Epoxyeicosatrienoic acids constrict isolated pressurized rabbit pulmonary arteries

DALING ZHU,1 MICHAEL BOUSAMRA II,2 DARRYL C. ZELDIN,3 JOHN R. FALCK,4 MARY TOWNSLEY,5 DAVID R. HARDER,1 RICHARD J. ROMAN,1 AND ELIZABETH R. JACOBS1,6

1Department of Physiology, Cardiovascular Research Center, 4Department of Medicine, and 2Department of Surgery, Medical College of Wisconsin, Milwaukee, Wisconsin 53226; 5Department of Biochemistry, University of Texas Southwestern Medical Center, Dallas, Texas 75235; 3Department of Physiology, University of South Alabama, Mobile, Alabama 36688; and 6Laboratory of Pulmonary Pathobiology, National Institute of Environmental Health Sciences, Research Park, North Carolina 27709

Zhu, Daling, Michael Bousamra II, Darryl C. Zeldin, John R. Falck, Mary Townsley, David R. Harder, Richard J. Roman, and Elizabeth R. Jacobs. Epoxyeicosatrienoic acids constrict isolated pressurized rabbit pulmonary arteries. Am. J. Physiol. Lung Cell. Mol. Physiol. 278: L335–L343, 2000.—Little information is available regarding the vasoactive effects of epoxyeicosatrienoic acids (EETs) in the lung. We demonstrate that 5,6-, 8,9-, 11,12-, and 14,15-EETs contract pressurized rabbit pulmonary arteries in a concentration-dependent manner. Constriction to 5,6-EET methyl ester or 14,15-EET is blocked by indomethacin or ibuprofen (10−5 M), SQ-29548, endothelial denuding, or submaximal preconstriction with the thromboxane mimetic U-46619. Constriction of pulmonary artery rings to phenylephrine is blunted by treatment with the epoxyenase inhibitor N-methylsulfonyl-6-(2-propargyloxyphenyl)hexanamide. Pulmonary arteries and peripheral lung microsomes metabolize arachidonic acid to products that comigrate on reverse-phase HPLC with authentic epoxyeicosatrienoic acids (EETs). Within the lung, the cytochrome P-450 monooxygenase pathway. Generally, the P-450 enzymes convert arachidonic acid into epoxyeicosatrienoic acid (EETs) and hydroxyeicosatetraenoic acids (HETEs), compounds that may themselves undergo further metabolism by cyclooxygenase or other enzymes (25). Within the lung, the cytochrome P-450 isoforms are thought to be important in metabolism and/or detoxification of xenobiotics (e.g., Refs. 13, 27). In many vascular beds, EETs cause vasorelaxation (1, 2, 12, 18). However, there are reports that EETs constrict renal arteries via cyclooxygenase-dependent mechanisms (40). We have recently shown that 20-HETE is synthesized from arachidonic acid by microsomal protein of human and rabbit lung; furthermore, 20-HETE dilates human pulmonary arteries in a concentration- and cyclooxygenase-dependent manner (6, 47). Zeldin and co-workers (44, 45) and Knickle and Bend (24) demonstrated that EETs are endogenous products of rabbit, guinea pig, human, and rat lungs and that they dilate guinea pig bronchi in a concentration-dependent manner. Cytochrome P-450s of the CYP2J subfamily are localized not only to the proximal airway cells but also appear in bronchial and pulmonary vascular smooth muscle cells of humans and rats. Their location in the lungs suggests that these metabolites could modulate pulmonary vascular tone (44). We hypothesized that 1) rabbit peripheral lung tissue and pulmonary arteries express P-450 isoforms that catalyze the formation of EETs, 2) EETs increase the tone of isolated pressurized pulmonary arteries, 3) the proconstrictive actions of EETs are endothelium and cyclooxygenase dependent, and 4) if endogenously formed EETs contribute to the intrinsic tone of pulmonary arteries, inhibition of epoxygenases should blunt the constrictive response to phenylephrine in pulmonary arterial rings. Our data demonstrate conversion of arachidonic acid to EET products from peripheral lung and pulmonary artery microsomes as well as immunospecific protein for five CYP450 isoforms known to catalyze the formation of EETs. All EETs regioisomers constrict isolated pressurized rabbit pulmonary arteries in a concentration-dependent manner. Constriction is blocked by pretreatment with indomethacin, ibuprofen,
EETs CONSTRICNT RABBIT PULMONARY ARTERIES

the thromboxane-receptor antagonist SQ-29548, or by endothelium removal. Arteries that are preconstricted with U-44196 and then exposed to 5,6-EET methyl ester (5,6-EET-ME) also exhibit blunt ed EET-induced vasoconstriction. These data are consistent with the interpretation that the vasoactive effects of EETs stimulate cyclooxygenase-dependent synthesis of a pressor prostanoid (such as thromboxane or a closely related compound), which causes vasoconstriction. Alternatively, EETs may be metabolized by cyclooxygenase to a vasoconstrictive substrate. Finally, we show that pretreatment of pulmonary artery rings with the epoxygenase inhibitor N-methanesulfonfyl-6-(2-propargyl-oxyphenyl)hexanamide (MS-PPOH) shifts the concentration-response curve to phenylephrine to the right, raising the suggestion that endogenously formed EETs may increase the tone of pulmonary arteries.

METHODS

Reagents. All unlabeled EETs, SQ-29548, and U-46619 were purchased from BIOMOL. [1-14C]Arachidonic acid was acquired from DuPont-NEN (Wilmington, DE). NADH, NADPH, isocitrate, and isocitrate dehydrogenase were purchased from Sigma Chemical (St. Louis, MO). Western blots were visualized using Renaissance Western Blot Chemiluminescence Reagent (DuPont-NEN). All chemicals were of analytic grade unless otherwise stated.

Animals. Adult male New Zealand White rabbits (weight 2–3 kg) were used in this study, which was approved by our Animal Experimentation Committee and conformed with recommendations of the Guide for the Care and Use of Laboratory Animals (Department of Health and Human Services). Rabbits were cared for in the Animal Resource Laboratory of the Medical College of Wisconsin, which is approved by the American Association for Accreditation of Laboratory Animal Care. The animals were maintained in our facility for a minimum of 2 days before use to acclimate and to identify potential signs of respiratory disease. Dogs were housed in the Animal Center at the University of South Alabama, also conforming with recommendations for use of laboratory animals. Dogs were anesthetized with ketamine and acepromazine and were free of microfilaria. Samples from the peripheral dog lungs, which were used for preparation of microsomes and cytochrome P-450 assays, were harvested by one of the investigators and rapidly frozen in liquid nitrogen. They were shipped on dry ice to the Medical College of Wisconsin, where microsomes were prepared and assays were performed as described below.

Isolated vessel protocol. Rabbits were anesthetized with intramuscular injections of xylazine (6 mg/kg), ketamine (55 mg/kg), and acepromazine (1.6 mg/kg). The heart and lungs were removed en bloc, and pulmonary arteries with an outer diameter of 300–500 µm were dissected under a microscope from excised lung tissue bathed in iced physiologic saline solution (PSS). The composition of the PSS was (in mM): NaCl 118, KCl 4.7, CaCl2 27, NaHCO3 10, glucose 2.5, MgSO4 0.57, and KH2PO4 1.2. The solution was gassed with 95% O2-5% CO2, both ends of an artery with all side branches tied off were secured over glass cannulas tapered to an outer diameter of ~80 µm. Vessels were stretched to remodeled constrictor to the outflow cannula and adjusting a reservoir attached to the inflow cannula to maintain 10 mmHg. Pressures were continuously monitored via a fluid-filled pressure transducer attached to the outflow line. Internal diameters of the vessels were determined using a stereomicroscope (Zeiss), charged-coupled device television system camera (KP-130 AU, Hitachi), a television monitor (CVM-1271, Sony), and a video-measuring system (Colorado Video). Effective magnification to the video monitor was approximately ×400. After an equilibration period of 90–120 min, reactivity was confirmed by quantitative constriction to 30 mM KCl. The effects of all drugs were tested with the superfusion system stopped. Some vessels were pretreated for 30 min with indomethacin (10 µM) or ibuprofen (10 µM) to inhibit cyclooxygenase (26), SQ-25548 (5 µM) to block thromboxane receptors, or U-46619 (5 µM; an effective constrictor of isolated pulmonary arteries, e.g., Ref. 37) and then tested for vasoactive effects of EETs. In other studies, time and vehicle control experiments were performed. In selected experiments, endothelium was denuded after control data were obtained by either passing a bubble through the vessel (17) or by exposure to a cocktail of complement and antibody against factor VIII-related antigen (23). Effective removal of the endothelium was verified by loss of relaxation to ACh (10–6 M) in vessels precontracted with phenylephrine (~20% magnitude of relaxation before endothelium removal). Reactivity to KCl confirmed viability of the denuded and intact vessels at the end of the experiment. To examine the effect of preconstriction and/or depolarization on the response to EETs, some vessels were studied before and after preconstriction with KCl.

Pulmonary artery ring studies. Pulmonary artery rings were obtained and examined for isometric contractile responses according to methods previously described by us for study of bronchial tone (22). Briefly, rabbits were anesthetized, the heart and lungs were removed as previously described, and pulmonary artery rings 1–2 mm in diameter were dissected free in ice-cold PSS. Rings were mounted on tungsten wire, one connected to a fixed holder and the other to a force displacement transducer (model FT03, Gould Electronics) for continuously measuring isometric tension, and immersed in pH-adjusted, oxygenated PSS solution at 37°C. Tension data were relayed from transducers to a signal amplifier (600 series eight-channel amplifier, Gould Electronics). Data were acquired and analyzed using CODAS software (DataQ Instruments). Rings were loaded with 0.75 g passive tension and then equilibrated for an additional 30 min before the studies were begun. Viability of the rings was determined by contraction to 12.5 and 25 mM KCl. We examined the concentration-response of rings to incremental concentrations of phenylephrine (10–10 to 10–6 M) pretreated with vehicle or the highly specific epoxygenase inhibitor MS-PPOH (2 × 10–5 M; Refs. 29, 42). In a separate set of experiments, four rings were contracted with the calcium ionophore A-23187 (10–6 M) for 2 h. Immediately thereafter, microsomes were made from the rings, and assays for arachidonic acid metabolites were performed.

Cytochrome P-450 metabolism of arachidonic acid by lung microsomes. Microsomes were prepared from homogenates of peripheral lungs, medium, and small (~<1-mm-diameter) pulmonary arteries, or pulmonary artery rings by differential centrifugation using a modification of methods previously reported by us (6). Sequential centrifugations at 9,000 g for 30 min and 100,000 g for 1.5 h were performed to generate a microsomal pellet. Protein was quantified according to the method of Bradford (7). Microsomal proteins (1 mg/ml, 200 µl final volume) were resuspended in assay buffer (100 mM potassium phosphate, 1 mM EDTA, and 10 mM MgCl2) and incubated for 30 min at 37°C with [14C]arachidonic acid (0.125 µCi/ml; 2 µM). NADPH (1 mM) and a NADPH-
regenerating system containing 10 mM isocitrate and 0.1 U/ml isocitrate dehydrogenase (19) were included in each assay unless otherwise specified. In cases where [1-14C]EETs rather than arachidonate were used as substrates, NADPH, isocitrate, and isocitrate dehydrogenase were omitted from the assays, and cofactors for cyclooxygenase (2.5 μM hemoglobin and 1 mM epinephrine) were included instead (5). Assays were performed in the presence of room air (PO2 \~ 140 mmHg). Reactions were terminated by acidification with formic acid, and products were isolated by two extractions with ethyl acetate. The organic phase was back extracted with 1 ml of distilled water, evaporated under nitrogen, and reconstituted in ethanol. Products were separated on a C18 reverse-phase HPLC column (Supelco, Bellefonte, PA) using a linear gradient ranging from 100% solvent A (acetonitrile-water-acetic acid, 30:70:1) to 100% solvent B (acetonitrile-acetic acid, 100:1) over 40 min. 14C-labeled products were detected using a radioactive detector (HPLC, Beckman System Gold Programmable Detector Module 171). Identification of metabolites was based on coelution with authentic standards.

Western blot identification of P-450 protein. Microsomal suspensions were separated by electrophoresis on 10% SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. Nonspecific binding was blocked by incubating the membrane overnight in Tris-buffered saline containing 0.05% Tween 20 plus 10% nonfat milk. The membrane was then incubated for 2 h at room temperature with one of the following antibodies: 1) a polyclonal antibody (1:2,000) to rat liver CYP2C11 epoxigenase (Ref. 19; sequence homology to rabbit CYP2C1, CYP2C2, and CYP2C14), 2) a polyclonal antibody (1:1,000) raised in goats against rat liver CYP2B1 (Genent, sequence homology in the rabbit CYP2B4) or CYP2E1 (1:1,000; Gentest, sequence homology in the rabbit CYP2E1), 3) a polyclonal antibody (1:1,000) raised in goats against human CYP1A1 (Genent, sequence homology CYP1A1 in rabbit), or 4) 2) 2 polyclonal antibody (1:1,000) raised in rabbit against recombinant human CYP2J2 (Ref. 44; sequence homology with rabbit CYP2J1). Membranes were incubated with horseradish peroxidase-labeled secondary antibody (1:2,000) and then visualized using enhanced chemiluminescence.

Preparation of labeled EETs. Radiolabeled EETs were prepared according to the method of Corey et al. (11). Briefly 2.5 μCi of [1-14C]arachidonic acid were evaporated under argon and resuspended in a solution of 50 μl of dichloromethane, 20 μl of water, 1 mM NaHCO3 (final concentration), and 0.5 mM m-chloroperoxybenzoic acid (PBA), and then stirred under argon for 45 min. Additional aliquots of 25 μl of dichloromethane, 0.37 mM NaHCO3, and 0.18 mM PBA solution were added. The solution was again capped under argon and stirred for 15 min. Triphenylphosphine (0.4 mM final concentration) was added, and the reaction was stirred again for 5 min under argon. Lipids were extracted with dichloromethane, and the organic layer was dried under argon. The extracted material was transferred to solid-phase extraction cartridges with a carrier of glacial acetic acid-hexane (0.5:99.5 by weight), and the column was rinsed with 10 ml of the same solution to elute arachidonic acid. The EETs were then eluted with 10 ml of ether-glacial acetic acid-hexane (20:0.5:79.5). The solvent was evaporated under argon, and the EETs were resuspended in 100% ethanol. Products were separated first on a C18 Nucleosil (5 μm, 4.6 × 250 mm) column using our standard EETs-HETEs gradient (see Cytochrome P-450 metabolism of arachidonic acid by lung microsomes; materials with an elution time between 24 and 28 min (retention time of EETs in our system) were collected and further separated into regioisomers by normal-phase HPLC to verify their identity (34).

Statistics. Data are presented as means ± SE. Differences between vessel diameters after treatment with different concentrations of EETs were assessed using one-way ANOVA for repeated measures followed by a Student-Newman-Keuls test when significant differences were identified. Differences between vessel diameters in response to EETs before and after treatment with indomethacin, SQ-29548, or U-46619 were compared using a two-tailed unpaired t-test. P < 0.05 was considered significant.

RESULTS

P-450/ EETs products. Figure 1, A and B, shows representative reverse-phase chromatograms of arachidonic acid products generated by incubations of microsomal proteins prepared from rabbit peripheral lung and small to medium vessels incubated with [14C]arachidonic acid. When products with retention times between 26 and 29 min are included, the average conversion rate of [14C]arachidonic acid into EETs by rabbit peripheral lung microsomes was 3.2 ± 1.5 (11,12- and 14,15-EET) and 0.9 ± 0.3 (5,6- and 8,9-EET) pmol·mg protein−1·min−1 (n = 5). Pulmonary artery microsomes from freshly isolated pulmonary arteries or pulmonary artery rings contracted for 2 h by A-23187 also have the capacity to synthesize EETs (Fig. 1B). In data not shown, synthesis of products that comigrated with authentic EET regioisomers was not observed in assays utilizing boiled (inactive) microsomes or those in which NADPH and isocitrate were omitted (n = 3 each). As we have previously demonstrated, pulmonary artery microsomes convert arachidonic into 20-HETE (47). Inclusion of indomethacin (10 μM) abolished all peaks, with elution times consistent with thromboxane, 6-keto-PGF₁α, PGE, or PGF₂α (n = 4; data not shown). Figure 2 shows a representative chromatogram of eicosanoid products of peripheral dog lung microsomes, demonstrating significant conversion of arachidonic acid into products that comigrate with EETs. Mean conversion rate of arachidonic acid into EETs for dog lung microsomes was 12 ± 4.7 pmol·mg protein−1·min−1 (n = 4).

Figure 3 shows results of assays in which rabbit peripheral lung microsomes were incubated with a mixture of [1-14C]EETs regio- and stereoisomers in the presence of vehicle or indomethacin (10−5 M final concentration). No products with retention times consistent with those of cyclooxygenase products or which were inhibited by the inclusion of indomethacin were observed, although substantial conversion of EETs to products which coelute with dihydroxyeicosatetraenoic acid standards was demonstrated.

P-450 immunospecific protein. Rabbit peripheral lung and pulmonary artery microsomes separated electrophoretically and probed with specific P-450 polyclonal antibodies demonstrated immunoreactive protein bands at ~58 kDa (see Fig. 4) with all primary antibodies investigated. In each case, the density of immunospecific protein was greater in peripheral lung than in pulmonary artery microsomes. Microsomal proteins from peripheral lung probed with anti-CYP2J antibody demonstrate two distinct bands, suggesting that rabbit
lung contains more than one CYP2J isoform, in contrast to samples from human and rat lung (44).

Isolated vessel studies. Pulmonary arteries had baseline internal diameters of 380 ± 13 µm (n = 30; range 243–510 µm) and did not change over the course of the equilibration period. Viability of vessels was determined by a brisk contractile response to 30 mM KCl before and after completion of the experiments. Exposure of vessels to KCl (30 mM) reduced diameters to 88 ± 1.2% of control diameters (n = 6). Pretreatment with 10 µM indomethacin did not change the baseline diameter of the vessels (diameter 100.4 ± 0.4% baseline values; n = 7; P > 0.4). However, treatment with the thromboxane-receptor antagonist SQ-29548 (5 µM) caused relaxation over that of baseline tension to 109.8 ± 3.6% pretreatment values (P = 0.01 by paired t-test; n = 7). Removal of endothelium (by either passing a bubble through the lumen of the vessel or exposure to antibody against factor VIII antigen and complement) resulted in a decrease in vessel diameter to 88.9 ± 2.7% (n = 6; P < 0.02). In vessels precontracted with phenylephrine (10^{-6} M), ACh (10^{-6} M) caused a biphasic response (relaxation followed by sustained contraction) as reported by Altiere and colleagues (3). Removal of the endothelium effectively abolished the initial relaxation phase; ACh-induced increase in diameter was 10.5 ± 3% of that observed before removal of endothelium (n = 6). Vehicle alone (ethanol) in concentrations exceeding that of the highest concentrations of EETs employed (1%) had no effect on vessel diameter of endothelium-intact or -denuded arteries (n = 4; Fig. 5).

The vasopressor response to EETs was concentration dependent (see Fig. 5). The maximum response (vessel diameters 80.3 ± 2.6% baseline; n = 4) was observed in the presence of 10^{-6} M 5,6-EET-ME; higher concentrations of other isomers were not tested due to confounding vehicle effect, nonphysiological concentrations, and high cost of the compounds. All EETs regioisomers (5,6-, 8,9-, 11,12-, and 14,15-EETs and 5,6-EET-ME) caused vasoconstriction. To facilitate comparison of vasoactive effects of low concentrations of these epoxides, percent of baseline diameters of the vessels exposed to 2 × 10^{-6} M EETs are shown in Fig. 5B. The greatest constriction was observed in arteries treated with 5,6-EETs. Vasoconstriction to the methylated derivative of 5,6-EETs (5,6-EET-ME) was less than that of...
5,6-EETs and similar to that of other EETs regioisomers.

Constriction to 5,6-EET-ME and 14,15-EETs was blocked by pretreatment with 10 μM indomethacin (Fig. 6) or by the structurally dissimilar cyclooxygenase inhibitor ibuprofen (10 μM; diameter reduced to 83 ± 5.3% baseline by 10⁻⁶ M 5,6-EET-ME with vehicle and 99.7 ± 0.2% baseline after pretreatment with ibuprofen; n = 3; data not shown). Constriction to KCl before and after indomethacin was not different (94 ± 1.2% with indomethacin, n = 6, and 92.4 ± 0.6 without indomethacin, n = 16). In addition, contraction to 5,6-EET-ME was inhibited by removal of endothelium, achieved either by passing a bubble through the lumen of the vessel (n = 4) or treatment with anti-factor VIII antigen and complement (Fig. 6; n = 4). In these vessels, vasoconstrictive response to 30 mM KCl after application of EETs was preserved, with diameters decreasing to 94.3 ± 0.95% (n = 8, data for both methods of removing endothelium combined) of the denuded baseline after application of KCl.

We also tested the effects of thromboxane-receptor mimetics and antagonists on EETs-associated decreases in vessel diameters (Figs. 7 and 8). 5,6-EET-ME-induced vasoconstriction was blunted by pretreatment with the thromboxane-receptor antagonist SQ-29548 (5 µM). In contrast, submaximal preconstriction with 30 mM KCl did not modify the vasoconstrictive response to EETs (Fig. 7). Finally, vasoconstriction to 5 × 10⁻⁶ M 5,6-EET-ME after submaximal preconstriction with 5 µM U-46619 was significantly blunted compared with response of the same arteries to EET in the absence of U-46619 (Fig. 8) or subsequent contraction to KCl (n = 4).

Pulmonary artery ring studies. To begin to investigate the contribution of endogenously formed EETs to pulmonary artery tone, we examined the concentration-response of pulmonary artery rings pretreated first with vehicle and then with the suicide substrate epoxygenase inhibitor MS-PPOH (20 µM) to the standard constricting agent phenylephrine. MS-PPOH decreased basal tone and shifted the concentration-response curve of pulmonary artery rings to phenylephrine to the right (Fig. 9), consistent with inhibition of a factor that increased the state of activation of our rings. In P-450 assays, MS-PPOH blocked conversion of arachidonic acid into EETs in a concentration-dependent manner, with IC₅₀ ~ 10 µM (data not shown), without significant inhibition of other metabolites at concentrations <50 µM.

DISCUSSION

Arachidonic acid is hydrolyzed from cell membrane phospholipids through phospholipases, whereupon it is metabolized by cyclooxygenase, lipoxygenase, or P-450.
monooxygenase enzyme systems to a variety of biologically active metabolites. In excess of 480 isoforms are encoded by the P-450 gene superfamily (e.g., Ref. 28). In the lung, many of these monooxygenases metabolize a wide variety of substrates, including drugs, xenobiotics, and steroids (8, 9). Among other potential products, P-450 enzymes in the lung form HETEs and EETs. The capacity of peripheral lung from rabbits, guinea pigs, and humans to form 20-HETE is well established (6, 32, 33, 43, 45, 47). Recent data have confirmed the ability of peripheral rabbit, rat, and human lungs to metabolize arachidonic acid into EETs (24, 44, 45). Immunohistochemical experiments demonstrate that P-450 of the CYP2J subfamily is expressed in ciliated airway epithelial cells, bronchial and vascular smooth muscle cells, endothelium, and alveolar macrophages of rat and humans (44). Endogenously formed 14,15-, 11,12-, and 8,9-EETs from rabbit, rat, and human lungs can also be identified by gas chromatography-mass spectrometry. Our data expand these observations by demonstrating epoxide formation in peripheral dog and rabbit lung tissues as well as in microsomes from rabbit pulmonary arteries. In addition to CYP2J protein, we found immunospecific bands for CYP2B, CYP2C, CYP2E, and CYP1A in rabbit peripheral lung and pulmonary artery microsomes. Zeldin et al. (45) demonstrated that CYP2B isoforms are primarily responsible for EET formation in normal adult rabbit lung.

![Graph A](image1)

**Fig. 5.** A: peak responses of isolated pressurized vessels to 14,15-, 11,12-, 8,9-, and 5,6-EET and 5,6-EET methyl ester (ME) are shown. Drug was applied to bath in stop-flow state, and vessel diameters were measured at 1-min intervals. All EET regioisomers caused concentration-dependent decrease in vessel diameter, with peak contraction being observed with 10^{-6} M 5,6-EET-ME. Number of vessels studied with each EET is in parentheses. B: constrictive response to single concentration of EETs (2 x 10^{-8} M) or vehicle was greatest with 5,6-EETs (P = 0.013 by ANOVA followed by Student-Newman-Keuls for 5,6-EET). Number of experiments with each regioisomer appear in or above bar. ID, internal diameter. * Different from baseline or vehicle control experiments.

![Graph B](image2)

**Fig. 6.** Constrictive response to 5,6-EET-ME and 14,15-EET was blocked by indomethacin or endothelial cell denudation. Diameters of vessels in presence of vehicle and EETs were determined first. Vessels were then treated for 30 min with indomethacin (10^{-5} M) or were deendothelialized, and diameters of vessels in response to same concentrations of EET were determined again (n = 8 for deendothelialized; n = 4 each for indomethacin followed by EET). * Different from ID values obtained in arteries pretreated with indomethacin or with endothelium removed.

![Graph C](image3)

**Fig. 7.** Decreases in vessel diameter in presence of EETs could be inhibited by submaximal preconstriction with thromboxane-receptor antagonist SQ-29548 (5 x 10^{-6} M; n = 4) but not by preconstriction with KCl (n = 4). * Different from ID values obtained in vessels pretreated with SQ-29548.
from rabbits and dogs but are specifically biosynthesized in pulmonary arteries and that a number of P-450 isoforms capable of catalyzing the formation of EETs are strategically located in peripheral lung and pulmonary artery microsomes.

One of the most important physiological functions of P-450 enzymes in other organ systems (including renal, cerebral, splanchic, and coronary) is postulated to be the formation of HETEs and EETs, which modulate smooth muscle tone. In the renal and cerebral circulations, 20-HETE is a potent vasconstrictor (18, 48). Generally, EETs dilate renal, coronary, cerebral, and other systemic arteries (4, 15, 18, 35). However, at least one group of investigators has reported cyclooxygenase-dependent constriction of renal arteries in the presence of EETs (40). Most germane to our work are the reports of two groups of investigators examining the effects of EETs in the pulmonary circulation. Tan and co-workers (41) described vasodilation of isolated rabbit lungs preconstricted with the thromboxane mimetic U-46619 to 5,6-EET but noted no change in perfusion pressure unless baseline tone was increased. In this model, the EET-associated decreases in perfusion pressure could be blocked by both indomethacin and the nitric oxide synthase inhibitor N^-nitro-L-arginine (41). Similarly, Stephenson et al. (38) observed that 5,6-EETs caused a decrease in pulmonary vascular resistance in isolated perfused canine lungs preconstricted with the thromboxane mimetic U-46619. EET-induced decreases in pulmonary vascular resistance were primarily due to a decrease in venous rather than arterial resistance and were accompanied by an increase in immunoreactive 6-keto-PGF\(_{1\alpha}\) in the perfusate. EET-associated pulmonary venous relaxation could be blocked by pretreatment with indomethacin and was not observed in pulmonary vessels that were not preconstricted. Furthermore, these investigators observed that isolated pulmonary artery rings (3–4 mm in length) preconstricted with 5-hydroxytryptamine (and pulmonary venous rings contracted with U-46619) relaxed to 3 × 10\(^{-6}\) M 5,6-EETs (38). They have also reported that canine pulmonary venous rings preconstricted with PGF\(_{2\alpha}\), relaxed to micromolar concentrations of 5,6-EET after treatment with indomethacin but not with vehicle (39). In contrast, our data demonstrate that all regioisomers of EETs constrict isolated pressurized rabbit pulmonary arteries from ~250 to 500 µm in internal diameter. Pulmonary arteries preconstricted with KCl also contracted to 5,6-EET-ME, suggesting that increased tone accomplished by partial depolarization did not block 5,6-EET-ME-induced constriction. Furthermore, we found that pretreatment with the highly specific epoxygenase inhibitor MS-PPOH (29, 42) decreases basal tone and shifts the concentration-response curve of pulmonary artery rings to phenylephrine to the right, consistent with a preconstrictive contribution of endogenously formed EETs to enhancing pulmonary arterial tone. The differences in our observations and those of Tan et al. (41) and Stephenson et al. (38) suggest that the pulmonary vasoactive effects of EETs are not straightforward but appear to depend on factors such...
as species (and perhaps intrinsic cyclooxygenase capacity or density of thromboxane receptors in lung vascular tissue), concentration of EETs, and mechanism of delivery, size, and other characteristics of the vessels tested (arteries vs. veins), precontractile state of the vessel, and perhaps influence of surrounding tissues. Additional studies to determine the reasons for qualitatively different effects of EETs in these models will undoubtedly shed light on the function(s) of epoxyenase metabolites in the lung.

Our data demonstrate that contraction to 5,6-EET (and its methyl ester) is concentration, endothelium, and cyclooxygenase dependent. In addition, constriction could be blocked by pretreatment with the thromboxane-receptor antagonist SQ-29548. Together, these data suggest that EET-induced vasoconstriction is indirect and depends on cyclooxygenase-dependent synthesis of a thromboxane-like compound. Both 5,6- and 14,15-EETs may be metabolized by cyclooxygenase to vasoactive products (30). Although EET-associated relaxation in coronary arteries is cyclooxygenase independent (34), there is precedent for endothelium- and cyclooxygenase-dependent effects of EETs on vascular tone in other vascular beds. In the rat tail artery and isolated perfused rabbit kidney, vasodilatory responses to EETs were found to be endothelium as well as cyclooxygenase dependent (9). Similarly, dilation of rabbit pial arterioles to arachidonic acid and EETs could be blocked by pretreatment with indomethacin (14). Our data demonstrate indomethacin-inhibitable constriction to 5,6- and 14,15-EETs. One interpretation of these observations is that both EETs may act as substrates for cyclooxygenase in the lung and that the vasoconstrictive effects we report are dependent on cyclooxygenase metabolites of these eicosanoids. We do not favor this explanation for several reasons. First, it is not generally believed that region isoforms other than 5,6-EET can serve as a substrate for cyclooxygenase enzymes (30), although one group of investigators has suggested that 8,9-EET can be metabolized by cyclooxygenase to 11(R)-hydroxy-8(S),9(R)-epoxyeicosatrienoic acid (46). Second, the fact that constriction can be inhibited by the thromboxane-receptor antagonist SQ-29548 and preconstriction with thromboxane mimetic U-46619 suggests that thromboxane (or a closely related compound that acts through the thromboxane receptor) is the immediate mediator of constriction in our model. Further evidence against the hypothesis that EETs are metabolized by cyclooxygenase to a thromboxane-like constricting agent is our inability to demonstrate the formation of any indomethacin-inhibitable products of EETs. An alternative explanation, which we believe is more likely, is that EETs stimulate the release and/or metabolism of arachidonic acid (by increased intracellular calcium or some other indirect mechanism), which is in turn metabolized to a vasoconstrictor prostanooid, probably an endoperoxide PGH or thromboxane-like product. EETs effectively increase intracellular calcium levels in vascular smooth muscle cells and cortical collecting ducts cells, and stimulated prostaglandin synthesis in primary cultures of rabbit cortical collecting duct cells (16, 36). Furthermore, they stimulate tyrosine kinase activity in endothelial cells (20). Finally, EETs transcriptionally stimulate the expression of PGG-PGH synthase in intestinal crypt epithelial cells (31). Each of these actions may activate phospholipases and/or stimulate releases of vasconstrictive endoperoxides.

We conclude that EETs are products of rabbit pulmonary arteries and effect concentration-, cyclooxygenase-, and endothelium-dependent contraction of isolated pulmonary arteries. These data, together with the observation that EETs-induced constriction could be blocked by the thromboxane-receptor antagonist SQ-29548, suggest that these P-450-derived eicosanoids act indirectly through the synthesis and release of an endoperoxide-thromboxane-like compound. Blunted constriction to phenylephrine in pulmonary artery rings after epoxygenase inhibition raises the possibility that EETs may contribute to pulmonary artery tone, but the contribution of these metabolites to vascular tone under various physiological or pathophysiological conditions remains to be established.

We are grateful to Ying Gao for the excellent work with isolated pressurized vessels and assistance with Western blots and cytochrome P-450-assays. We also thank Jayashree Narayanan for assistance with HPLC studies and Michael Aebly for help with imaging.

The work was supported by the National Institutes of Health Grants HL-49294 (E. R. Jacobs) and GM-31278 (J. R. Falck). Address for reprint requests and other correspondence: E. R. Jacobs, Cardiovascular Research Center, 8701 Watertown Plank Rd, Milwaukee, WI 53226 (E-mail: ejacobs@post.its.mcm.edu). Received 18 March 1999; accepted in final form 13 September 1999.

REFERENCES