left anterior descending coronary artery; KETE, ketoicosenoic acid; HETE, hydroxyicosatetraenoic acid.

### Table 3. Synthesis of 12- and 15-HETE by Normal Circumflex or Stenosed LAD Canine Coronary Arteries

<table>
<thead>
<tr>
<th></th>
<th>Normal circumflex (ng/g)</th>
<th>Stenosed LAD (ng/g)</th>
</tr>
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<tbody>
<tr>
<td>12-HETE</td>
<td>162±68</td>
<td>17±3</td>
</tr>
<tr>
<td>15-HETE</td>
<td>1,102±154*</td>
<td>93±14*</td>
</tr>
</tbody>
</table>

Values (ng/g) are expressed as mean±SEM for n=10. Vessel segments were incubated with 20 µM A23187 in 1 ml of 10 mM HEPES buffer at 37°C for 30 minutes. Hydroxyicosatetraenoic acids (HETEs) were measured in the media by radioimmunoassay as described in "Materials and Methods.”

*Significantly different from normal circumflex, p<0.001.
products represented major metabolites of $^{14}$C-arachidonic acid in the stenosed LAD but were only minor products in the normal left circumflex sample (data not shown). Consistent with their assignment as DHETs, UV absorbance was not detected for any of the five peaks. Since the DHETs are catabolites of the EETs, the presence of 5,6-DHET and 5,6-DHET-$\Delta$-lactone suggests that the stenosed coronary artery synthesizes 5,6-EET even though its presence was not detected in Figure 4.

Incubation buffer of normal left circumflex coronary arteries that were treated with inhibitors of arachidonic acid metabolism was extracted and analyzed by reverse phase HPLC (solvent program 1). Indomethacin inhibited the synthesis of material comigrating with 6-keto-PGF$_{1\alpha}$ and increased the release of $^{14}$C metabolites in the HETE and EET fractions (Figure 7B). BW755c, a nonselective inhibitor of cyclooxygenase and lipoxygenase pathways, reduced the synthesis of products in the PG, HETE, and EET fractions (Figure 7C). Metyrapone, an inhibitor of cytochrome P-450, inhibited the synthesis of compounds migrating in the PG and EET fraction but increased those comigrating with the HETEs (Figure 7D).

Incubation media from the stenosed LAD and normal left circumflex coronary arteries were extracted under conditions known to extract LTs and then analyzed by reverse phase HPLC (solvent program 6). The chromatograms in Figure 8 show radioactive peaks comigrated with LTC$_4$, in the normal artery and LTB$_4$ and LTC$_4$ in the stenosed artery. Although LTs have a UV absorbance maximum of 280 nm, no absorbance was detected that corresponded to these radioactive peaks.

### Biological Activity of Arachidonic Acid Metabolites

Collagen-induced aggregation of canine platelet-rich plasma was not affected by pretreatment with either 12-HPETE, 15-HPETE, or 15-KETE (7.5×10$^{-9}$ to 1×10$^{-6}$ M). Collagen stimulated the release of TXB$_2$ by platelets; however, this was also not affected by pretreatment with either 12-HPETE or 15-HPETE (data not shown). Aggregation of platelet-rich plasma was not altered by any of the four EETs between 10$^{-9}$ and 10$^{-4}$ M. PGI$_2$ (70 nM) completely abolished platelet aggregation.

Canine coronary artery rings were used to test the effect of EETs, HPETEs, and HETEs on vascular reactivity. The EETs had no effect on basal tension. However, they induced a concentration-related relaxation of canine coronary arteries that were contracted with U46619, a thromboxane-mimetic (Figure 9). All four EETs were equipotent in inducing relaxation. The EETs were eight to 10 times less potent than acetylcholine but significantly more potent than PGI$_2$ in eliciting relaxation of the coronary artery. Neither indomethacin (10$^{-5}$ M) nor propranolol (5×10$^{-5}$ M) altered the vasorelaxation produced by the EETs. The 14,15-epithioicosatrienoic acid, which has a sulfur atom substituted for the oxygen bonded to carbons 14 and 15, was at least as potent as the 14,15-EET in relaxing dog coronary arteries (data not shown, n=5). The 14,15- and 11,12-EETs also relaxed vessels contracted with 5 nM porcine endothelin (n=10). The 8,9- and 5,6-EETs were not tested. 12- or 15-HPETE (10$^{-9}$–10$^{-6}$ M) did not affect basal tension of vessels and only relaxed precontracted vessels at the highest concentration tested. 15-HPETE and 12-HPETE at 10$^{-6}$ M caused...
25±6% and 28±7% relaxation, respectively. The corresponding HETEs had little effect (15±3% relaxation). 15-KETE and the saturated 20-carbon fatty acid arachidic acid (1 μM) had no effect on vascular reactivity.

Discussion

Previously, we found that normal canine coronary arteries metabolized arachidonic acid to 6-keto-PGF₁α, the major metabolite of PG₁₂. Other investigators have reported similar findings. We also examined arachidonic acid metabolism in an experimental model of concentric coronary artery stenosis.

This model is characterized by cyclic flow variations that are spontaneous decreases in coronary blood flow followed by sudden restorations of flow. These flow variations are due to the accumulation of platelet aggregates at the site of stenosis and vasoconstriction distal to the stenosis. We have established with light and electron micrographs that there is endothelial damage at the site of the stenosis, adherence of platelets to the lumen of the vessel and infiltration of leukocytes into the media. In contrast to the normal vessel, the stenosed segments of coronary arteries from dogs undergoing cyclic flow variations synthesize TXB₂, the major metabolite of TXA₂, and produce lesser amounts of 6-keto-PGF₁α from arachidonic acid.

Since vascular cells, platelets, and inflammatory cells may synthesize lipoxigenase and epoxygenase metabolites of arachidonic acid that are biologically active, the present study was designed to identify these metabolites and compare their production in normal and stenosed, endothelially injured coronary arteries. We have shown in this study that normal or stenosed vessels synthesized primarily 12- and 15-HETE with lesser amounts of 11-, 9-, 8-, and 5-HETE. Radiochemical and radioimmunoassay data indicated that approximately fivefold to 10-fold more HETEs were produced by the stenosed vessel than by the normal coronary artery. In addition, the stenosed vessels, in particular, appeared to synthesize 15-KETE. While a complete mass spectrum was not obtained for this metabolite, several lines of evidence are consistent with the 15-KETE structure, including UV absorbance maximum at 278 nm, comigration on HPLC with an authentic 15-KETE standard, and its conversion to 15-HETE upon treatment with sodium borohydride.
Similarly, Piomelli et al\textsuperscript{24} reported that canine and human coronary arteries produced 12-, 15-, and 5-HETEs; however, this conclusion was based only on comigration with HETE standards on HPLC. Blood vessels from other species and vascular beds have been found to synthesize HETEs. Funk and Powell\textsuperscript{25} found that fetal bovine aorta produced 11- and 15-HETE and that these metabolites were products of cyclooxygenase rather than lipoxigenase. Rat aorta was also found to synthesize 12-, 15-, and 5-HETE.\textsuperscript{26} The synthesis of 15-KETE has not been previously reported in blood vessels or vascular cells. This metabolite may arise directly from 15-HPETE\textsuperscript{27} or through the action of prostaglandin-15-dehydrogenase on 15-HETE.\textsuperscript{28}

Similar experiments have been performed on vascular cells in culture. Rabbit aortic smooth muscle cells were found to synthesize 5-, 8-, 9-, and 12-HETE,\textsuperscript{29} whereas rat aortic smooth muscle cells synthesized only 15-HETE and 11-HETE. In addition, Revtyak et al\textsuperscript{17} demonstrated that bovine coronary artery endothelial cells synthesized 12-, 15-, and 11-HETE, whereas several investigators using umbilical venous endothelial cells found 12-, 15-, 11-, and 5-HETEs.\textsuperscript{30,32} The increased synthesis of HETEs by the stenosed coronary artery compared with the normal vessel may reflect the accumulation of inflammatory cells or activation of lipoxynogenase enzymes by vascular membrane damage. There is precedent for both possibilities. Mullane et al\textsuperscript{33} reported that 12-HETE production was increased 10-fold in ischemic myocardium and attributed this increase to infiltration of inflammatory cells. On the other hand, cell damage was found to unmask 15-lipoxigenase activity in polymorphonuclear cells.\textsuperscript{34} Similarly, Black et al\textsuperscript{35} found that injury by UV light led to an increase in 12-HETE production by human skin. We have found that vascular damage in vitro under circumstances in which inflammatory cells could not collect will enhance HETE synthesis (authors' unpublished observation). Therefore, it seems possible that platelet and leukocyte accumulation or cell damage could account for the excess HETE synthesis by the stenosed coronary artery.

Small radioactive peaks comigrating with LTB\textsubscript{4} and LTC\textsubscript{4} were observed in incubations from the stenosed LAD; however, this was not associated with the characteristic UV absorbance at 280 nm of these compounds. It is possible that inadequate quantities of the LTs were synthesized and escaped UV detection. Piomelli and colleagues\textsuperscript{24} reported the synthesis of small amounts of LTC\textsubscript{4}, LTD\textsubscript{4}, and LTE\textsubscript{4} by normal canine coronary arteries. The cellular source of these LTs is not known; however, it is apparently not from endothelial or smooth muscle cells alone.\textsuperscript{36,37} Vascular mast cells may contribute to the production of LTs or, in our studies, infiltrating leukocytes or mast cells may be involved.\textsuperscript{38}

The present study indicates that canine coronary arteries synthesize 14,15-; 11,12-; 8,9-; and 5,6-EET. This identification was based on comigration on HPLC and GC, the absence of UV absorbance, and

![Figure 7. Separation of ^14C-metabolites from normal circumflex coronary artery. Vessels were pretreated with indomethacin (10 μM), BW755c (50 μM), or metyrapone (50 μM) before being stimulated with ^14C-arachidonic acid and A23187 for 10 minutes at 37° C. Incubation buffer was extracted and chromatographed as in Figure 1. Migration times of known standards are shown above the chromatogram. PG, prostaglandin; DHET, dihydroxyeicosatrienoic acid; HETE, hydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid.](http://circres.ahajournals.org/lookup/doi/10.1161/01.RES.87.12.617)
been reported mainly in hepatic and renal tissue. Prior to this report, the synthesis of these compounds has not been described in blood vessels. In recent studies, we have presented preliminary data indicating that human endothelial cells synthesize EETs. It has also been shown that human and dog, but not rat, polymorphonuclear leukocytes contained a cytochrome P-450 monooxygenase that converted arachidonic acid to several unidentified compounds. Human platelets synthesize 14,15-EET; however, this synthesis is by a non-cytochrome P-450 enzyme that acts on esterified arachidonic acid. Therefore, it is possible that infiltrating leukocytes or adherent platelets contribute to the enhanced production of the EETs and DHETs by the stenosed vessel. However, it is also possible that a cytochrome P-450 epoxygenase is present in vascular tissue and that injury promotes its activation.

The effect of EETs on vascular reactivity has only been recently studied. Pinto and coworkers showed that cytochrome P-450 was present in canine coronary arteries. When cytochrome P-450 was induced, endothelium-dependent relaxations by arachidonic acid were enhanced, whereas inhibition of the enzyme reduced these relaxations, suggesting that a cytochrome P-450 metabolite was responsible for the relaxations. However, no metabolite was identified. In the present study, the four EET regioisomers relaxed coronary artery rings in a concentration-related manner. Why the four regioisomers are equipotent is unknown, but there is probably not a specific receptor for an EET in dog coronary artery. However, the epoxide structure of the EET must be important because the HETEs, HPETEs, 15-KETE, and arachidonic acid gave a much reduced relaxant effect or none at all. Also, the relaxation by the EETs did not appear to be specific for U46619-contracted vessels because they also relaxed vessels that were precontracted with endothelin. These arachidonic acid metabolites were more potent than PG\(_I\)\(_2\) as a vasorelaxant of canine coronary arteries. Thus, the EETs may be the cytochrome P-450 metabolites responsible for the relaxations produced by arachidonic acid. There have been other reports of vasorelaxation induced by EETs. Microgram amounts of 11,12- or 8,9-EET produced vasodilation in the microcirculation of the rat intestine. In addition, Carroll et al demonstrated that 5,6-EET dilated the isolated, perfused rat tail artery, whereas 8,9-; 11,12-; and 14,15-EET did not.

In contrast to the EETs, 12- and 15-HETE or 12- and 15 HPETE had no effect on basal tension and had very little effect on vessels contracted with U46619 in concentrations up to 1 \(\mu\)M. In contrast to our findings, 15-HPETE and 15-HETE caused endothelium-independent contractions of canine coronary arteries; however, no doses were reported. When canine coronary arteries were contracted, 15-HPETE or 15 HETE produced relaxation.

We were unable to demonstrate an effect of HPETEs, HETEs, 15-KETE, or EETs on aggrega-
tion of canine platelet-rich plasma. Fitzpatrick et al. found that 14,15- or 8,9-EET inhibited platelet cyclooxygenase and aggregation. Inhibition of cyclooxygenase by HPETEs has also been shown in human platelets. However, these studies used washed platelets. In the present study, the plasma proteins present in the platelet-rich plasma may have bound the EETs and HPETEs, effectively reducing their concentrations. Indeed, while aggregation of human washed platelets was inhibited by 1–3 μM EETs, much greater concentrations (50–200 μM) were needed to inhibit aggregation if platelet-rich plasma was tested.

In summary, stenosed or normal canine coronary arteries produce primarily 12- and 15-HETE with lesser amounts of 11-, 9-, 8-, and 5-HETE. 15-KETE was also synthesized. Radiochemical and radioimmunoassay data indicated that the stenosed arteries synthesized fivefold to 10-fold more product than normal canine coronary arteries. 14,15-; 11,12-; 8,9-; and 5,6-EET were produced by canine coronary vessels. These EETs were also made in greater amounts by the stenosed dog coronary artery. The four EET regioisomers produced equipotent, endothelium-independent, concentration-related relaxations of dog coronary artery rings.

We believe that the development of cyclic flow variations in this canine model closely mimics the pathophysiology of unstable angina in humans. Both conditions are characterized by platelet deposition and aggregation, coronary artery narrowing and endothelial injury, increases in plasma transcardiac thromboxane concentrations, and reductions in coronary blood flow. An evaluation of lipoxygenase and epoxygenase products in platelets with unstable angina has not been conducted. However, the results of this study suggest that the lipoxygenase and epoxygenase pathways are amplified during cyclic flow variations. The consequence of this may be the production of vasodilator EETs that may antagonize the vasoconstrictor effects of TXA2.

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**KEY WORDS**
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- lipoxygenase
- arachidonic acid
- epoxygenase
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