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Research Article

Cytochrome P450 metabolizes arachidonic acid to several unique and biologically active compounds in rabbit liver and kidney. Microsomal fractions prepared from rabbit lung homogenates metabolized arachidonic acid through cytochrome P450 pathways, yielding cis-epoxyeicosatrienoic acids (EETs) and their hydration products, vic-dihydroxyeicosatrienoic acids, mid-chain cis-trans conjugated dienols, and 19- and 20-hydroxyeicosatetraenoic acids. Inhibition studies using polyclonal antibodies prepared against purified CYP2B4 demonstrated 100% inhibition of arachidonic acid epoxide formation. Purified CYP2B4, reconstituted in the presence of NADPH-cytochrome P450 reductase and cytochrome b5, metabolized arachidonic acid, producing primarily EETs. EETs were detected in lung homogenate using gas chromatography/mass spectroscopy, providing evidence for the in vivo pulmonary cytochrome P450 epoxidation of arachidonic acid. Chiral analysis of these lung EETs demonstrated a preference for the 14(R),15(S)-, 11(S),12(R)-, and 8(S),9(R)-EET enantiomers. Both EETs and vic-dihydroxyeicosatrienoic acids were detected in bronchoalveolar lavage fluid. At micromolar concentrations, methylated 5,6-EET and 8,9-EET significantly relaxed histamine-contracted guinea pig hilar bronchi in vitro. In contrast, 20-hydroxyeicosatetraenoic acid caused contraction to near maximal tension. We conclude that CYP2B4, an abundant rabbit lung cytochrome P450 enzyme, is the primary constitutive pulmonary arachidonic acid epoxygenase and that these locally produced, biologically active eicosanoids may be involved in maintaining homeostasis within the lung.

The Rabbit Pulmonary Cytochrome P450 Arachidonic Acid Metabolic Pathway: Characterization and Significance

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Abstract

Cytochrome P450 metabolizes arachidonic acid to several unique and biologically active compounds in rabbit liver and kidney. Microsomal fractions prepared from rabbit lung homogenates metabolized arachidonic acid through cytochrome P450 pathways, yielding *cis*-epoxyeicosatrienoic acids (EETs) and their hydration products, *vic*-dihydroxyeicosatrienoic acids, mid-chain *cis–trans* conjugated dienols, and 19- and 20-hydroxyeicosatetraenoic acids. Inhibition studies using polyclonal antibodies prepared against purified CYP2B4 demonstrated 100% inhibition of arachidonic acid epoxide formation. Purified CYP2B4, reconstituted in the presence of NADPH-cytochrome P450 reductase and cytochrome b_5, metabolized arachidonic acid, producing primarily EETs. EETs were detected in lung homogenate using gas chromatography/mass spectroscopy, providing evidence for the in vivo pulmonary cytochrome P450 epoxidation of arachidonic acid. Chiral analysis of these lung EETs demonstrated a preference for the 14(*R*),15(*S*)-, 11(*S*),12(*R*)-, and 8(*S*),9(*R*)-EET enantiomers. Both EETs and *vic*-dihydroxyeicosatrienoic acids were detected in bronchoalveolar lavage fluid. At micromolar concentrations, methylated 5,6-EET and 8,9-EET significantly relaxed histamine-contracted guinea pig hilar bronchi in vitro. In contrast, 20-hydroxyeicosatetraenoic acid caused contraction to near maximal tension. We conclude that CYP2B4, an abundant rabbit lung cytochrome P450 enzyme, is the primary constitutive pulmonary arachidonic acid epoxygenase and that these locally produced, biologically active eicosanoids may be involved in maintaining homeostasis within the lung. (*J. Clin. Invest.* 1995. 95:2150–2160.)

Key words: eicosanoid • epoxygenase • epoxyeicosatrienoic acid • dihydroxyeicosatrienoic acid • hydroxyeicosatetraenoic acid

Introduction

Arachidonic acid is present in vivo esterified to the *sn*-2 position of cellular glycerophospholipids (1). Activation of hormonally sensitive phospholipases releases arachidonic acid (AA)[1] from the phospholipid pools, thus making it available for oxygenation by three different enzyme systems (1–3). PGH_2 synthases metabolize arachidonic acid, yielding PGH_2, which serves as the precursor of the prostaglandins, thromboxane, and prostacycline (2, 3). Lipoxygenases convert arachidonic acid to unstable hydroperoxy intermediates that go on to form the leukotrienes and hydroxyeicosatetraenoic acids (HETEs) (2, 3). Cytochrome P450 arachidonic acid monooxygenases catalyze the formation of epoxides (EETs), mid-chain *cis–trans* conjugated dienols (HETEs), and C-19/C-20 alcohols (19-OH-AA and 20-OH-AA) (2, 3).

The role of cytochrome P450 in the NADPH-dependent oxygenation of arachidonic acid in extrapulmonary tissues is well established (4–6). The P450-derived arachidonic acid metabolites have numerous biological activities, including effects on vascular tissues (7–10), modulation of membrane ion fluxes (11–13), stimulation of peptide hormone release (14, 15), inhibition of cyclooxygenase activity and platelet aggregation (16), angiogenic effects (17), and effects on neutrophil chemotaxis (18). McGiff (11) has recently proposed that the renal arachidonic acid monooxygenase is involved in the pathophysiology of hypertension. Using chiral-phase HPLC techniques, our laboratory has demonstrated that EET production by rat liver and kidney microsomal fractions is stereoselective and under P450 enzyme control (19, 20). Furthermore, chiral analysis of endogenous EET pools in rat liver (21), kidney (7), and plasma (22) demonstrates that the 14(*R*),15(*S*)-, 11(*S*),12(*R*)-, and 8(*S*),9(*R*)-EET enantiomers predominate. The EETs are enzymatically hydrated by epoxide hydrolases to produce the corresponding *vic*-dihydroxyeicosatrienoic acids (DHETs) (23, 24). The cytosolic epoxide hydrolase shows high affinity for the EETs ($K_m < 5\ \mu M$) and a selectivity for the endogenous enanti-

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1. *Abbreviations used in this paper:* AA, arachidonic acid; BAL, bronchoalveolar lavage; DHET, *vic*-dihydroxyeicosatrienoci acid; EET, *cis*-epoxyeicosatrienoic acid; GC, gas chromatography; HETE, hydroxyeicosatetraenoic acid; Me, methyl; MS, mass spectroscopy; NICI, negative-ion chemical ionization; P450, cytochrome P450 monooxygenase; PFB, pentafluorobenzyl; PSS, physiologic salt solution; R_t, retention time; TMS, trimethylsilyl.

omers (23). Importantly, the urinary excretion of DHETs is increased in pregnancy-induced hypertension (25).

Much of what we know about the cytochrome P450 monooxygenase system is derived from studies in hepatic tissues. Less is known about the pulmonary P450 monooxygenase system. Rabbit lung contains significant quantities of cytochromes P450 (26, 27), NADPH-cytochrome P450 reductase (27, 28), and epoxide hydrolases (29). These components have been shown by in situ hybridization and immunolocalization to be present primarily within Clara cells and type II pneumocytes, although they have also been detected in alveolar macrophages, goblet cells, and endothelial cells (26–28). Despite studies on the components of the pulmonary P450 system and their localization, the physiological significance of this enzyme system is unknown (30–32). Pulmonary P450 has long been thought to function primarily in the metabolism of exogenous compounds (including drugs and inhaled carcinogens) (30). Only recently has the pulmonary P450 metabolism of endogenous substances been reported (33, 34). We demonstrate here an active role for this enzyme system in the regio- and stereoselective oxygenation of arachidonic acid and suggest that its products may be of significance to lung physiology.

Methods

Preparation of microsomal fractions. Male New Zealand White rabbits (3–4 kg) were killed by lethal intravenous injection of sodium pentobarbital (50–60 mg/kg) and then exsanguinated by perforation of the right ventricle. The lungs were perfused in situ via the main pulmonary artery with ice-cold 0.15 M KCl and then removed, weighed, minced, washed twice with the perfusing solution, and immediately homogenized in 0.01 M Tris-Cl buffer, pH 7.5, containing 0.25 M sucrose (20 g of wet tissue per 100 ml). Microsomal fractions were obtained by differential centrifugation at 4°C as previously described (35) and used within 48 h of preparation. Protein determinations were performed according to the method of Bradford (36).

Microsomal incubations. Reaction mixtures containing 0.05 M Tris-Cl buffer, pH 7.5, 0.15 M KCl, 0.01 M $MgCl_2$, 8 mM sodium isocitrate, 0.5 IU/ml isocitrate dehydrogenase, and 1.0 mg/ml microsomal protein were constantly stirred at 30°C. After temperature equilibration, [1-^{14}C]arachidonic acid (0.8–1.2 μCi/μmol) was added to a final concentration of 75–100 μM. 1 min later, NADPH (1 mM, final concentration) was added. At 15-min intervals, aliquots were withdrawn, and the reaction products were extracted into ethyl ether, dried under a nitrogen stream, and analyzed by HPLC (35).

Incubations in reconstituted systems using purified pulmonary P450 enzymes. CYP2B4 and CYP4B1 were purified from rabbit lung microsomal fractions according to published procedures (37). The enzymes were judged homogeneous based on their electrophoretic properties (37). NADPH-cytochrome P450 reductase and cytochrome b_5 were purified as described (38, 39). The metabolism of arachidonic acid by purified P450s was reconstituted as previously described (19). Briefly, purified P450, NADPH-cytochrome P450 reductase, and cytochrome b_5 (1 μM each, final concentration) were mixed in the presence of sonicated L-α-dilauroyl-*sn*-glycero-3-phosphocholine (100–200 μg/ml). After 15 min at 4°C, the mixture was diluted fivefold with 0.05 M Tris-Cl buffer, pH 7.5, containing 0.15 M KCl, 0.01 M $MgCl_2$, 8 mM sodium isocitrate, and 0.5 IU/ml isocitrate dehydrogenase. Addition of [1-^{14}C]-arachidonic acid (75–100 μM, final concentration) was followed by initiation with NADPH (1 mM, final concentration). Incubations were done with constant mixing at 30°C. At different time points, samples were withdrawn, extracted into ethyl ether, dried under a nitrogen stream, and analyzed by HPLC (35).

Antibody inhibition studies. Polyclonal antibodies against purified rabbit lung P450 enzymes CYP2B4 and CYP4B1 and against NADPH-cytochrome P450 reductase were prepared as described (40). The immunospecificity of the anti-CYP2B4, anti-CYP4B1, and anti-reductase antibodies have been validated by Ouchterlony double immunodiffusion (40) and protein immunoblotting of lung microsomal fractions (41). Purified immune IgG or the corresponding control, nonimmune IgG fractions were added to the microsomal incubates at immunoglobulin/hemeprotein ratios ranging from 0.5 to 5 mg of IgG per mg of microsomal protein. After 20 min at 22°C, [1-^{14}C]arachidonic acid was added (75–100 μM, final concentration), the temperature was raised to 30°C, and the reaction was initiated by adding NADPH. Metabolites were extracted and resolved by HPLC (35).

Quantification of endogenous EETs in lung homogenate. Lungs were removed, perfused as previously described, and immediately homogenized in methanol/water (2:3, 20 ml per g of wet tissue) containing triphenylphosphine (0.1 mM, final concentration). An aliquot of the homogenate, corresponding to 0.5 g of lung wet tissue, was transferred to a test tube containing an equivalent volume of a 2-mM solution of triphenylphosphine in chloroform and a mixture of [1-^{14}C]8,9- 11,12-, and 14,15-EET (52–55 μCi/μmol, 30 ng each) as internal standards. After mixing, HCl was added to a final concentration of 0.75 M, and the suspension was centrifuged to effect phase separation. The aqueous phase was extracted once more with 2 vol of chloroform/methanol (2:1), the organic mixture was neutralized by the addition of 6 M NaOH, and the combined organic phases were evaporated under a nitrogen stream. Phospholipid-bound EETs were recovered by saponification (21), and the resulting material was dissolved in acetic acid/hexane (0.5:99.5 [vol/vol]) and applied to a SiO_2 column (200–400 mesh, 5 × 20 mm) (21). The EETs were eluted with acetic acid/ethyl ether/hexane (0.5:50:49.5 [vol/vol/vol]), and the eluent, containing a mixture of radiolabeled internal standards and total endogenous EETs, was resolved into individual regio- and stereoisomers by HPLC as described (21, 42, 43).

Quantification of endogenous EETs and DHETs in bronchoalveolar lavage fluid. Rabbits were anesthetized with sodium pentobarbital (40–50 mg/kg), and the thoracic cavity was entered to effect lung collapse and to allow direct visualization during lavage. The trachea was dissected free and cannulated. Bronchoalveolar lavage (BAL) was performed by instillation and withdrawal of three 60-ml aliquots of normal saline. The BAL fluid was immediately placed on ice in a flask containing triphenylphosphine (1 mg per ml of BAL fluid). BAL samples were extracted twice with 2 vol of chloroform/methanol (2:1) and once more with an equal volume of chloroform. The combined organic phases were concentrated and evaporated in tubes containing mixtures of [1-^{14}C]8,9-, 11,12-, and 14,15-EET and [1-^{14}C]5,6-, 8,9-, 11,12-, and 14,15-DHET internal standards (52–55 μCi/μmol, 30 ng each). Saponification to recover phospholipid-bound EETs (21) was followed by SiO_2 column purification (21). The eluent, containing a mixture of radiolabeled internal standards and total endogenous EETs and DHETs, was resolved into individual regioisomers by HPLC (21, 35). In some experiments, BAL fluid cells, obtained by centrifugation at 600 *g* for 15 min, were analyzed separately. Phospholipid-bound EETs were quantified as described previously (22, 44).

Gas chromatography/mass spectroscopy analysis. For analysis of EETs, aliquots of the individual EET–pentafluorobenzyl esters (EET-PFBs) were dissolved in dodecane and analyzed by gas chromatography/negative-ion chemical ionization/mass spectroscopy (GC/NICI/MS) on a Nermag R1010C quadrupole instrument (Nermag Corp., Malmarson, France) as described (21). Quantifications were made by GC/NICI/selected ion monitoring at m/z 319 (loss of PFB from endogenous EET-PFB) and m/z 321 (loss of PFB from [1-^{14}C]EET-PFB internal standard) (Fig. 1). The EET-PFB/[1-^{14}C]EET-PFB ratios were calculated from the integrated values of the corresponding ion current intensities. For analysis of DHETs, aliquots of the individual DHET-PFB-trimethylsilyl ether (DHET-PFB-TMS) derivatives were dissolved in dodecane and analyzed by GC/NICI/MS as described (20). Quantifications were made by GC/NICI/selected ion monitoring at m/z 481 (loss of PFB from endogenous DHET-PFB-TMS) and m/z 483 (loss of PFB from [1-^{14}C]DHET-PFB-TMS internal standard). The DHET-

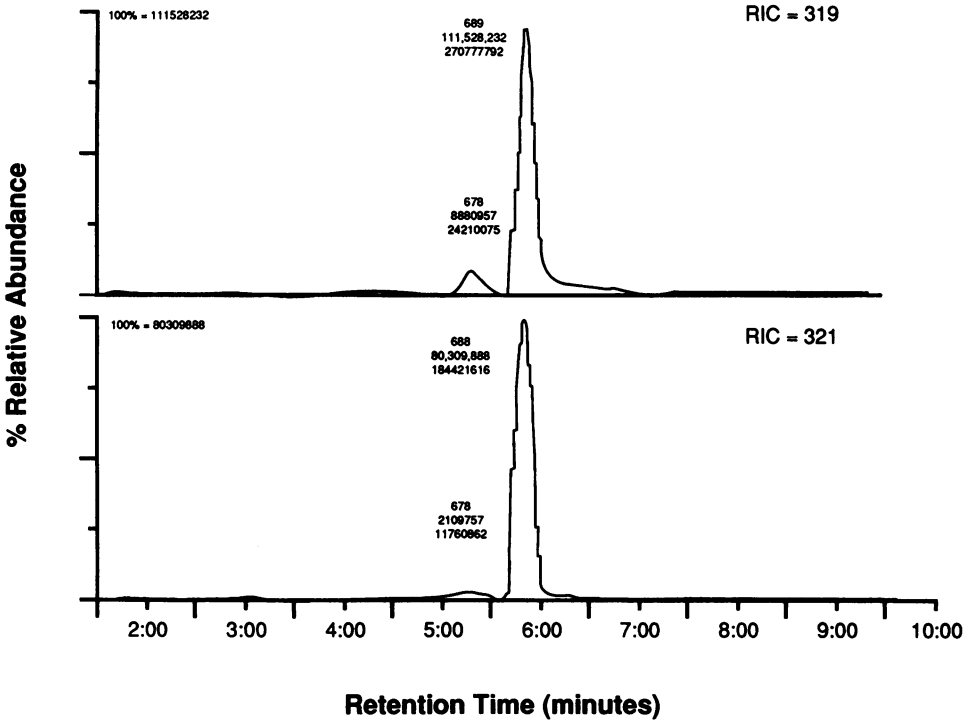

Figure 1. Selected ion current profile under NICI conditions for 14,15-EET extracted from rabbit BAL fluid. Purified rabbit BAL fluid 14,15-EET-PFB was analyzed by GC/NICI/selected ion monitoring at m/z 321 and 319 as described in Methods. The samples' $^{12}C/^{14}C$ ratios were calculated from the integration of the area under the observed peaks.

PFB-TMS/[1-^{14}C]DHET-PFB-TMS ratios were calculated from the integrated values of the corresponding ion current intensities.

Synthetic procedures. Racemic EETs were prepared as previously described (45, 46). The enantiomers of 14,15- and 8,9-EET were prepared by total asymmetric synthesis according to published procedures (47, 48). [1-^{14}C]EETs were synthesized from [1-^{14}C]arachidonic acid (52–57 μCi/μmol) by nonselective epoxidation (49). Enantiomerically pure [1-^{14}C]EETs were obtained by chiral-phase HPLC separation of racemic [1-^{14}C]EETs (42). DHETs and [1-^{14}C]DHETs were prepared by chemical hydration of individual EETs as described (23). All synthetic EETs and DHETs were purified by reverse-phase HPLC (35). 20-HETE was synthesized as described (50) and purified by reverse-phase HPLC (51). PGE_2 was obtained from Sigma Chemical Co. (St. Louis, MO) and was used without further purification. Methylations were performed using an ethereal solution of diazomethane (52). PFB esters were formed by reaction with PFB bromide as described (21). TMS ethers were prepared using 25% (vol/vol) bis(trimethylsilyl)-trifluoroacetamide in anhydrous pyridine as previously described (53).

Isolation of poly(A)$^+$ mRNA and Northern analysis. Rabbits were sacrificed as previously described, and their livers, lungs, kidneys, hearts, and brains were removed, immediately frozen in liquid nitrogen, and stored at −80°C. Within the next 48 h, total RNA was extracted by homogenization of frozen tissue in 5.5 M guanidinium thiocyanate containing 25 mM sodium citrate, 0.5% (wt/vol) sodium *N*-lauryl sarcosine, and 0.2 M 2-mercaptoethanol, shearing of DNA by repeated passage through a 20-gauge needle, and density gradient centrifugation on a cushion of 5.7 M CsCl containing 0.1 M EDTA (54). Poly(A)$^+$ mRNA was prepared by the oligo(dT)–cellulose method using an mRNA purification kit (Pharmacia LKB Biotechnology Inc., Almeda, CA). After ethanol precipitation and quantification by spectrophotometry, the poly(A)$^+$ mRNA (2–5 μg) was denatured and electrophoresed in a 1.2% agarose gel containing 0.2 M formaldehyde as described (54). After transfer to a nitrocellulose membrane, the blots were hybridized with the following sequence-specific oligonucleotide probes: CYP1A1 (5′-ACACCTGGACGTTGGCATTCTCGTCCAACC-3′, complementary to residues 674–703 of the CYP1A1 cDNA clone) (55); CYP2B4 and CYP2B5 (5′-AGGAAAGCCAGGAGGAGGAGCAGGCTGAACTCCAT-3′, complementary to residues 16–50 of the CYP2B4 cDNA clone and residues 38–72 of the CYP2B5 cDNA clone) (56); CYP4B1 (5′-CCCACAGGCCCAGGCGGGAGAGGAAGCCGAGCATC-3′, complementary to residues 24–58 of the CYP4B1 cDNA clone) (57); and a consensus oligonucleotide probe to CYP2C1, CYP2C2, CYP2C3, CYP2C4, CYP2C5, CYP2C14, CYP2C15, and CYP2C16 (5′-GGG-(C)TCCTGTCCTGCATGCAGGGGC-3′, complementary to residues 1063–1086 of the CYP2C16 cDNA clone) (58). Hybridizations were performed at 42°C in 50% formamide containing 0.75 M NaCl, 0.1% (wt/vol) SDS, 0.1% (wt/vol) sodium pyrophosphate, 4× Denhardt's solution, and 100 μg/ml salmon sperm DNA. Oligonucleotides were synthesized using the Cyclone Oligonucleotide Synthesizer (Waters Assoc., Marlborough, MA), purified by the Poly-Pak Cartridge Purification System (Glen Research Co., Sterling, VA), and end labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP (59).

Measurement of airway smooth muscle contractile force. Male Hartley guinea pigs (480–590 g) were sacrificed with an intraperitoneal overdose of urethane. Their lungs were removed and placed in physiological salt solution (PSS; composition: 117 mM NaCl, 4.75 mM KCl, 2.8 mM $CaCl_2$, 1.19 mM KH_2PO_4, 1.19 mM $MgSO_4$, 24.6 mM $NaHCO_3$, 5 mM glucose, 0.027 mM $CaNa_2EDTA$) aerated with 95% air/5% CO_2 at 22°C. Using a dissecting microscope, cylindrical segments of hilar bronchi (1.5–2.0 mm in length) were isolated from both lungs and cleaned of adherent tissue. Similar to methods used to study isolated rat airways (60), the segments were threaded onto two tungsten wires 50 μm in diameter. One of the wires was fixed to a plastic holder that was attached to a microdisplacement device. The other wire was fixed to a holder that was mounted on an isometric transducer (Harvard Apparatus, South Natick, MA) to measure contractile force (F). The airway segments were placed in 5-ml tissue baths containing PSS at 37°C and aerated with a mixture of air and CO_2 such that the P_{CO2} and P_{O2} were maintained at 40 and 140 mmHg, respectively. The internal circumference (C_I) of each tissue was incrementally changed by increasing the distance between the wires using the microdisplacement device to achieve an equilibrated resting force (F_R) of 100 mg. This C_I was maintained throughout the experiment. Mechanical responses were recorded on a four-channel strip chart recorder (Linearecorder WR3101; Western Graphtec, Irvine, CA).

The airway segments were allowed to equilibrate for 30–60 min at

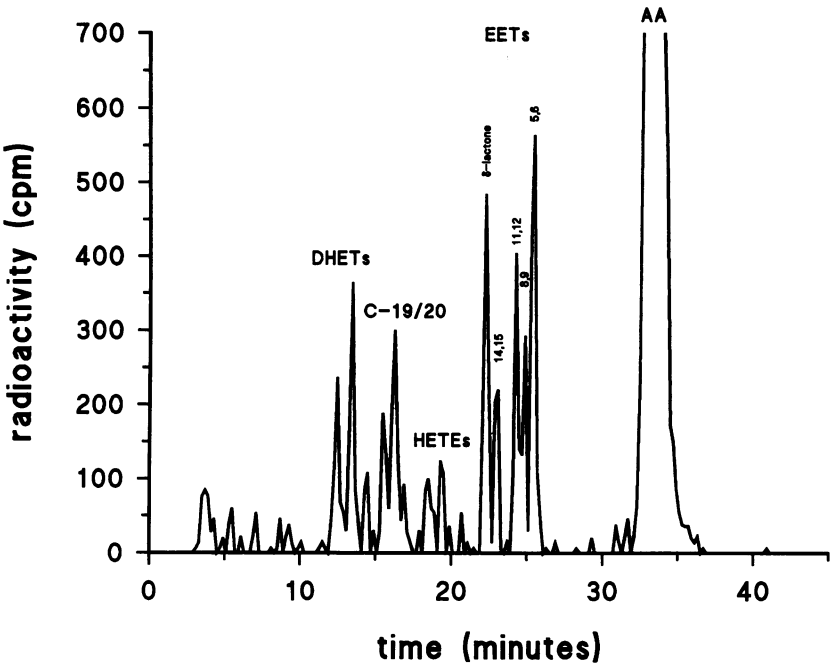

Figure 2. Reverse-phase HPLC chromatogram of organic soluble metabolites generated by incubations of rabbit lung microsomal fractions with arachidonic acid. Microsomal fractions (1 mg/ml protein) were incubated with [1-^{14}C]arachidonic acid (100 μM) at 30°C for 30 min in the presence of NADPH (1 mM) and an NADPH-regenerating system. The ethyl ether–soluble products were resolved by reverse-phase HPLC, and the eluent radioactivity was monitored with an on-line radioactive flow detector.

the appropriate C_I, during which time they were washed with fresh PSS every 15 min. Reference contractile force of each tissue to 1 mM bethanechol (F_{MAX}) was then determined. Baths were then washed with fresh PSS, and the tissues were allowed to equilibrate again at F_R over 45–60 min with washes of fresh PSS every 15 min. At this point, concentrations of histamine sufficient to produce a stable contractile force of ~ 50% of F_{MAX} (F_{HIST}) were incrementally added to the baths. Methylated 5,6-EET (5,6-EET-Me), 8,9-, 11,12- and 14,15-EET, 20-OH-AA, and PGE_2 were dissolved in ethanol (1 mM, final concentration) and serially diluted with PSS to allow concentration–response studies on the contractile force of the tissues at test compound concentrations ranging from 10^{-10} to 10^{-5} M. Control tissues were studied using the ethanol/PSS vehicle in equivalent volumes (1 mM, final ethanol concentration in the baths). After each incremental change in test compound concentration, F was monitored for at least 3 min or until a stable response was reached. At the end of the protocol, 1 μM isoproterenol was added to the baths, and F was monitored until a stable decrement in F_{HIST} was achieved. The maximum responses of the tissues to each alteration of the bath composition were expressed as $\%\Delta F_{HIST}$ and analyzed by repeated measures analysis of variance with contrast analysis of the planned comparisons (Statistica/W; StatSoft, Tulsa, OK).

Results

In vitro metabolism of arachidonic acid by pulmonary P450. Incubations of rabbit lung microsomal fractions with [1-^{14}C]-arachidonic acid in the presence of NADPH resulted in the formation of EETs, DHETs, HETEs, and C-19/C-20 alcohols (Fig. 2). We identified these metabolites by comparing their HPLC properties with those of authentic standards and by GC/MS analysis (35). None of these metabolites were formed in the absence of NADPH, showing that the reaction was P450 mediated. The predominant metabolites, accounting for 70% of the total products, were EETs and their hydration products, the

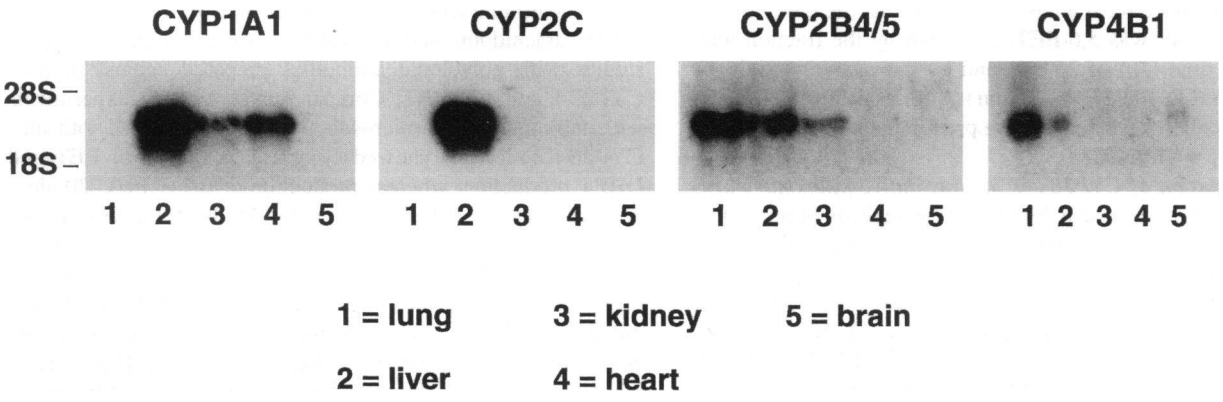

Figure 3. Northern blot analysis of poly(A)$^+$ mRNA extracted from rabbit tissues. 5 μg of poly(A)$^+$ mRNA isolated from rabbit liver, lung, kidney, heart, and brain was submitted to electrophoresis and blot hybridized with 5′ end sequence–specific oligonucleotide probes to CYP1A1, CYP2B4/5, and CYP4B1, or with a consensus oligonucleotide probe to CYP2C1, CYP2C2, CYP2C3, CYP2C4, CYP2C5, CYP2C14, CYP2C15, and CYP2C16 as described in Methods.

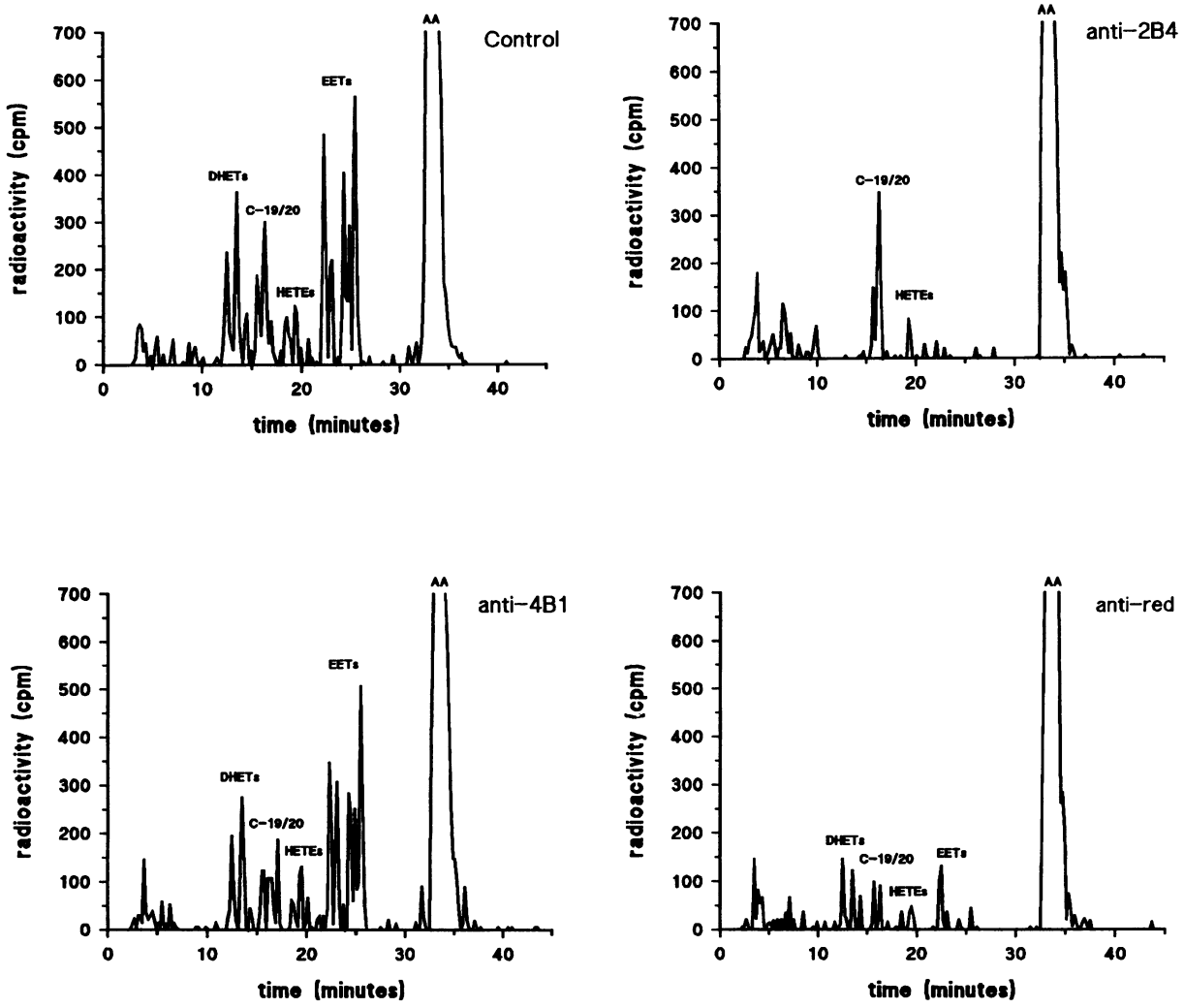

Figure 4. Antibody inhibition studies. Polyclonal IgG antibodies against purified rabbit lung CYP2B4, CYP4B1, and NADPH-cytochrome P450 reductase, or the corresponding control, nonimmune IgG fractions (2–3 mg of IgG per mg of microsomal protein), were added to the microsomal incubates as described in Methods. [1-^{14}C] arachidonic acid was added, and the reaction was initiated with addition of NADPH. The ethyl ether–soluble metabolites were separated by reverse-phase HPLC as described.

DHETs (Fig. 2). The C-19/C-20 alcohols and HETEs accounted for 16% and 14% of the total products, respectively (Fig. 2). Regiochemical analysis revealed that the predominant EET regioisomer was 5,6-EET, as shown by the fraction with a retention time (R_t) of 26 min and by the recovery of the δ-lactone of 5,6-DHET (R_t = 22 min). Similar analysis of the C-19/C-20 alcohols revealed that the predominant isomer was 20-OH-AA (R_t = 16 min).

Identification of CYP2B4 as a constitutive pulmonary arachidonic acid epoxygenase. Northern blot analysis of poly(A)$^+$ mRNA extracted from rabbit lung, liver, kidney, heart, and brain was performed using radiolabeled, sequence-specific oligonucleotide probes to CYP1A1, CYP2B4/5, CYP4B1 and a consensus oligonucleotide probe to several 2C subfamily members (including CYP2C1, CYP2C2, CYP2C3, CYP2C4, CYP2C5, CYP2C14, CYP2C15, and CYP2C16). Message for CYP1A1 was abundant in liver, less abundant in heart and kidney, and present at very low levels in lung (Fig. 3). Message for the 2C subfamily P450s was also most abundant in liver and undetectable in lung (Fig. 3). In contrast, messages for CYP2B4/5 and CYP4B1 were present at high levels in lung but were less abundant in extrapulmonary tissues (Fig. 3).

To determine which pulmonary P450 isoform was responsible for arachidonic acid epoxidation, we performed inhibition studies with polyclonal antibodies prepared against purified CYP2B4 and CYP4B1. Compared with control experiments with nonimmune IgG, microsomal fractions incubated with anti-CYP2B4 antibodies showed complete inhibition of EET and DHET production, whereas production of the C-19/C-20 alcohols was largely unchanged (Fig. 4). Antibody titration studies demonstrated that complete inhibition of epoxygenase activity occurred at antibody concentrations as low as 1 mg of IgG per mg of microsomal protein. In contrast, anti-CYP4B1 antibodies did not significantly alter P450-mediated metabolism of arachidonic acid at concentrations as high as 3 mg of IgG per mg of microsomal protein (Fig. 4). Experiments using antibodies to the NADPH-cytochrome P450 reductase showed a marked (85–90%) inhibition of arachidonic acid metabolism (Fig. 4), thus confirming the role of cytochrome P450 in arachidonic acid epoxidation and C-19/C-20 oxidation. As shown in Fig. 4,

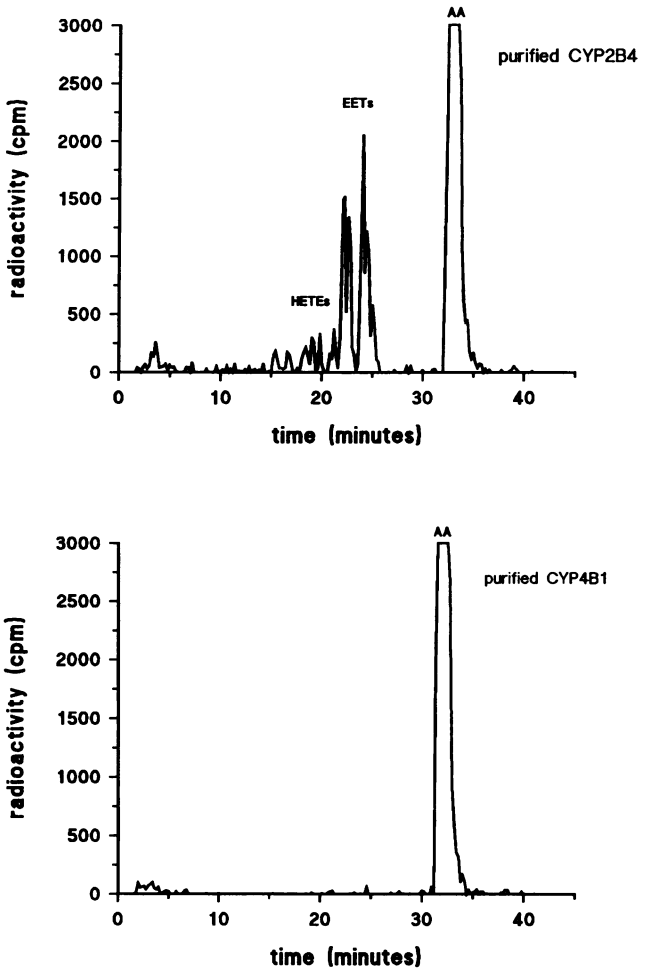

Figure 5. Reverse-phase HPLC chromatogram of organic soluble metabolites generated during incubations of purified CYP2B4 and CYP4B1 with [1-^{14}C]arachidonic acid. Purified CYP2B4 or CYP4B1, NADPH-cytochrome P450 reductase, and cytochrome b_5 were incubated with [1-^{14}C]arachidonic acid, NADPH, and an NADPH-regenerating system as described in Methods. Reaction products were resolved by reverse-phase HPLC as described.

HETE production was minimally altered in the presence of anti-reductase antibodies, suggesting that HETE formation may be P450 independent.

To evaluate further the P450 arachidonic acid monooxygenase activity of CYP2B4 and CYP4B1, electrophoretically homogeneous lung P450s (CYP2B4 or CYP4B1), cytochrome b_5, and NADPH-cytochrome P450 reductase were reconstituted in the presence of L-α-dilauroyl-*sn*-glycero-3-phosphocholine and incubated with arachidonic acid in the presence of NADPH. CYP2B4 metabolized arachidonic acid at a rate of 53 pmol of product formed per nmol of P450 per min, producing primarily EETs (80% of total) (Fig. 5). Regiochemical analysis of the EET products demonstrated a unique preference for the 5,6-EET, as shown by the recovery of 5,6-EET (R_t = 25 min) and the δ-lactone of 5,6-DHET (R_t = 22 min) (Fig. 5). The regiochemistry of the epoxygenase products formed by CYP2B4 (Fig. 5) was similar to that formed during incubations of rabbit lung microsomal fractions with arachidonic acid (Fig. 2). In contrast, the metabolism of arachidonic acid by CYP4B1 was undetectable (Fig. 5). The relative abundance of CYP2B4 message in rabbit lung (Fig. 3), together with the documented epoxygenase activity of this hemeprotein (Figs. 4 and 5), indicates that CYP2B4 is the predominant constitutive pulmonary arachidonic acid epoxygenase.

Table I. Regio- and Stereochemical Composition of Rabbit Lung EETs

Regioisomer	Concentration	Distribution	Enantioselectivity	
			R,S	*S,R*
	ng/g lung	*% total*	*%*	*%*
14,15-EET	147	40	68	32
11,12-EET	103	28	38	62
8,9-EET	116	32	41	59

Values are the means of at least four different experiments with SE < 15% of the mean. Distribution is expressed as a percentage of the total lung EETs (5,6-EET not measured).

Detection of EETs and DHETs in rabbit lung homogenate and BAL fluid. Rabbit lung homogenate contained ~ 350 ng of total EET per g of lung (Table I). The major EET regioisomer present in lung homogenate was 14,15-EET (40% of total), followed by roughly equal amounts of 11,12- and 8,9-EET (28 and 32% of total, respectively) (Table I). The labile 5,6-EET suffered extensive decomposition during the extraction and purification process used and therefore could not be quantified. Chiral analysis of rabbit lung EETs revealed that the 14(*R*),15(*S*)-, 11(*S*),12(*R*)-, and 8(*S*),9(*R*)-EET were the predominant antipods (Table I).

BAL fluid contained ~ 400 pg of total EET per ml of lavage and 150 pg of total DHET per ml of lavage (Table II). The major EET regioisomer present in BAL fluid was 14,15-EET (47% of total), followed by roughly equal amounts of 11,12- and 8,9-EET (24 and 29% of total, respectively) (Table II). As before, the labile 5,6-EET could not be quantified. The major DHET regioisomer present in BAL fluid was 5,6-DHET (41% of total) (Table II). BAL fluid contained smaller amounts of 14,15-, 11,12-, and 8,9-DHET (22, 15, and 22% of total, respectively) (Table II). The majority (69%) of the total EETs were present within the cellular compartment of BAL fluid. Further-

Table II. Regioisomeric Composition of Rabbit BAL Fluid EETs and DHETs

Regioisomer	Concentration	Distribution
	pg/ml lavage	*% total*
14,15-EET	182	47
11,12-EET	90	24
8,9-EET	107	29
14,15-DHET	31	22
11,12-DHET	21	15
8,9-DHET	31	22
5,6-DHET	57	41

Values are the means of at least five different experiments with SE < 15% of the mean. Distribution is expressed as a percentage of the total lung EETs (5,6-EET not measured) or DHETs.

more, most (> 80%) of the EETs in both the cellular and extracellular compartments were esterified to phospholipids. Control experiments were performed to confirm that artifactual EET or DHET formation did not occur during the BAL extraction and purification process. Using 0.5 mg of HPLC-purified arachidonic acid added to BAL fluid, we were able to demonstrate that artifactual EET or DHET formation was negligible under the conditions of analysis described in Methods.

Biological activities of P450 arachidonic acid metabolites. Tissue bath studies were performed on 24 cylindrical segments of hilar bronchi from 17 guinea pigs. From an F_R of 111.0±11.6 mg (mean±SE), F_{MAX} to 1 mM bethanechol was 782.1±81.8 mg. F_{HIST} was 51.1±2.3% of F_{MAX}, requiring a concentration of 2.4±0.3 μM histamine. 1 μM isoproterenol consistently relaxed the tissues from F_{HIST} (ΔF_{HIST} = −83.0±2.9%). Both 5,6-EET-Me (n = 3) and 8,9-EET (n = 3) caused significant concentration-related relaxation of the tissues below F_{HIST} at concentrations of 10^{-5}–10^{-6} M (ΔF_{HIST} = −37.4±13.0% and −24.9±4.9% at 10^{-5} M for 5,6-EET-Me and 8,9-EET, respectively, $P < 0.05$) (Fig. 6, *A* and *B*). Compared with the eicosanoid smooth muscle relaxant PGE_2 (n = 3), 5,6-EET-Me and 8,9-EET were ~ 6–6.5-fold less potent at 10^{-6} M and 1.5–2-fold less potent at 10^{-5} M in this system (data not shown). 11,12-EET (n = 3) caused a mild reduction in contractile force (ΔF_{HIST} = −7.3±3.8% at 10^{-5} M, P = 0.09), whereas 14,15-EET (n = 3) was without effect (ΔF_{HIST} = 7.7±7.1% at 10^{-5} M, $P > 0.10$). 20-OH-AA (n = 4) caused a marked concentration-related contraction above F_{HIST} (ΔF_{HIST} = +55.0±23.6% at 10^{-5} M, $P < 0.05$) (Fig. 6 *C*). The ethanol/PSS vehicle (n = 5) had no significant effect on F_{HIST} at any tested concentration (ΔF_{HIST} = −3.9±3.2% at 10^{-3} M ethanol, $P > 0.10$). Thus, arachidonic acid is metabolized by the pulmonary cytochrome P450 monooxygenase enzyme system into compounds that both reduce (5,6-EET and 8,9-EET) and increase (20-OH-AA) bronchial smooth muscle tone.

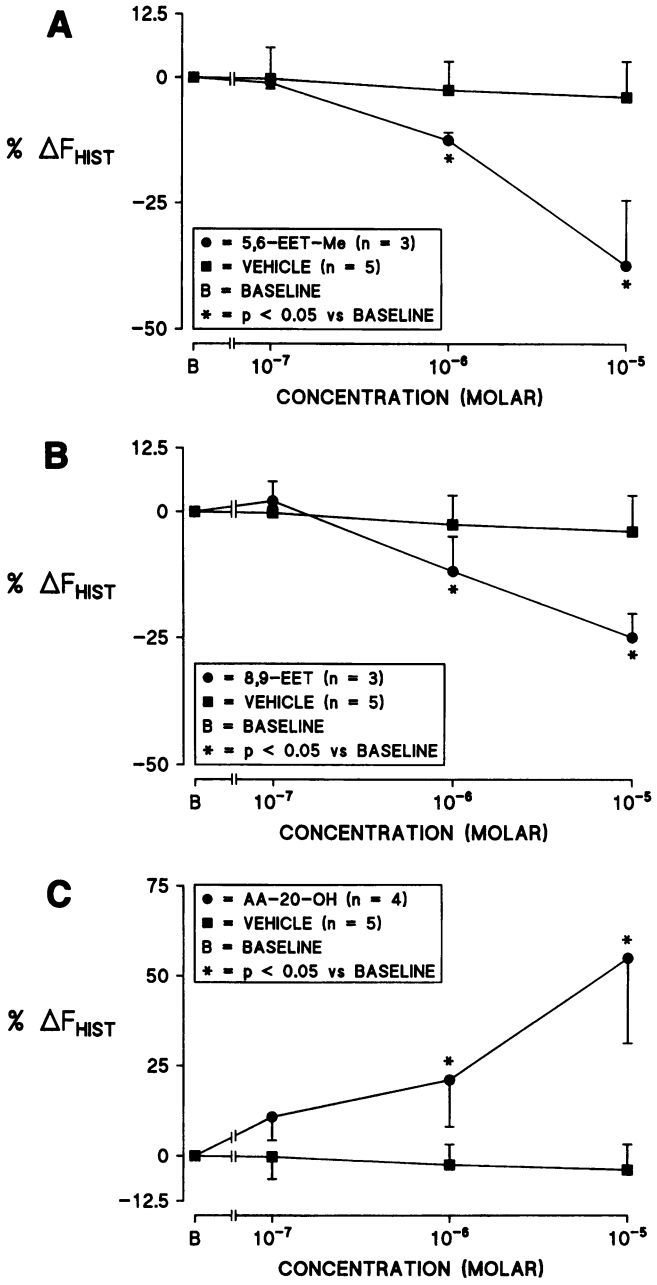

Figure 6. Effects of 5,6-EET-Me, 8.9-ET, and 20-OH-AA on guinea pig bronchial smooth muscle tone. Cylindrical segments of guinea pig hilar bronchi were mounted on a myograph as described in Methods. Tissues were precontracted to 50% maximal force with histamine, and concentration–response curves were determined using synthetic HPLC-purified P450 arachidonic acid metabolites (10^{-7}–10^{-5} M). Values shown are the means of at least three different experiments ±SE.

Discussion

Although several studies have suggested a possible link between P450 and hypoxic pulmonary vasoconstriction (31, 61), pulmonary P450 has long been thought to function primarily in the metabolism of exogenous compounds, including xenobiotics and inhaled carcinogens (30). Oxygenation of these chemicals has been shown to cause either activation or detoxification (30). Only recently has the pulmonary P450 metabolism of endogenous substances been reported (33, 34). We have provided data to suggest an active role of the pulmonary P450 system in the regio- and stereoselective oxygenation of endogenous substrates (arachidonic acid). Incubations of rabbit lung microsomal fractions with radiolabeled arachidonic acid resulted in the formation primarily of EETs (Fig. 2). Regiochemical analysis revealed that the 5,6-EET was the predominant EET regioisomer formed (Fig. 2). The profile of metabolites formed by rabbit lung microsomal fractions was qualitatively different from that reported for rat and rabbit liver microsomal fractions, in which 11,12- and 14,15-EET were the predominant regioisomers (19, 62). The rabbit lung product profile was also different from that observed for rat and rabbit kidney, in which C-19/C-20 alcohols constituted > 50% of the total products and 11,12-EET was the predominant EET regioisomer produced (5, 6, 20). In fact, rabbit lungs are unique in their selectivity for epoxidation of arachidonic acid at the 5,6 position. This is particularly interesting given the important and potent biologic activities attributed to 5,6-EET and/or its methyl ester (11, 63). Of note, Oliw and Moldeus (64) have previously observed the rabbit lung microsomal metabolism of arachidonic acid into primarily C-19/C-20 alcohols and PGs, with only small amounts of DHETs produced. The inability of these investigators to detect significant pulmonary microsomal epoxygenase activity may be related to differences in experimental conditions. More recently, Knickle and Bend (65) have demonstrated

that microsomal fractions prepared from guinea pig lungs metabolize arachidonic acid to C-16 through C-20 alcohols and EETs.

Northern blot analysis revealed that messages for CYP2B4/5 and CYP4B1 were present at high levels in rabbit lung but were less abundant in extrapulmonary tissues (Fig. 3). Previous work has shown that message for CYP2B4 is abundant in both rabbit liver and lung (56). Protein immunoblotting studies have shown that, together, the CYP2B4 and CYP4B1 proteins account for better than two-thirds of total rabbit lung microsomal P450 content (30). Additionally, rabbit lung CYP2B4 protein has been immunoquantitated (27). To determine which of the abundant pulmonary P450 isoforms is responsible for arachidonic acid epoxidation in rabbit lung, we performed antibody inhibition studies and purified enzyme reconstitution experiments. Anti-CYP2B4 antibodies completely inhibited EET and DHET production without affecting the production of C-19/C-20 alcohols (Fig. 4). Furthermore, electrophoretically homogeneous lung CYP2B4 actively metabolized arachidonic acid, producing primarily EETs (Fig. 5). Importantly, the regiochemistry of the epoxygenase products formed by purified CYP2B4 was strikingly similar to that of the products formed during incubations of rabbit lung microsomal fractions with arachidonic acid (Figs. 2 and 5). The relative abundance of CYP2B4 message and protein in rabbit lung (Fig. 3) (30, 56), together with the documented epoxygenase activity of this hemeprotein (Figs. 4 and 5), indicates that CYP2B4 is the predominant constitutive pulmonary arachidonic acid epoxygenase. Previous work has demonstrated that the predominant arachidonic acid epoxygenases present in rat kidney (20), rabbit kidney (66), and guinea pig lung (65) microsomal fractions belong to the CYP2B or CYP2C subfamilies. Additionally, rat liver P450s belonging to the CYP1A and CYP2B subfamilies have been shown to generate EETs (19). Although our data strongly suggest that the predominant constitutive epoxygenase present in rabbit lung is CYP2B4, the additional role of other inducible P450s (e.g., CYP1A1) cannot be excluded based on the data presented.

Whereas in vitro studies are an indispensable tool for the enzymatic characterization of metabolic pathways, they provide only limited information with regard to the in vivo production and concentration of formed metabolites. The documentation of EETs and DHETs as endogenous constituents of rabbit lung provided further evidence to support the in vivo pulmonary P450 metabolism of arachidonic acid. Using a combination of HPLC and GC/MS, we detected substantial amounts of EETs in rabbit lung homogenate (Table I). However, exposure to oxidizing gases can lead to the accumulation of lipid epoxides that originate from nonenzymatic processes (67). A decisive criterion for the enzymatic origin of the lung EETs would be the demonstration of their stereoselective biosynthesis (21). Therefore, we performed chiral analysis of the rabbit lung EETs and demonstrated that the 14(*R*),15(*S*)-, 11(*S*),12(*R*)-, and 8(*S*),9(*R*)-EET enantiomers were the predominant antipods (Table I). This stereochemical preference was similar to that previously reported in rat liver (21), kidney (7), and plasma (22). Importantly, since the endogenous enantiomers (i.e., 14(*R*),15(*S*)-, 11(*S*),12(*R*)-, and 8(*S*),9(*R*)-EET) are also the preferred substrates for lung cytosolic epoxide hydrolase (23), the observed EET stereochemical composition (Table I) may underestimate the stereoselectivity of in vivo EET production by the pulmonary epoxygenase(s).

BAL fluid also contained significant quantities of 14,15-, 11,12-, and 8,9-EET (Table II). Unfortunately, the primary metabolite produced during rabbit lung microsomal incubations with arachidonic acid, 5,6-EET (Fig. 2), suffers extensive decomposition during the extraction and purification process and therefore cannot be quantified. However, the concentration of 5,6-DHET, the more stable metabolite of 5,6-EET, can be measured using the techniques described in Methods. Importantly, the major DHET regioisomer present in BAL fluid was 5,6-DHET (Table II), thus confirming the in vivo biosynthesis of 5,6-EET and supporting the results obtained from microsomal incubations and purified enzyme reconstitution studies. Perdik et al. (68) have recently reported, in preliminary form, that isolated, perfused rabbit lungs produced arachidonic acid metabolites ex vivo that coeluted with synthetic EETs on reverse-phase HPLC. They also noted that the production of these metabolites was inhibited by pretreatment with the cytochrome P450 inhibitor, SKF 525-A (68). Their findings are consistent with the demonstration here of EETs in lung homogenate and BAL fluid.

Previous work has shown that, once formed, the EETs may undergo a variety of further metabolic transformations (2, 3). First, the EETs can be metabolized by cytosolic or microsomal epoxide hydrolases to produce DHETs (23, 24). The cytosolic enzyme not only has high affinity for the EETs ($K_m < 5\ \mu M$), but also demonstrates a substrate preference for the endogenous enantiomers (23). The detection of DHETs in BAL fluid suggests the in vivo pulmonary hydration of EETs and represents a potential pathway for the metabolism and disposition of these biologically active compounds. Second, the EETs are found esterified to the *sn*-2 position of hepatic glycerophospholipids (44). Stereochemical analysis of hepatic EET–phospholipid pools shows that acylation is stereoselective, with preference for the endogenous enantiomers (44). Our finding that > 80% of the EETs in BAL fluid were esterified to phospholipids suggests that a similar process occurs in the lung. The presence of membrane phospholipids containing oxidized moieties has been shown to alter cell membrane physicochemical properties, including fluidity (69, 70), and may also alter their surface active properties. Thus, one possible role of EET–phospholipids within the airspace may be to modulate the properties of surfactant. Finally, the EETs can be conjugated to glutathione (2) or be further oxidized by either cyclooxygenase (71, 72) or by cytochrome P450 monooxygenase (2). Together, these metabolic pathways tightly control the steady-state concentration of the EETs.

The cytochrome P450 arachidonic acid metabolites have been shown to be biologically active both in vivo and in numerous in vitro animal models (2, 3, 11, 63). For example, the EETs (*a*) stimulate glucagon and insulin release from isolated rat pancreatic islets (14) and somatostatin release from the median eminence (15); (*b*) activate Na^+/H^+ exchange and are mitogenic in rat renal mesangial cells (12); (*c*) inhibit cyclooxygenase activity and platelet aggregation (16); and (*d*) cause stereoselective renal artery vasoconstriction (7) and mesenteric (9) and cerebral (73) artery vasodilation. Most of the biologic effects of EETs occur at concentrations of 10^{-7}–10^{-5} M (11, 63), with the exception of their effects on peptide hormone secretion, which occur in the nanomolar range (15). The effects of the EETs on vascular smooth muscle suggested that cytochrome P450 arachidonic acid metabolites might also affect lung bronchial smooth muscle tone. We examined the effects

of synthetic, HPLC-purified P450 arachidonic acid metabolites on airway smooth muscle contractile force using cylindrical segments of guinea pig hilar bronchi. Guinea pigs have been extensively used to study the response of airway smooth muscle to a variety of contractile and relaxant substances, largely because isolated guinea pig airways react in a fashion similar to that of isolated human airways (74, 75). Furthermore, the similarities between the guinea pig and the rabbit pulmonary cytochrome P450 arachidonic acid monooxygenase pathways (65) justify the use of guinea pig bronchi for these studies. We found that both 5,6-EET-Me and 8,9-EET caused significant relaxation of the bronchial tissues, whereas 20-OH-AA caused marked contraction (Fig. 6). Moreover, the EETs affected bronchial smooth muscle tone in vitro at concentrations of 10^{-6}–10^{-5} M (Fig. 6), consistent with the known potency of these compounds on mesenteric vascular smooth muscle (9). However, the observed potency of the EETs on bronchial smooth muscle in the present study was less than that of other lipid mediators (e.g., PGE_2) in this system. The observed biologic actions of the EETs likely depend on their local concentration at the site of activity (i.e., within the microenvironment of the effector cells). Our measurements of EETs in lung homogenate and BAL fluid do not allow for an accurate estimation of local concentrations. Though we cannot rule out that the effects of the EETs on airway smooth muscle are only pharmacologic, the localization of CYP2B4 by immunohistochemistry to cells within the rabbit airway (including Clara and goblet cells) (26–28), together with the known arachidonic acid epoxygenase activity of this hemeprotein (Figs. 4 and 5), suggests that high levels of the EETs might be attainable in the vicinity of airway smooth muscle.

We studied the biologic effects of 5,6-EET-Me because, in our hands, 5,6-EET is quite labile in aqueous media. Tsubosaka et al. (76) have recently reported in preliminary form that 5,6-EET relaxed carbachol-contracted tracheal smooth muscle in vitro in a dose-dependent manner. They further noted that pretreatment with indomethacin, a potent cyclooxygenase inhibitor, reduced the 5,6-EET–dependent tracheal smooth muscle relaxation (76). Whereas 5,6-EET is known to be a substrate for cyclooxygenase, resulting in 5,6-epoxy PGs (71, 72), esterified fatty acids (such as 5,6-EET-Me) are poor substrates for this enzyme system (77). Further investigation is required to determine whether 5,6-EET is the biologically active arachidonic acid metabolite, or whether further metabolism by cyclooxygenase is necessary for optimal activity. Cytochrome P450 metabolites of arachidonic acid are known to have effects on Na^+/K^+-ATPase (11, 13) and calcium ion flux (78), processes that are intimately involved in excitation–contraction coupling. Determining whether the effects of the EETs and/or 20-OH-AA on bronchial smooth muscle tone observed in the present study (Fig. 6) are secondary to effects of these metabolites on ion transport or are a consequence of the direct effects of these compounds on the airway smooth muscle will also require further investigation.

In summary, we provide evidence that rabbit lung microsomal fractions metabolize arachidonic acid via cytochrome P450 monooxygenase to form EETs, their hydration products (DHETs), HETEs, and C-19/C-20 alcohols. Antibody inhibition studies and P450 reconstitution experiments demonstrate that CYP2B4 is a constitutive pulmonary arachidonic acid epoxygenase. We show that both EETs and DHETs are detectable in significant amounts in rabbit lung homogenate and BAL fluid. Chiral analysis of the endogenous EETs confirms their biosynthetic origin. Finally, we show that several of the cytochrome P450 arachidonic acid metabolites alter airway smooth muscle tone in vitro at micromolar concentrations.

In conclusion, our data suggest that, in addition to the cyclooxygenase and lipoxygenase pathways, the cytochrome P450 monooxygenase pathway is an important member of the pulmonary arachidonic acid metabolic cascade. The regio- and stereoselective generation of these unique, biologically active eicosanoids may play an important role in the control of lung homeostasis under normal conditions. Furthermore, altered local concentration of these compounds may contribute to lung dysfunction in selected pathophysiological states.

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