0026-895X/96/051111-07$3.00/0

MOLECULAR PHARMACOLOGY, 50:1111–1117 (1996).

CYP2J Subfamily P450s in the Lung: Expression, Localization, and Potential Functional Significance

DARRYL C. ZELDIN, JULIE FOLEY, JIXIANG MA, JAMES E. BOYLE, JORGE M. S. PASCUAL, CINDY R. MOOMAW, KENNETH B. TOMER, CHARLES STEENBERGEN, and SHU WU

Laboratories of Pulmonary Pathobiology (D.C.Z., J.M., J.E.B., J.M.S.P., C.R.M., S.W.), Experimental Pathology (J.F.), and Molecular Biophysics (K.B.T.), National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709, Department of Medicine (J.E.B.), University of North Carolina, Chapel Hill, North Carolina 27599, and the Departments of Medicine (J.M.S.P.) and Pathology (C.S.), Duke University Medical Center, Durham, North Carolina 27719

Received July 2, 1996; Accepted August 7, 1996

SUMMARY

Cytochrome P450 (P450) monooxygenases catalyze the epoxidation of arachidonic acid to form epoxyeicosatrienoic acids, which modulate bronchial smooth muscle tone and airway transepithelial ion transport. We recently described a new human P450 arachidonic acid epoxygenase (CYP2J2) and the corresponding rat homologue (CYP2J3). Northern analysis of lung RNA using CYP2J cDNA probes demonstrated that CYP2J2 and CYP2J3 mRNAs were expressed in the lung. Immunoblotting of microsomal fractions prepared from human and rat lungs using a polyclonal antibody raised against recombinant human CYP2J2 revealed a single 56-kDa band confirming abundant pulmonary CYP2J2 and CYP2J3 protein expression. Immunohistochemical analysis of formalin-fixed paraffin-embedded human and rat lung sections using the anti-human CYP2J2 IgG and avidin/biotin/peroxidase detection showed that CYP2J proteins were primarily expressed in ciliated epithelial cells lining the airway. Prominent staining was also noted in nonciliated airway epithelial cells, bronchial and pulmonary vascular smooth muscle cells, pulmonary vascular endothelium, and alveolar macrophages, whereas less intense staining was noted in alveolar epithelial cells. Endogenous epoxyeicosatrienoic acids were detected in both human and rat lung using gas chromatography/mass spectrometry, thus providing direct evidence for the *in vivo* human and rat pulmonary P450 metabolism of arachidonic acid. Based on these data, we conclude that CYP2J2 and CYP2J3 are abundant pulmonary arachidonic acid epoxygenases and that CYP2J products, the epoxyeicosatrienoic acids, are endogenous constituents of human and rat lung. In addition to known effects on airway smooth muscle tone and transepithelial electrolyte transport, the localization of CYP2J proteins to vascular smooth muscle and endothelium suggests that epoxyeicosatrienoic acids may also be involved in the modulation of pulmonary vascular tone.

Pulmonary P450s have been proposed to play important roles in the biotransformation of xenobiotics and in the activation/detoxification of inhaled chemical carcinogens and lung toxins (1–5). A number of P450 enzymes have been shown to be constitutively expressed in the lung, including members of the CYP1A, CYP2A, CYP2B, CYP2E, CYP2F, and CYP4B subfamilies (6–12).[1] NADPH-cytochrome P450 reductase, another component of the mixed-function oxidase enzyme system, has also been shown to be expressed in the lung (1, 8, 13). Immunohistochemical studies and *in situ* hybridization have demonstrated that these P450 enzyme components are primarily present within nonciliated bronchiolar epithelial (Clara) cells and type II pneumocytes, although low levels of expression have been described in alveolar macrophages, goblet cells, ciliated epithelial cells, and vascular endothelial cells (8, 14–16). Despite extensive studies describing the components of the pulmonary P450 system and their cellular localization, little is known about the function of these enzymes with respect to normal lung physiology or pathophysiology. Several studies have suggested a link between lung P450 and hypoxic pulmonary vasoconstriction (17, 18). More recently, pulmonary P450s have been shown to catalyze the oxidation of arachidonic acid and other eicosanoids, thus suggesting a potential functional role of these hemoproteins in the bioactivation of endogenous fatty acid substrates in the lung (19–23).

Arachidonic acid can be oxidized by at least three distinct enzymatic pathways (24–27). Prostaglandin H_2 synthases metabolize arachidonic acid to prostaglandins, thromboxane, and prostacyclin. Lipoxygenases convert arachidonic acid to leukotrienes and hydroxyeicosatetraenoic acids. P450 mo-

This work was supported in part by National Institutes of Health Contract N01-ES35356 and the Bryan Alzheimer Disease Research Center Rapid Autopsy Program (National Institutes of Health Grant P50-AG05128).

[1] The cytochrome P450 nomenclature of Nelson *et al.* (58) is used throughout the article.

ABBREVIATIONS: P450, cytochrome P450; EET, *cis*-epoxyeicosatrienoic acid; HPLC, high performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; PFB, pentafluorobenzyl; SDS, sodium dodecyl sulfate.

nooxygenases catalyze the formation of EETs, hydroxyeicosatetraenoic acids, and C19/C20 alcohols of arachidonic acid. The cyclooxygenase and lipoxygenase metabolites of arachidonic acid are endogenous constituents of the lung and have been shown to have potent effects on airway and vascular smooth muscle tone, pulmonary vascular permeability, mucus secretion, and lung cell growth and differentiation (28–31). Much less information is available about the presence and/or biological actions of P450-derived arachidonic acid metabolites in the lung. Microsomal fractions prepared from guinea pig and rabbit lungs metabolize arachidonic acid to EETs as the principal reaction products (21, 22). Furthermore, CYP2B4 seems to be the primary constitutive pulmonary arachidonic acid epoxygenase present in rabbit and guinea pig lung (21, 22). Our group and others have demonstrated that several of the EET regioisomers cause a dose-dependent relaxation of guinea pig airway smooth muscle *in vitro* (22, 32). More recently, we have shown that 11,12-EET causes a dose-dependent increase in rat tracheal transepithelial voltage variation and decrease in transepithelial short circuit current variation, likely mediated through inhibition of a conductive Cl^- pathway (33). The effects of EETs on vascular smooth muscle tone in extrapulmonary tissues suggest that these arachidonic acid metabolites may also possess vasoactive properties in the lung (34–37). Importantly, lung lavage fluid arachidonic acid epoxygenase metabolites are increased after exposure to high levels of oxygen in both infants and lambs, suggesting that EETs may be involved in the pathogenesis of hyperoxic lung injury (38, 39).

Researchers in our laboratory recently cloned a new human P450 cDNA (CYP2J2) (40) and the corresponding rat homologue (CYP2J3),[2] both of which were highly expressed in extrahepatic tissues. The recombinant rat and human CYP2J proteins were active in the metabolism of arachidonic acid to EETs (40).[2] The purpose of this investigation was to (a) examine the expression and immunohistochemical localization of CYP2J P450s in human and rat lung and (b) determine whether CYP2J products, the EETs, were produced *in vivo* in human and rat lung. Based on molecular, immunological, and biochemical data, we conclude that (a) human and rat lungs contain an active arachidonic acid epoxygenase of the CYP2J subfamily that is primarily localized to airway epithelial cells, bronchial and vascular smooth muscle cells, vascular endothelium, and alveolar macrophages and (b) in addition to the cyclooxygenase and lipoxygenase pathways, the P450 epoxygenase pathway is an important member of the pulmonary arachidonic acid metabolic cascade.

Experimental Procedures

Materials. [α-^{32}P]dATP and [1-^{14}C]arachidonic acid were purchased from DuPont-New England Nuclear (Boston, MA). *Escherichia coli* polymerase I was purchased from New England Biolabs (Beverly, MA). Triphenylphosphine, α-bromo-2,3,4,5,6-pentafluorotoluene, *N*,*N*-diisopropylethylamine, *N*,*N*-dimethylformamide, and diazald were purchased from Aldrich Chemical (Milwaukee, WI). All other chemicals and reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise specified. Normal human lung was obtained through the Cooperative Human Tissue Network (National Disease Research Interchange, Philadelphia, PA) or from local tissue donors. Rat lung tissues were obtained from male Fischer 344 rats that had been fed *ad libitum* and killed by lethal CO_2 inhalation.

Isolation of mRNA and nucleic acid blot hybridization analysis. Normal human and rat lung tissues were snap-frozen in liquid nitrogen immediately after collection and stored at −80° until use. Total RNA was extracted using the guanidinium thiocyanate/cesium chloride density gradient centrifugation method as described previously (41). Poly(A)$^+$ mRNA was prepared by the oligo(dT)-cellulose method using reagents supplied by Pharmacia (Piscataway, NJ). For Northern blot analysis, lung mRNA (5 μg) was denatured and electrophoresed in 1.2% agarose gels containing 0.2 M formaldehyde. After capillary-pressure transfer to GeneScreen Plus nylon membranes (New England Nuclear Research Products, Boston, MA), the blots were hybridized with either the 1.9-kb CYP2J2 cDNA probe (human) or the 1.8-kb CYP2J3 cDNA probe (rat), both labeled by nick translation using *E. coli* polymerase I and [α-^{32}P]dATP (40).[2] Hybridizations were performed at 42° in 50% formamide containing 1 M NaCl, 1% (w/v) SDS, 10% (w/v) dextran sulfate, and 0.1 mg/ml heat-denatured salmon sperm DNA.

Protein immunoblotting and immunohistochemistry. Microsomal fractions were prepared from frozen normal human and rat lung tissues by differential centrifugation at 4° as described previously (22). For some experiments, rats were pretreated with either phenobarbital (80 mg/kg/day intraperitoneally for 3 days followed by the addition of 0.05% phenobarbital sodium salt to drinking water for 10 days), β-naphthoflavone (40 mg/kg/day intraperitoneally for 4 days), clofibrate (250 mg/kg/day intraperitoneally for 4 days), or acetone (1% in drinking water for 7 days). Polyclonal anti-human CYP2J2 IgG was raised in New Zealand White rabbits against the purified, recombinant CYP2J2 protein and affinity purified as described previously (40). For immunoblotting, lung microsomal fractions were electrophoresed in SDS-10% (w/v) polyacrylamide gels (80 × 80 × 1 mm), and the resolved proteins were transferred electrophoretically onto nitrocellulose membranes. Membranes were immunoblotted using rabbit anti-human CYP2J2 IgG, goat anti-rabbit IgG conjugated to horseradish peroxidase (BioRad, Richmond, CA) and the ECL Western Blotting Detection System (Amersham International, Buckinghamshire, UK) as described previously (40). Neither preimmune IgG nor rabbit non-immune IgG (Biogenex, San Ramon, CA) significantly cross-reacted with microsomal fractions prepared from human or rat lung. The anti-CYP2J2 IgG recognized a single protein band corresponding to purified recombinant human CYP2J2 in microsomal fractions prepared from several different human tissue samples and did not cross-react with the following human CYP1- and CYP2-family P450s: CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, and CYP2E1 (40). The anti-CYP2J2 IgG also immunoreacted with a single protein band corresponding to recombinant rat CYP2J3 in microsomal fractions prepared from different rat tissues but did not cross-react with the following rat P450s: CYP1A1, CYP2A1, CYP2B1, CYP2B2, CYP2C11, CYP2C13, CYP2C23, and CYP2E1.[3] Antibodies to rat CYP1A1, CYP2B1, CYP2E1, and CYP4A1 were purchased from GENTEST (Woburn, MA) and used according to the manufacturer's instructions.

For immunohistochemistry, rat and human lung tissues were fixed in 10% neutral buffered formalin, processed routinely, and embedded in paraffin. Localization of CYP2J2 and CYP2J3 protein expression was investigated using the anti-CYP2J2 IgG (1:200 dilution) on serial sections (5–6 μm) of human and rat lung. Slides were deparaffinized in xylene and hydrated through a graded series of ethanol to 1× Automation buffer (25 mM Tris · HCl, pH 7.5, 150 mM NaCl, plus nonhazardous proprietary reagents) (Biomeda, Burlingame, CA) washes. Endogenous peroxidase activity was blocked with 3% (v/v) hydrogen peroxide for 15 min. After rinsing in 1× automation buffer, slides were microwave treated, cooled, and blocked with

[2] S. Wu, E. Murphy, S. Gabel, K. B. Tomer, J. Foley, C. Steenbergen, J. R. Falck, C. R. Moomaw, and D. C. Zeldin. Molecular cloning, expression, and functional significance of a cytochrome P450 highly expressed in rat heart myocytes. Submitted for publication.

normal goat serum, and the primary antibody was applied for 30 min. Preimmune rabbit IgG was used as the negative control in place of the primary antibody. The bound primary antibody was visualized by avidin-biotin-peroxidase detection using the Vectastain Rabbit Elite Kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions and using 3,3′-diaminobenzidine as the color-developing reagent. Slides were counterstained with Harris hematoxylin, dehydrated through a graded series of ethanol-to-xylene washes, and cover-slipped with Permount^{M} (Fisher, Springfield, NJ). Specific CYP2J2 and CYP2J3 immunohistochemical staining was confirmed by prestaining adsorption of the anti-CYP2J2 IgG with a 300-fold molar excess of the purified, recombinant CYP2J2 antigen in 140 mM NaCl, 4 mM KCl, and 10 mM sodium phosphate buffer, pH 7.4, at 4° for 12 hr.

Detection of endogenous EETs in human and rat lung. Methods used to quantify endogenous EETs present in human and rat lung were similar to those used to quantify endogenous EETs in rat liver (42) and human heart (40). Briefly, freshly obtained lung tissues (~0.5 g each) were frozen in liquid nitrogen and immediately homogenized in 10–15 ml of 10 mM sodium phosphate buffer, pH 7.4, containing 140 mM NaCl, 4 mM KCl, and 1 mg/ml triphenylphosphine, a hydroperoxide-reducing agent. The lung homogenate was extracted twice, under acidic conditions, with two volumes of chloroform/methanol (2:1) and once again with an equal volume of chloroform, and the combined organic phases were evaporated in tubes containing mixtures of [1-^{14}C]8,9-EET, [1-^{14}C]11,12-EET, and [1-^{14}C]14,15-EET (55–57 μCi/μmol, 80 ng each) internal standards. Saponification to recover phospholipid-bound EETs was followed by SiO_2 column purification. The eluent, containing a mixture of radiolabeled internal standards and total endogenous lung EETs, was resolved into individual regioisomers and enantiomers by HPLC as described previously (42–44). For analysis, aliquots of individual EET-PFB esters were dissolved in dodecane and analyzed by GC/MS on a VG TRIO-1 quadrupole mass spectrometer (Fisons/VG, Altrincham, Manchester, UK) operating under negative-ion chemical ionization conditions (source temperature, 100°; ionization potential, 75 eV; filament current, 500 μA) at unit mass resolution and using methane as a bath gas. Quantification was made through selected ion monitoring of 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). The EET-PFB/[1-^{14}C]EET-PFB ratios were calculated from the integrated values of the corresponding ion current intensities.

Other methods. EETs were prepared by total chemical synthesis according to published procedures (45, 46). [1-^{14}C]EET internal standards were synthesized from [1-^{14}C]arachidonic acid (55–57 μCi/μmol) by nonselective epoxidation as described previously (47). All synthetic EETs were purified by reverse-phase HPLC. Methylations were performed using an ethereal solution of diazomethane (48). PFB esters were formed by reaction with PFB bromide as described previously (42). Protein determinations were performed according to the method of Bradford (49).

Results

Expression of CYP2J2 and CYP2J3 in the lung by Northern blot analysis and protein immunoblotting. High stringency blot hybridization of poly(A)$^+$ mRNA extracted from different rat and human lung tissues using the radiolabeled CYP2J3 and CYP2J2 cDNA probes, respectively, produced a 1.8–2.0-kb band in each lane, demonstrating that CYP2J message was expressed in the lung (Fig. 1). The CYP2J3 cDNA also hybridized with a 4.4-kb band in rat lung RNA (Fig. 1). The identity of this larger transcript remains unknown, but it may represent an alternate splice variant of CYP2J3 or a new rat P450 that shares nucleic acid sequence homology with CYP2J3. Immunoblotting studies using polyclonal antibodies raised against recombinant human CYP2J2 that cross-reacted with rat CYP2J3 but not with other human or rat P450s demonstrated an electrophoretically distinct band at ~56 kDa in microsomal fractions prepared from human and rat lung, indicating that CYP2J2 and CYP2J3 proteins were abundantly expressed in the pulmonary tissues (Fig. 2). There was remarkably little interindividual or interanimal variation in the expression of CYP2J2 or CYP2J3 mRNA or protein in different lung specimens (Figs. 1 and 2). Pretreatment of animals with phenobarbital, β-naphthoflavone, clofibrate, or acetone induced CYP2B-, CYP1A-, CYP4A-, and CYP2E-subfamily P450s, respectively, in rat liver but had no effect on the pulmonary expression of CYP2J3 (data not shown). Based on these data, we conclude that (a) CYP2J2 mRNA and protein are expressed in human lung, (b) CYP2J3 mRNA and protein are expressed in rat lung, (c) there is relatively low interindividual and interanimal differences in expression of CYP2J2 and CYP2J3 in the lung, and (d) pulmonary CYP2J3 expression is not inducible with phenobarbital, β-naphthoflavone, clofibrate, or acetone.

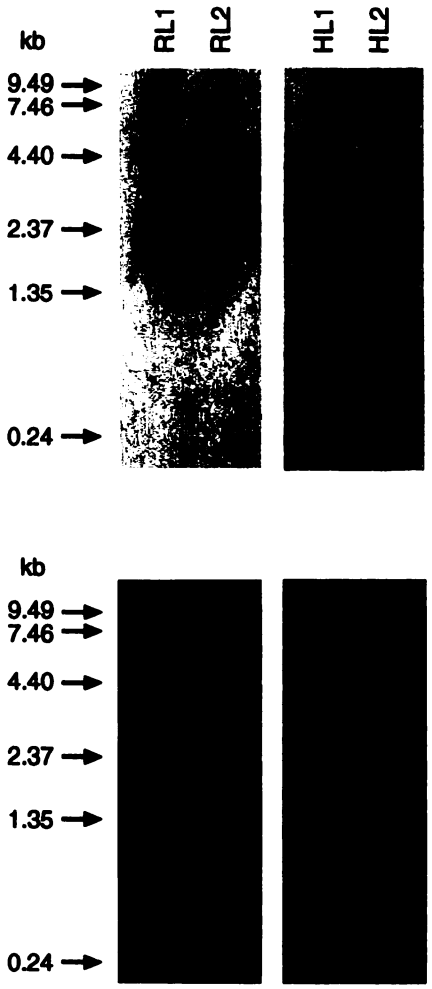

Fig. 1. Northern blot analysis of mRNA extracted from rat (*RL1 and RL2*) and human (*HL1 and HL2*) lung tissues. Poly(A)$^+$ mRNA (5 μg) isolated from rat and human lung was denatured, electrophoresed in a 1.2% agarose gel containing 0.2 M formaldehyde, transferred to a nylon membrane, and blot hybridized with radiolabeled (rat) CYP2J3 or (human) CYP2J2 cDNA probes as described in Experimental Procedures. *Top*, after a 48-hr exposure time. *Bottom*, ethidium bromide-stained gel before transfer.

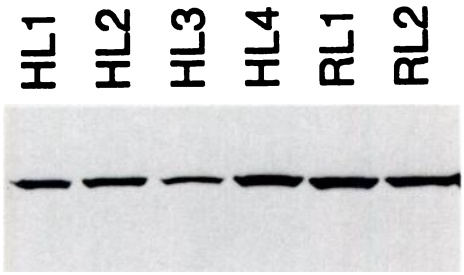

Fig. 2. Pulmonary expression of CYP2J2 and CYP2J3 by protein immunoblotting. Microsomal fractions prepared from four different human lung (*HL1–HL4*) and two different rat lung (*RL1–RL2*) specimens (40 μg of microsomal protein/lane) were electrophoresed on SDS-10% polyacrylamide gels, and the resolved proteins were transferred to nitrocellulose, immunoblotted with affinity purified rabbit anti-human CYP2J2 IgG and goat anti-rabbit IgG conjugated to horseradish peroxidase, and visualized using the ECL detection system and autoradiography as described in Experimental Procedures.

Localization of CYP2J2 and CYP2J3 proteins in the lung by immunohistochemistry. To determine the distribution of CYP2J proteins in the lung, we stained formalin-fixed paraffin-embedded human and rat lung tissue sections using the anti-CYP2J2 IgG. As shown in Fig. 3, human CYP2J2 immunoreactivity was primarily present in epithelial cells lining the airway. Positive staining was present in both ciliated and nonciliated epithelial cells but seemed to be more prominent in the ciliated cells (Fig. 3A). Prominent epithelial staining was present throughout the airway from trachea to bronchioles (data not shown). Intense staining was also present in bronchial smooth muscle cells (Fig. 3A), pulmonary vascular smooth muscle cells (Fig. 3C), endothelium lining both large and small pulmonary vessels (Fig. 3, A and C), and alveolar macrophages (Fig. 3E). Less-intense staining was present in alveolar epithelial cells (Fig. 3E), whereas connective tissue cells did not stain (Fig. 3, A, C, and E). Both preimmune IgG and rabbit nonimmune IgG produced negative staining throughout the human lung (Fig. 3, B, D, and F). Furthermore, prestaining absorption of anti-CYP2J2 IgG with excess purified, recombinant CYP2J2 abolished the positive reaction, thus demonstrating the specificity of the immunostaining for the CYP2J2 protein (data not shown).

The anti-CYP2J2 IgG produced a similar staining pattern in sections of rat lung (Fig. 4). Thus, prominent staining was present in ciliated and nonciliated epithelial cells lining the large (data not shown) and small airways (Fig. 4, A, C, and E). Strong positive staining was also present in alveolar macrophages and vascular endothelial cells, whereas less-intense staining was present in bronchial and vascular smooth muscle cells and alveolar lining cells (Fig. 4, A, C, and E). Both preimmune IgG and rabbit nonimmune IgG produced negative staining throughout the rat lung (Fig. 4, B, D, and F). Furthermore, prestaining absorption of anti-CYP2J2 IgG with excess purified, recombinant CYP2J2 abolished the positive reaction, thus demonstrating the specificity of the immunostaining for the CYP2J3 protein (data not shown).

Detection of EETs in human and rat lung by GC/MS. Using a combination of HPLC and GC/MS, we detected substantial amounts of EETs in both human and rat lung tissue.

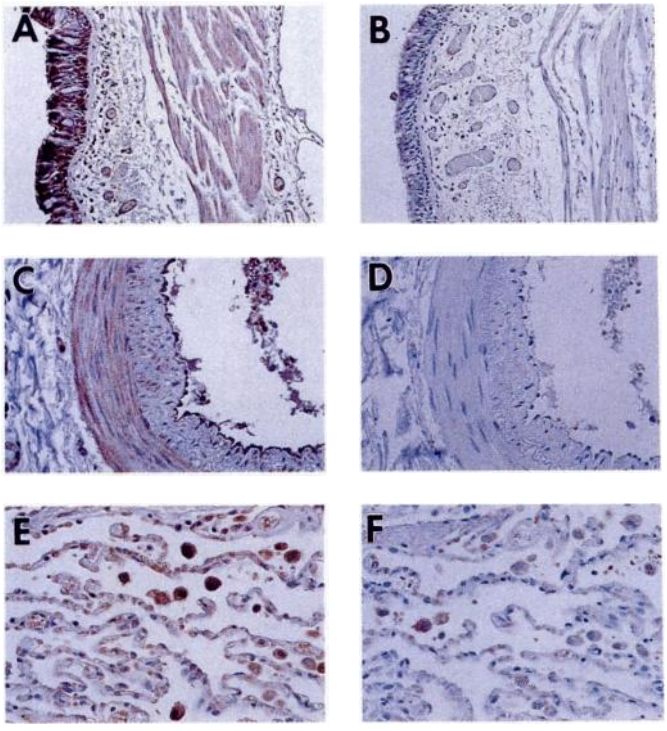

Fig. 3. Localization of CYP2J2 in human lung by immunohistochemistry. Adjacent sections of human lung immunostained with (A, C, and E) rabbit anti-human CYP2J2 IgG or (B, D, and F) preimmune IgG. A, Large airway with strongly positive luminal epithelial cells and positive staining in vascular endothelial cells and bronchial smooth muscle cells. B, Major pulmonary artery with strong endothelial staining and positive staining in vascular smooth muscle cells. C, Alveolar area with intense staining of macrophages and less-intense staining of alveolar lining cells. Magnification: A and B, 13.2×; C–F, 26.4×.

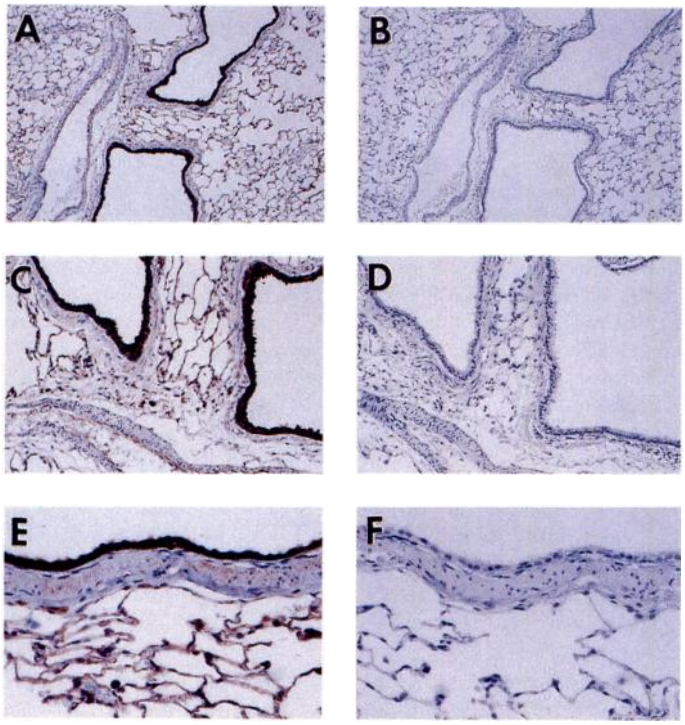

Fig. 4. Localization of CYP2J3 in rat lung by immunohistochemistry. Photomicrographs of adjacent sections of rat lung immunostained with (A, C, and E) rabbit anti-human CYP2J2 IgG or (B, D, and F) preimmune IgG. Intense staining in small airway epithelial cells. Positive staining is also present in vascular endothelial cells, vascular and bronchial smooth muscle cells, alveolar macrophages, and alveolar lining cells. Magnification: A and B, 6.6×; C and D, 13.2×; E and F, 26.4×.

TABLE 1

Regiochemical and stereochemical composition of human lung EETs

The enantiomers of human lung 14,15-EET, 11,12-EET, and 8,9-EET were extracted, purified, and quantified as described in Experimental Procedures. Concentration values shown are mean ± standard error of three determinations on lung specimens from different patients. For enantioselectivity, the standard error was <10% of the mean.

Regioisomer	Concentration	Distribution	Enantioselectivity	
			R,S	*S,R*
	ng/g of lung	*% total*		
14,15-EET	48 ± 10	43	64	36
11,12-EET	29 ± 5	26	62	38
8,9-EET	34 ± 8	31	47	53
5,6-EET	ND	ND	ND	ND

N.D., not determined.

As shown in Table 1, human lung contained ~110 ng of total EET/g of lung. The predominant regioisomer present was 14,15-EET (43% of the total), followed by approximately equal amounts of 11,12-EET and 8,9-EET (26% and 31% of the total, respectively) (Table 1). The labile 5,6-EET underwent extensive decomposition during the extraction and purification process and therefore could not be quantified. Chiral analysis of human lung EETs revealed that the 14(*R*),15(*S*)-EET and 11(*R*),12(*S*)-EET were the predominant antipodes (optical purity, 64% and 62%, respectively) (Table 1). In contrast, 8,9-EET was recovered in a nearly racemic mixture (Table 1). Importantly, the regiochemistry of endogenous human lung EETs was similar to that of recombinant CYP2J2 products (40), thus suggesting that CYP2J2 was one of the predominant enzymes responsible for epoxidation of arachidonic acid pools in human lung.

Rat lung contained ~130 ng of total EET/g of lung (Table 2). As was the case in human lung, the predominant regioisomer present in rat lung was 14,15-EET (57% of the total), followed by approximately equal amounts of 11,12-EET and 8,9-EET (17% and 26% of the total, respectively) (Table 2). As before, the labile 5,6-EET could not be quantified. Chiral analysis of rat lung EETs revealed that 14(*R*),15(*S*)-EET and 11(*S*),12(*R*)-EET were the predominant antipodes (optical purity, 64% and 60%, respectively) (Table 2). In contrast, 8,9-EET was recovered from rat lung in a nearly racemic mixture (Table 2). Importantly, the regiochemical properties of rat lung EETs were similar to those of the recombinant CYP2J3 enzyme,[2] suggesting that CYP2J3 was one of the primary enzymes responsible for epoxidation of endogenous arachidonic acid pools in rat lung.

TABLE 2

Regiochemical and stereochemical composition of rat lung EETs

The enantiomers of rat lung 14,15-EET, 11,12-EET, and 8,9-EET were extracted, purified, and quantified as described in Experimental Procedures. Concentration values shown are mean ± standard error of six determinations on lung specimens from different animals. For enantioselectivity, standard error was <5% of the mean.

Regioisomer	Concentration	Distribution	Enantioselectivity	
			R,S	*S,R*
	ng/g of lung	*% of total*		
14,15-EET	73 ± 6	57	64	36
11,12-EET	22 ± 2	17	40	60
8,9-EET	34 ± 2	26	50	50
5,6-EET	ND	ND	ND	ND

N.D., not determined.

Discussion

The pulmonary P450 system has long been thought to function primarily in the metabolism of exogenous compounds, including inhaled drugs and carcinogens (1–5). Oxidation of these chemicals can cause either activation or detoxification (1–5). During the past decade, there has been an increased awareness that this ubiquitous enzyme system may also be involved in the bioactivation of endogenous substrates such as arachidonic acid (19–23). In this report, we provide molecular and immunological evidence to show that CYP2J-subfamily P450s are highly expressed in both human and rat lung and that expression is localized to specific cell types within the airway, pulmonary vasculature, and lung parenchyma. Furthermore, we provide biochemical data to demonstrate that CYP2J products, the EETs, are present *in vivo* in human and rat lung.

Previous work has shown constitutive expression of a number of P450 monooxygenases in the lung, including members of the CYP1A, CYP2A, CYP2B, CYP2E, CYP2F, and CYP4B subfamilies (6–12). Our data demonstrate that in addition to these well-characterized P450s, a newly described P450 belonging to the CYP2J subfamily is constitutively expressed in rat and human lung at both the mRNA and protein levels (Figs. 1 and 2). A number of factors are known to alter the expression of lung P450s, including induction of enzyme synthesis by exogenous chemicals (4). In this regard, several investigators have reported relatively large interindividual variation in the pulmonary expression of CYP2B-, CYP2F-, and CYP4B-subfamily P450s (9, 11, 50). In addition, exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin has been shown to induce CYP1A1 in rabbit lung (7, 8, 12). The remarkably constant expression of CYP2J2 in different human lung specimens, together with the fact that pretreatment of rats with common P450 inducers does not alter the expression of CYP2J3, suggests that the CYP2J enzymes may be less susceptible to induction by environmental factors.

Immunolocalization studies in rabbits, hamsters, rats, and mice have demonstrated that the lung cells with the highest levels P450 proteins are Clara cells and type II pneumocytes (8, 14–16, 51, 52). Furthermore, P450 monooxygenase activities have been demonstrated *in situ* in Clara cells and in isolated preparations of both Clara cells and type II pneumocytes (8, 52–54). P450 proteins have also been documented, albeit at markedly lower levels, in alveolar macrophages, ciliated epithelial cells, goblet cells, and vascular endothelial cells (8, 15, 16). The distribution of CYP2J2 and CYP2J3 in human and rat lung, respectively, seems to be unique to this subfamily of hemoproteins (Figs. 3 and 4). Thus, high expression levels were evident throughout the airway epithelium, in both ciliated and nonciliated epithelial cells, but with highest expression in the ciliated cells. Prominent expression was also present in smooth muscle cells surrounding the bronchi and pulmonary artery branches, vascular endothelium, and alveolar macrophages. To our knowledge, this is the first report documenting the presence of a P450 enzyme in either pulmonary artery vascular or bronchial smooth muscle cells. Coceani *et al.* (55) recently showed that a P450

belonging to the CYP3A subfamily is expressed in vascular smooth muscle cells of the aorta and ductus arteriosus.

The cellular localization of CYP2J proteins in the lung may have important functional implications. For example, the expression of CYP2J2 and CYP2J3 in airway epithelial cells suggests a potential role for the EETs in regulating the volume and composition of the airway surface liquids, which affect lung mucociliary clearance. In this regard, our group recently demonstrated that 11,12-EET causes a dose-dependent increase in rat tracheal transepithelial voltage variation and a corresponding decrease in transepithelial short circuit current variation, likely mediated through inhibition of a conductive Cl^- pathway (33). The localization of CYP2J2 and CYP2J3 in airway smooth muscle cells has important implications given that CYP2J products, the EETs, were recently shown to have potent effects on bronchial smooth muscle tone (22). The localization of CYP2J2 and CYP2J3 in pulmonary vascular smooth muscle and endothelium suggests that the EETs may also be involved in modulation of lung vascular tone, especially given the well-documented effects of the EETs in controlling vascular tone in extrapulmonary tissues (34–37). Further work will be necessary to better define the functional significance of CYP2J products in lung physiology and pathophysiology.

Although *in vitro* studies are important for the enzymatic characterization of metabolic pathways, they provide limited information with respect to the *in vivo* production and concentration of the formed metabolites. The demonstration of EETs as endogenous constituents of human and rat lung provided further evidence to support the *in vivo* pulmonary P450 metabolism of arachidonic acid. Compared with liver and kidney cortex, lung contained ~3-6-fold fewer total EETs/g of tissue (36, 42, 56, 57). However, compared with heart, lung contained ~2-fold more total EETs/g of tissue (40).[2] The regiochemical and stereochemical profile of human lung EETs was similar to that reported for human kidney cortex and human liver but different from that reported for human heart (which produced racemic mixtures of 11,12-EET and 8,9-EET) (40, 56, 57). Although the regiochemical profile of rat lung EETs was similar to that of both rat liver and kidney, the stereochemistry of rat lung EETs was unique to this tissue (36, 42). These findings have important implications given that some of the biological actions of the EETs in the lung are both regioselective and stereoselective. For example, the effects on airway epithelial cell electrophysiology were highly stereospecific for 11(*R*),12(*S*)-EET, the least abundant rat lung enantiomer (33). Because stereoselective formation of eicosanoids is a sufficient criterion to establish their enzymatic origin (42), we conclude, based on the data in Tables 1 and 2, that 14,15-EET and 11,12-EET were produced, *in vivo*, by the human and rat lung epoxygenase(s). The presence of CYP2J proteins and CYP2J products (the EETs) in human and rat lung does not prove that the EETs detected *in vivo* were formed by the CYP2J enzymes. In fact, other lung P450s (e.g., CYP2B4) have also been shown to metabolize arachidonic acid to EETs (22, 23). However, the fact that the regioselectivity of EETs recovered from human and rat lung resembles the regioselectivity of recombinant CYP2J2 and CYP2J3 products (40)[2] suggests that these hemoproteins are among the predominant enzymes responsible for epoxidation of arachidonic acid pools in the lung.

The biological effects of the EETs on airway smooth muscle tone and on airway epithelial cell ion transport occur at concentrations of 10^{-9} to 10^{-6} M (22, 23). These physiological actions likely depend on the local concentration of the EETs at the site of activity (i.e., within the microenvironment of the effector cells). Our measurement of EETs in rat and human lung homogenate do not allow an accurate estimation of local concentrations. Although we cannot rule out that the effects of the EETs in the airway are only pharmacological, the localization of CYP2J enzymes by immunohistochemistry to airway epithelial cells and to bronchial smooth muscle cells, together with the known arachidonic acid epoxygenase activity of these hemoproteins, suggests that high levels of EETs might be attainable in the vicinity of the effector cells.

In summary, we provided molecular and immunological data that demonstrate that CYP2J2 and CYP2J3, newly described arachidonic acid epoxygenases, are abundantly expressed in the lung. Furthermore, we show that CYP2J protein expression is localized to specific cell types in the lung, including ciliated and nonciliated airway epithelium, bronchial and pulmonary vascular smooth muscle, vascular endothelium, and alveolar macrophages. We also report that CYP2J products, the EETs, are found *in vivo* in human and rat lung and that the regioselectivity of EETs recovered from the lung matches the regioselectivity of recombinant CYP2J products. We conclude that (a) in addition to the cyclooxygenase and lipoxygenase pathways, the P450 epoxygenase pathway is an important member of the human and rat pulmonary arachidonic acid metabolic cascade, and (b) CYP2J enzymes are among the principal constitutive arachidonic acid epoxygenases expressed in the lung. We speculate that in addition to known effects on airway smooth muscle tone and transepithelial electrolyte transport, the localization of CYP2J proteins to vascular smooth muscle and endothelium suggests that EETs may also be involved in the modulation of pulmonary vascular tone.

Acknowledgments

We thank Drs. Richard Philpot, Masahiko Negishi, Paul Nettesheim, and Robert Maronpot for their helpful comments during preparation of the manuscript.

References

1. Guengerich, F. P. Preparation and properties of highly purified cytochrome P450 and NADPH-cytochrome P450 reductase from pulmonary microsomes of untreated rabbits. *Mol. Pharmacol.* **13:**911–923 (1977).
2. Serabjit-Singh, C. J., C. R. Wolf, and R. M. Philpot. The rabbit pulmonary monooxygenase system: immunochemical and biochemical characterization of enzyme components. *J. Biol. Chem.* **254:**9901–9907 (1979).
3. Boyd, M. R., C. N. Statham, and N. S. Longo. The pulmonary Clara cell as a target for toxic chemicals requiring metabolic activation: studies with carbon tetrachloride. *J. Pharmacol. Exp. Ther.* **212:**109–114 (1980).
4. Philpot, R. M., and B. R. Smith. Role of cytochrome P450 and related enzymes in the pulmonary metabolism of xenobiotics. *Environ. Health Perspect.* **55:**359–367 (1984).
5. Devereux, T. R., B. A. Domin, and R. M. Philpot. Xenobiotic metabolism by isolated pulmonary cells. *Pharmacol. Ther.* **41:**243–256 (1989).
6. de Waziers, I., P. H. Cugnenc, C. S. Yang, J.-P. Leroux, and P. H. Beaune. Cytochrome P450 isoenzymes, epoxide hydrolase and glutathione transferases in rat and human hepatic and extrahepatic tissues. *J. Pharmacol. Exp. Ther.* **253:**387–394 (1990).
7. Domin, B. A., and R. M. Philpot. The effect of substrate on the expression of activity catalyzed by cytochrome P450: metabolism mediated by rabbit isoenzyme 6 in pulmonary microsomal and reconstituted monooxygenase systems. *Arch. Biochem. Biophys.* **246:**128–142 (1986).
8. Domin, B. A., T. R. Devereux, and R. M. Philpot. The cytochrome P450 monooxygenase system of rabbit lung: enzyme components, activities,, and induction in the nonciliated bronchiolar epithelial (Clara) cell, alveolar type II cell, and alveolar macrophage. *Mol. Pharmacol.* **30:**296–303 (1986).
9. Gonzalez, F. J., C. L. Crespi, M. Czerwinski, and H. V. Gelboin. Analysis

of human cytochrome P450 catalytic activities and expression. *Tohoku J. Exp. Med.* **168:**67–72 (1992).
10. Ueno, T., and F. J. Gonzalez. Complete sequence of the rat CYP2A3 gene specifically transcribed in lung. *Nucleic Acids Res.* **18:**4623 (1990).
11. Nhamburo, P. T., S. Kimura, O. W. McBride, C. A. Kozak, H. V. Gelboin, and F. J. Gonzalez. The human CYP2F gene subfamily: identification of a cDNA encoding a new cytochrome P450, cDNA-directed expression, and chromosome mapping. *Biochemistry* **29:**5491–5499 (1990).
12. Dees, J. H., B. S. S. Masters, U. Muller-Eberhard, and E. F. Johnson. Effect of 2,3,7,8-tetrachlorodibenzy-p-dioxin and phenobarbital on the occurrence and distribution of four cytochrome P450 isozymes in rabbit kidney, lung, and liver. *Cancer Res.* **42:**1423–1432 (1982).
13. Dees, J. H., L. D. Coe,, Y. Yasukochi,, and B. S. Masters. Immunofluorescence of NADPH-cytochrome c (P-450) reductase in rat and minipig tissues injected with phenobarbital. *Science (Washington D. C.)* **208:**1473–1475 (1980).
14. Serabjit-Singh, C. J., C. R. Wolf, and R. M. Philpot. Cytochrome P450: localization in rabbit lung. *Science (Washington D. C.)* **207:**1469–1470 (1980).
15. Serabjit-Singh, C. J., S. J. Nishio, R. M. Philpot, and C. G. Plopper. The distribution of cytochrome P450 monooxygenase in cells of the rabbit lung: an ultrastructural immunocytochemical characterization. *Mol. Pharmacol.* **33:**279–289 (1988).
16. Strum, J. M., T. Ito, R. M. Philpot, A. M. DeSanti, and E. M. McDowell. The immunocytochemical detection of cytochrome P450 monooxygenase in the lungs of fetal, neonatal, and adult hamsters. *Am. J. Respir. Cell Mol. Biol.* **2:**493–501 (1990).
17. Miller, M. A., and C. A. Hales. Role of cytochrome P450 in alveolar hypoxic pulmonary vasoconstriction in dogs. *J. Clin. Invest.* **64:**666–673 (1979).
18. Sylvester, J. T., and C. McGowan. The effects of agents that bind to cytochrome P450 on hypoxic pulmonary vasoconstriction. *Circ. Res.* **43:** 429–437 (1978).
19. Okita, R., R. Soberman, J. Bergholte, B. S. Masters, R. Hayes, and R. C. Murphy. ω-Hydroxylation of 15-HETE by lung microsomes from pregnant rabbits. *Mol. Pharmacol.* **32:**706–709 (1988).
20. Masters, B. S., R. T. Okita, A. S. Muerhoff, M. T. Leithauser, A. Gee, S. Winquist, D. L. Roerig, J. E. Clark, R. C. Murphy, and P. Ortiz de Montellano. Pulmonary P450 mediated eicosanoid metabolism and regulation in the pregnant rabbit. *Adv. Prostaglandin Thromboxane Leukotriene Res.* **19:**335–338 (1989).
21. Knickle, L. C., and J. R. Bend. Bioactivation of arachidonic acid by cytochrome P450 monooxygenases of guinea pig lung: the orthologue of cytochrome P450 2B4 is solely responsible for formation of epoxyeicosatrienoic acids. *Mol. Pharmacol.* **45:**1273–1280 (1994).
22. Zeldin, D. C., J. D. Plitman, J. Kobayashi, R. F. Miller, J. R. Snapper, J. R. Falck, J. L. Szarek, R. M. Philpot, and J. C. Capdevila. The rabbit pulmonary cytochrome P450 arachidonic acid metabolic pathway: characterization and significance. *J. Clin. Invest.* **95:**2150–2160 (1995).
23. Perdik, P., K. A. Pritchard, J. Z. Tan, and G. H. Gurtner. Arachidonic acid is metabolized by cytochrome P450 in the rabbit lung. *Am. Rev. Respir. Dis.* **147:**A920 (1993).
24. McGiff, J. C. Cytochrome P450 metabolism of arachidonic acid. *Annu. Rev. Pharmacol. Toxicol.* **31:**339–69 (1991).
25. Capdevila, J. H., J. R. Falck, and R. W. Estabrook. Cytochrome P450 and the arachidonic acid cascade. *FASEB J.* **6:**731–736 (1992).
26. Capdevila, J. H., D. C. Zeldin, K. Makita, A. Karara, and J. R. Falck. Cytochrome P450 and the metabolism of arachidonic acid and oxygenated eicosanoids, in *Cytochrome P450: Structure, Mechanism, and Biochemistry* (P. R. Ortiz de Montellano, ed.). 2nd Ed. Plenum Press, New York, 443–471 (1995).
27. Oliw, E. H. Oxygenation of polyunsaturated fatty acids by cytochrome P450 monooxygenases. *Prog. Lipid Res.* **33:**329–354 (1994).
28. Voelkel, N. F., K. R. Stenmark, J. Y. Wescott, and S.-W. Chang. Lung eicosanoid metabolism. *Clin Chest Med.* **10:**95–105 (1989).
29. Lewis, R. A., K. F. Austen, and R. J. Soberman. Leukotrienes and other products of the 5-lipoxygenase pathway: biochemistry and relation to pathobiology in human disease. *N. Engl. J. Med.* **323:**645–655 (1990).
30. Holtzman, M. J. Arachidonic acid metabolism: implications of biological chemistry for lung function and disease. *Am. Rev. Respir. Dis.* **143:**188–203 (1991).
31. Henderson, W. R. Eicosanoids and platelet-activating factor in allergic respiratory diseases. *Am. Rev. Respir. Dis.* **143:**S86–S90 (1991).
32. Tsubosaka, S., F. Sasaki,, T. Ishizaki, Y. Saitoh, T. Nakai, and S. Miyabo. In vitro effects of 5,6-epoxyeicosatrienoic acid (5,6-EET) on tracheal smooth muscle tone in guinea pigs. *Am. Rev. Respir. Dis.* **147:**A661 (1993).
33. Pascual, J. M. S., J. R. Yankaskas, and D. C. Zeldin. Arachidonic acid epoxide inhibits transepithelial voltage in rat tracheal epithelial cells. *Am. Rev. Respir. Dis.* **153:**A506 (1996).
34. Proctor, K., J. Falck, and J. Capdevila. Intestinal vasodilation by epoxyeicosatrienoic acids: arachidonic acid metabolites produced by a cytochrome P450 monooxygenase. *Circ. Res.* **60:**50–59 (1987).
35. Carroll, M. A., M. Schwartzman, J. Capdevila, J. R. Falck, and J. C. McGiff. Vasoactivity of arachidonic acid epoxides. *Eur. J. Pharmacol.* **138:**281–283 (1987).
36. Katoh, T., K. Takahashi, J. Capdevila, A. Karara, J. R Falck, H. R. Jacobson, and K. F. Badr. Glomerular stereospecific synthesis and hemodynamic actions of 8,9-epoxyeicosatrienoic acid in the rat kidney. *Am J. Physiol.* **261**: F578–F586 (1991).
37. Gebremedhin, D., Y.-H. Ma, J. R. Falck, R. J. Roman, M. VanRollins, and D. R. Harder. Mechanism of action of cerebral epoxyeicosatrienoic acids on cerebral arterial smooth muscle. *Am. J. Physiol.* **263:**H519–H525 (1992).
38. Cotton, R. B., T. A. Hazinski, S. Steele, J. H. Capdevila, and D. C. Zeldin. O_2 exposure is associated with the release of P450-derived arachidonic acid metabolites in the lung of premature infants at risk for bronchopulmonary dysplasia. *Am. J. Respir. Crit. Care Med.* **153:**A665 (1996).
39. Hazinski, T. A., R. B. Cotton, J. Morrow, E. Noisin, A. DeMatteo, and D. Zeldin. Cimetidine reduces lung oxidant stress and P450-derived oxidation products of arachidonic acid during hyperoxic lung injury in lambs. *Am. J. Respir. Crit. Care Med.* **153:**A810 (1996).
40. Wu, S., C. R. Moomaw, K. B. Tomer, J. R. Falck, and D. C. Zeldin. Molecular cloning and expression of CYP2J2, a human cytochrome P450 arachidonic acid epoxygenase highly expressed in heart. *J. Biol. Chem.* **271:**3460–3468 (1996).
41. Maniatis, T., J. Sambrook, and E. F. Fritsch. *Molecular Cloning: A Laboratory Manual*. 2nd Ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY (1989).
42. Karara, A., E. Dishman, I. Blair, J. R. Falck, and J. H. Capdevila. Endogenous epoxyeicosatrienoic acids: cytochrome P450 controlled stereoselectivity of the hepatic arachidonic acid epoxygenase. *J. Biol. Chem.* **264:** 19822–19827 (1989).
43. Capdevila, J. H., E. Dishman, A. Karara, and J. R. Falck. Cytochrome P450 arachidonic acid epoxygenase: stereochemical characterization of epoxyeicosatrienoic acids. *Methods Enzymol.* **206:**441–453 (1991).
44. Hammonds, T. D., I. A. Blair, J. R. Falck, and J. H. Capdevila. Resolution of epoxyeicosatrienoate enantiomers by chiral phase chromatography. *Anal. Biochem.* **182:**300–303 (1989).
45. Corey, E. J., A. Marfat, J. R. Falck, and J. O. Albright. Controlled chemical synthesis of enzymatically produced eicosanoids 11-, 12- and 15-HETE from arachidonic acid and conversion to corresponding HPETEs. *J. Am. Chem. Soc.* **102:**1433–1435 (1980).
46. Falck, J. R., and S. Manna. 8,9-Epoxy arachidonic acid: a cytochrome P450 metabolite. *Tetrahedron Lett.* **23:**1755–1756 (1982).
47. Falck, J. R., P. Yadagiri, and J. H. Capdevila. Synthesis of epoxyeicosatrienoic acids and heteroatom analogs. *Methods Enzymol.* **187:**357–364 (1990).
48. Capdevila, J. H., B. Pranamik, J. L. Napoli, S. Manna, and J. R. Falck. Arachidonic acid epoxidation: epoxyeicosatrienoic acids are endogenous constituents of rat liver. *Arch. Biochem. Biophys.* **243:**8–19 (1986).
49. Branford, M. M. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:**248–254 (1976).
50. Nhamburo, P. T., F. J. Gonzalez, O. W. McBride, H. V. Gelboin, and S. Kimura. Identification of a new P450 expressed in human lung: complete cDNA sequence, cDNA-directed expression, and chromosome mapping. *Biochemistry* **28:**8060–8066 (1989).
51. Rich, K. J., D. Sesardic, J. R. Foster, D. S. Davies, and A. R. Boobis. Immunohistochemical localization of cytochrome P450b/e in hepatic and extrahepatic tissues of the rat. *Biochem. Pharmacol.* **38:**3305–3322 (1989).
52. Chichester, C. H., R. M. Philpot, A. J. Weir, A. R. Buckpitt, and C. G. Plopper. Characterization of the cytochrome P450 monooxygenase system in nonciliated bronchiolar epithelial (Clara) cells isolated from mouse lung. *Am. J. Respir. Cell Mol. Biol.* **4:**179–186 (1991).
53. Boyd, M. R. Evidence for the Clara cell as a site of cytochrome P450-dependent mixed-function oxidase activity in lung. *Nature (Lond.).* **269:** 713–715 (1977).
54. Devereux, T. R., J. J. Diliberto, and J. R. Fouts. Cytochrome P450 monooxygenase, epoxide hydrolase and flavin monooxygenase activities in Clara cells and alveolar type II cells isolated from rabbit. *Cell Biol. Toxicol.* **1:**57–65 (1985).
55. Coceani, F., L. Kelsey, C. Ackerley, M. Rabinovitch, and H. Gelboin. Cytochrome P450 during ontogenic development: occurrence in the ductus arteriosus and other tissues. *Can. J. Physiol. Pharmacol.* **72:**217–226 (1994).
56. Zeldin, D. C., C. R. Moomaw, N. Jesse, K. B. Tomer, J. Beetham, B. D. Hammock, and S. Wu. Biochemical characterization of the human liver cytochrome P450 arachidonic acid epoxygenase pathway. *Arch. Biochem. Biophys.* **330:**87–96 (1996).
57. Karara, A., E. Dishman, J. R. Falck, and J. H. Capdevila. Stereochemical analysis of the endogenous epoxyeicosatrienoic acids of human kidney cortex. *FEBS Lett.* **268:**227–230 (1990).
58. Nelson, D. R., T. Kamataki, D. J. Waxman, F. P. Guengerich, R. W. Estabrook, R. Feyereisen, F. J. Gonzalez, M. J. Coon, I. C. Gunsalus, O. Gotoh, K. Okuda, and D. W. Nebert. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. *DNA Cell Biol.* **12:**1–51 (1993).

Send reprint requests to: Dr. Darryl C. Zeldin, Laboratory of Pulmonary Pathobiology, NIH, NIEHS, P.O. Box 12233, Research Triangle Park, NC 27709. E-mail: zeldin@niehs.nih.gov