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Bioactivation of Arachidonic Acid by the Cytochrome P450 Monooxygenases of Guinea Pig Lung: The Orthologue of Cytochrome P450 2B4 Is Solely Responsible for Formation of Epoxyeicosatrienoic Acids

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SUMMARY

Guinea pig lung microsomes converted arachidonic acid (AA) to two classes of cytochrome P450 (P450)-dependent metabolites, 16- through 20-hydroxyeicosatetraenoic acids [(16–20)-OH-AA] and epoxyeicosatrienoic acids (EETs). The rate of formation of (16–20)-OH-AA was ~3-fold higher in microsomes from β-naphthoflavone-induced versus untreated animals. In microsomes from untreated or induced animals EETs, the major class of P450 metabolites in guinea pig lung, were formed in a regioselective manner, with 8,9-, 11,12-, and 14,15-regioisomers accounting for ≥90% of the total EETs. With isozyme-selective inhibitors and inhibitory antibodies the role of individual pulmonary P450 isozymes in AA metabolism was examined. Metyrapone and SKF-525A (P450 2B selective) inhibited EET formation by ≥85% with little effect on (16–20)-OH-AA formation. 1-Aminobenzotriazole (1 mM), a mechanism-based inhibitor with low isozyme selectivity, inhibited the formation of both classes of metabolites by >95%, whereas *N*-α-methylbenzyl-1-aminobenzotriazole (1 μM), a P450 2B-selective mechanism-based inhibitor, abolished EET formation with little effect on (16–20)-OH-AA formation. Antibodies to rabbit P450 2B4 also abolished EET formation without inhibiting the formation of (16–20)-OH-AA, whereas antibodies to rabbit P450 4B1 did not inhibit the formation of either class of metabolites. α-Naphthoflavone (P450 1A1 selective in lung) did not inhibit the formation of either class of metabolites. These data demonstrate that the guinea pig orthologue of P450 2B4 is solely responsible for the bioactivation of AA to EETs in guinea pig lung and that a form of P450 other than a 2B, 4B, or 1A isozyme, which is inducible by β-naphthoflavone, is responsible for (16–20)-OH-AA formation.

The P450 monooxygenase system converts AA to three classes of primary metabolites (reviewed in Refs. 1 and 2); four regioisomeric *cis*-EETs, i.e., 5,6-EET, 8,9-EET, 11,12-EET, and 14,15-EET, are formed by olefin epoxidation, six regioisomeric *cis,trans*-conjugated HETEs, i.e., 5-, 8-, 9-, 11-, 12-, and 15-HETE, are formed by allylic oxidation, and (16–20)-OH-AAs are formed by C16 through C20 hydroxylation. These primary metabolites may undergo further metabolism; for example, EETs can be hydrolyzed by cytosolic epoxide hydrolase to form the corresponding diols (DiHETEs) (3) and 20-OH-AA can be oxidized to 20-COOH-AA (1, 2).

P450 metabolites of AA have a wide variety of potent biological effects, including stimulation of peptide hormone release, vasodilation, vasoconstriction, inhibition or stimulation of Na^+/K^+-ATPase, and inhibition of platelet aggregation (reviewed in Refs. 1 and 2). They apparently have important roles in physiology and pathophysiology, as in the development of hypertension (1, 2).

AA metabolites are produced by purified P450s in reconstituted monooxygenase systems and by P450 in microsomal fractions or isolated cell preparations from numerous tissues, including kidney (4, 5), liver (4, 6), hypothalamus (7), and cornea (8). EETs are endogenous to kidney (9) and liver (10) and occur in plasma (11) and urine (12). In both liver and plasma, >90% of the EETs are esterified to the *sn*-2 position of phospholipids (10, 11).

The microsomal P450 monooxygenase system is composed of the flavoprotein NADPH-P450 reductase and multiple isozymes of the hemoprotein P450 (13). The profile of P450-dependent metabolites of AA produced by a tissue is dependent

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ABBREVIATIONS: P450, cytochrome P450; AA, arachidonic acid; ABP, 4-aminobiphenyl *N*-hydroxylation; ABT, 1-aminobenzotriazole; αMB, *N*-α-methylbenzyl-1-aminobenzotriazole; α-NF, α-naphthoflavone; BBT, *N*-benzyl-1-aminobenzotriazole; β-NF, β-naphthoflavone; DETAPAC, diethylenetriaminepentaacetic acid; DiHETE, dihydroxyeicosatrienoic acid; EET, epoxyeicosatrienoic acid; ERF, 7-ethoxyresorufin *O*-deethylation; PB, phenobarbital; PRF, 7-pentoxyresorufin *O*-dependentylation; 20-COOH-AA, 1,20-eicosatetraenedioic acid; (16–20)-OH-AA, 16- through 20-hydroxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; HPLC, high performance liquid chromatography.

on its P450 isozyme composition, because individual isozymes vary in their ability to oxidize AA, the type of oxidation (i.e., epoxidation, allylic oxidation, or C16–C20 hydroxylation), and the regio- and stereoselectivity of attack (6).

The lung of many species, including rabbit, rat, mouse, hamster, guinea pig, and human, contains catalytically active P450. Microsomal P450 has been well characterized in the adult rabbit lung, where it is composed largely of three P450 isozymes, i.e., 1A1, 2B4, and 4B1 (14). Guinea pig lung contains immuno-orthologues of these three P450 isozymes, referred to as 1A1, 2Bx, and 4Bx, respectively (14, 15).[1] P450 1A1 is induced in lung by polycyclic aromatic hydrocarbons including β-NF (14). PB, which induces P450 2Bx in guinea pig liver, has no inductive effect in the lung (14, 16).

Pulmonary P450 from rabbit metabolizes AA to 19/20-OH-AA, with a small amount of DiHETEs also being formed (4). This is the only species in which P450-dependent AA metabolism has been examined in lung.

The primary objective of this study was to characterize the P450 metabolites of AA in guinea pig lung microsomes and to elucidate the role of individual P450 isozymes in their formation. Guinea pigs were used in this investigation for two reasons. First, we have previously characterized (5, 16, 17) the response of guinea pig hepatic, pulmonary, and renal P450 monooxygenase systems to ABT and its *N*-aralkylated derivatives, which are potent, isozyme-selective, mechanism-based inhibitors of P450. Second, in collaboration with Drs. Moffat and Karmazyn of the Department of Pharmacology and Toxicology, University of Western Ontario, London, Ontario, we have demonstrated potent effects of EETs in perfused guinea pig hearts and isolated myocytes (18).

Materials and Methods

Reagents. [1-^{14}C]AA (50–60 mCi/mmol, >99% radiochemical purity) was purchased from Amersham Canada Ltd. (Oakville, Canada). AA, NADPH, metyrapone, and nordihydroguaiaretic acid were purchased from Sigma Chemical Co. (St. Louis, MO); 7-ethoxyresorufin, 7-pentoxyresorufin, and resorufin from Molecular Probes (Eugene, OR); β-NF, α-NF, 4-aminobiphenyl, and 2,4,6-tri(2-pyridyl)-1,3,5-triazine from Aldrich Chemical Co. (Milwaukee, WI); Emulphor EL-620 from GAF Corp. (New York, NY); and SKF-525A from Biomol Research Lab. (Plymouth Meeting, PA). Goat antibodies to rabbit P450 2B4 and P450 4B1 were generously provided by Dr. R. M. Philpot of the National Institute of Environmental Health Sciences (Research Triangle Park, NC). ABT, BBT, and αMB were synthesized and purified as described previously (17).

Synthesis and separation of ^{14}C-radiolabeled 5,6-, 8,9-, 11,12-, and 14,15-EETs. All solvents were dried over molecular sieves (type 4A; Aldrich Chemical Co.). Crude *m*-chloroperoxybenzoic acid (50–60%; Aldrich Chemical Co.) was dissolved in diethyl ether and washed twice with 0.5 M potassium phosphate buffer, pH 7.5, to remove *m*-chlorobenzoic acid. The ether layer was taken to dryness under N_2 and the resulting *m*-chloroperoxybenzoic acid was dessicated under vacuum.

AA (1 mg, 3.3 mmol) in absolute ethanol and 0.25 μCi (1.39 μg, 4.5 μmol) of [^{14}C]AA in toluene were concentrated to an oil under N_2. To this oil was added 0.2 ml of *m*-chloroperoxybenzoic acid in CH_2Cl_2 (2.86 mg/ml, 3.3 mmol) and the reaction was allowed to proceed for 24 hr, with constant stirring, in a N_2 atmosphere (19).

Reaction products were dried under a gentle stream of N_2 and made up in 100 μl of methanol. Regioisomers were partially separated on an isocratic reverse phase HPLC system using a Waters μBondapak C_{18}, 10-μm, 8- × 100-mm radial-pak column (4, 20). The eluant was methanol/water/acetic acid (73:27:0.2, v/v/v) at 2 ml/min, and products were monitored by UV spectroscopy at 210 nm. Fractions were collected every 1 min and aliquots were taken for liquid scintillation counting. Retention times were observed as 29, 33, and 37 min for 14,15-EET, 8,9- plus 11,12-EETs, and 5,6-EET, respectively.

The 8,9- plus 11,12-EETs were further separated by isocratic normal phase HPLC on a Waters μPorasil C_{18}, 10-μm, 8- × 100-mm radial-pak column (21). The eluant was hexane/2-propanol/acetic acid (100:0.3:0.1, v/v/v) at 2 ml/min. Fractions were collected every 1 min and aliquots were taken for liquid scintillation counting. Retention times observed were 7 min for 11,12-EET and 10 min for 8,9-EET. These purified synthetic EETs had retention times identical to those of biologically produced EETs analyzed on the HPLC system that we routinely use to analyze P450 metabolism of AA (Fig. 1). The overall radioactive yield of purified products from the synthesis was 30.4%, distributed as 14,15-EET (11.7%), 11,12-EET (9.7%), 8,9-EET (4.1%), and 5,6-EET (4.9%).

Animal treatment. Male Hartley guinea pigs (300–375 g) were used for these experiments. Some guinea pigs were treated intraperitoneally with 80 mg/kg β-NF (2% in corn oil) or 80 mg/kg PB (2% in saline solution) daily for 4 days. In experiments studying the ability of the mechanism-based inhibitors to inactivate the pulmonary and hepatic systems *in vivo*, urethane (1.5 g/kg) was administered intraperitonally 24 hr after the last injection of PB. The animals used for these *in vivo* studies were pretreated with PB to increase P450 2Bx levels in the liver. Once animals were anesthetized, the jugular vein was exposed and ABT, BBT, αMB, or the drug vehicle (for 100% control values) was injected into it. ABT, BBT, and αMB were suspended in vehicle (5% bovine serum albumin/dimethylsulfoxide/Emulphor, 6:0.15:0.3), by sonication, at concentrations so that each animal received an equivalent volume of vehicle (2.15 ml/kg). BBT and αMB were dissolved in dimethylsulfoxide before addition to the Emulphor/bovine serum albumin mixture. The animals were sacrificed 4 hr after the

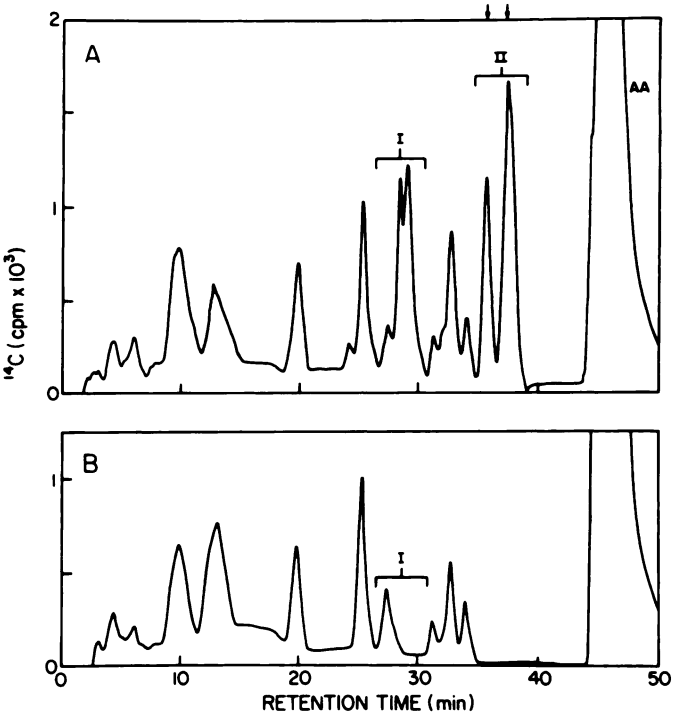

Fig. 1. HPLC chromatograms of the [^{14}C]AA metabolites formed by pulmonary microsomes from β-NF-induced guinea pigs in the presence (A) and absence (B) of NADPH. *Arrows*, retention times of synthetic 14,15-EET (*left*) and 5,6-, 8,9-, and 11,12-EET (*right*).

[1] In guinea pig only P450 1A1 has been sequenced and assigned to the P450 nomenclature family (26); therefore, we use P450 2Bx and 4Bx to refer to the orthologues of rabbit P450 2B4 and 4B1, respectively, in this manuscript.

inhibitor was administered. The lungs and livers were removed and stored at −80°.

Enzyme assays. Washed pulmonary and hepatic microsomes were prepared by differential centrifugation (16). Protein concentration was determined by the method of Lowry *et al.* (22), using bovine serum albumin as standard.

One substrate/monooxygenase pathway was monitored for each of the three isozymes known to be present in guinea pig lung, i.e., ERF for P450 1A1, PRF for P450 2Bx, and ABP for P450 4Bx (16, 17). ERF, PRF, and ABP activities, as well as erythromycin *N*-demethylation activity, were determined as described previously (16).

For the determination of AA metabolites, washed microsomal protein (1 mg of pulmonary protein or 0.25 mg of hepatic protein) was incubated with 100 μM [1-^{14}C]AA (0.5 or 0.1 μCi for pulmonary or hepatic microsomes, respectively) at 37° for 5 min in 0.1 M potassium phosphate buffer, pH 7.4, in the presence or absence of 1 mM NADPH. The total reaction volume was 1 ml. Before use, [^{14}C]AA was analyzed by HPLC to ensure >99% purity. Where indicated, 10^{-5} M indomethacin, 10^{-5} M nordihydroguaiaretic acid, 1 mM DETAPAC, or 1 mM DETAPAC plus 0.1 mM H_2O_2 was present in the incubations without NADPH and 10^{-5} M SKF-525A, 10^{-5} M metyrapone, or 3×10^{-7} M α-NF was present in the incubations with NADPH. The α-NF was added to the microsomal protein (on ice) at least 30 min before the addition of the protein to the reaction vial containing AA. For SKF-525A, in some instances the microsomal protein was incubated with SKF-525A and NADPH for 5 min at 37° before addition to the reaction vial containing AA. After incubation with AA, the metabolites were extracted with ethyl acetate containing 0.01% butylated hydroxytoluene at acidic pH, dried under a stream of N_2, and reconstituted in ethanol. The metabolites were separated by reverse phase HPLC on a 5-μm, 8- × 100-mm, Resolve C_{18} column (Waters, Mississauga, Canada) by a modification of the method of Capdevila *et al.* (21), using a linear gradient from acetonitrile/water/acetic acid (37.95:61.95:0.10, v/v/v) to acetonitrile/acetic acid (99.9:0.01, v/v) over 50 min, at a flow rate of 1 ml/min. This modification in mobile phase is routinely used in our laboratory to allow for the separation and quantification of cyclooxygenase and lipooxygenase metabolites in addition to the P450-dependent metabolites. Radioactivity was monitored with a flow-through detector [model 171; Beckman (Canada) Inc., Mississauga, Canada].

Initially, the AA metabolite classes were identified based on their retention times in the HPLC system described by Capdevila *et al.* (21). Resolution of aliquots of the same reaction mixtures on our modified system provided the retention times in this system. Moreover, the retention times of synthetic radiolabeled 5,6-, 8,9-, 11,12-, and 14,15-EET were determined experimentally in both systems for comparison.

For determination of the regioisomeric composition of the EETs, fractions containing these metabolites were collected from the initial reverse phase HPLC separation, which allows quantitation of 14,15-EET, and pooled. The acetonitrile was removed under N_2 and 2 ml of water were added. The aqueous phase was extracted three times with an equal volume of hexane containing 0.01% butylated hydroxytoluene. The organic phases were pooled, taken to dryness under N_2, and stored in 1 ml of acetonitrile at −20°. Immediately before the second separation of the regioisomers, aliquots were taken to dryness under N_2 and 3% isopropyl alcohol in hexane containing 0.1% acetic acid (100 μl) was added. Regioisomers of the EETs were separated isocratically by HPLC on a Waters 10-μm, 8- × 100-mm, radial-pak μPorasil column with a mobile phase of 0.35% isopropyl alcohol in hexane containing 0.1% acetic acid, at a flow rate of 1 ml/min (20). Radioactivity was monitored with the on-line flow-through radiochemical detector described above. The regioisomers were identified based on the retention times of synthetic radiolabeled EETs in this system (35.8, 39.4, 53.6, and 77.2 min for 14,15-, 11,12-, 8,9-, and 5,6-EET, respectively) and were quantitated radiochemically.

***In vitro* incubations with ABT, BBT, and αMB.** Microsomal protein (~11 mg) was incubated with 1 mM NADPH (no NADPH in controls) and various concentrations of ABT, BBT, or αMB (no inhibitor in controls) at 37° for 45 min, as described previously (17). After incubation, the microsomal protein was resedimented by centrifugation and then washed (by resuspension and resedimentation) to remove excess inhibitor. The microsomal pellets were resuspended and stored at −80° until ERF, PRF, and ABP activities and AA metabolism were determined.

Antibody inhibition. Washed microsomal protein was incubated on ice for 30 min with goat polyclonal antibodies to rabbit P450 2B4 or P450 4B1 or preimmune serum before determination of AA metabolism and monooxygenase activities. The antibodies used were those described previously by Serabjit-Singh *et al.* (23).

Results

HPLC chromatograms (Fig. 1) of the [^{14}C]AA metabolites formed by pulmonary microsomes from β-NF-induced guinea pigs in the presence (Fig. 1A) and absence (Fig. 1B) of NADPH show the presence of two classes of NADPH-dependent primary metabolites, indicated as area I and area II. Area II contains EETs, and the NADPH-dependent component of area I is (16–20)-OH-AA. The majority of the peaks present in the HPLC chromatogram obtained from microsomal incubations without NADPH, including the peak that co-chromatographs with (16–20)-OH-AA, were abolished by boiling of the microsomes before the determination of AA metabolism but were not affected by the inclusion of a cyclooxygenase inhibitor (10^{-5} M indomethacin), a lipooxygenase inhibitor (10^{-5} M nordihydroguaiaretic acid), DETAPAC (1 mM), or DETAPAC (1 mM) plus H_2O_2 (0.1 mM) in the AA incubation (data not shown). The formation of trace amounts of secondary metabolites (group III) of AA such as the DiHETEs (from EETs) or 20-COOH-AA (from 20-OH-AA) may also occur in lung but be obscured by a small NADPH-independent peak migrating in this area of the chromatograph.

In pulmonary microsomes from untreated animals the mean rates of formation of EETs (the major class of metabolites) and (16–20)-OH-AA were 0.18 and 0.04 nmol/min/mg of protein, respectively (Table 1). β-NF induction increased the rate of formation of (16–20)-OH-AA by ~3-fold but had litte effect on EET formation (0.23 nmol/min/mg of protein). 8,9-, 14,15-, and 11,12-EET represented 44.5%, 25.8%, and 22.3%, respectively, of the total EETs formed by microsomes from control animals (Table 2). The relative amounts of the EET regioisomers formed by microsomes from β-NF-induced animals were virtually identical to those of untreated guinea pigs, being 43.9%, 22.0%, and 22.0%, respectively, for 8,9-, 14,15-, and 11,12-EET.

The ability of classical P450 inhibitors to attentuate the formation of the NADPH-dependent AA metabolites in pulmonary microsomes was examined (Table 3). Metyrapone (10^{-5} M) and SKF-525A (10^{-5} M) inhibited the formation of EETs by ≥85% in pulmonary microsomes from both untreated and β-NF-induced animals. The majority of the inhibition by SKF-

TABLE 1

NADPH-dependent formation of AA metabolites in pulmonary microsomes from untreated and β-NF-induced guinea pigs

Data were obtained from two sets of microsomes, each pooled from at least eight animals.

	(16-20)-OH-AA	EETs
	nmol/min/mg	*nmol/min/mg*
Untreated	0.03, 0.05	0.17, 0.18
β-NF-induced	0.11, 0.13	0.22, 0.23

TABLE 2

Regioisomeric composition of EETs produced by pulmonary microsomes from untreated and β-NF-induced guinea pigs

Data were obtained from one set of microsomes pooled from at least eight animals.

	Composition	
	Untreated	β-NF-induced
	% of total EETs	
5,6-EET	7.4	11.0
8,9-EET	44.5	43.9
11,12-EET	22.3	22.0
14,15-EET	25.8	22.0

TABLE 3

Inhibition of AA metabolism by various P450 inhibitors in pulmonary microsomes from untreated and β-NF-induced guinea pigs *in vitro*

Data were obtained from one set of microsomes pooled from at least eight animals.

	Rate of formation			
	Untreated		β-NF-induced	
	Peak II (EETs)	Peak I[a]	Peak II (EETs)	Peak I[a]
	nmol/min/mg of protein			
No preincubation				
No NADPH	—[b]	0.05	—	0.01
NADPH	0.19	0.09	0.21	0.14
Metyrapone (10^{-5} M)	0.03 (85)[c]	0.11	0.01 (>95)	0.14
SKF-525A (10^{-5} M)	0.14 (26)	0.10	0.17 (19)	0.14
α-NF (3×10^{-7} M)	0.25 (<10)	0.11	0.21 (<10)	0.12
Preincubation[d]				
No SKF-525A	0.15	0.03	0.13	0.09
SKF-525A (10^{-5} M)	0.02 (87)	0.05	0.02 (85)	0.07

[a] (16-20)-OH-AA and/or NADPH-independent metabolite.
[b] —, Below detection limit.
[c] Values in parentheses, percentage.
[d] Microsomes were incubated for 5 min at 37° with SKF-525A in the presence of 1 mM NADPH immediately before determination of AA metabolism.

525A required preincubation with microsomal protein and NADPH, inasmuch as SKF-525A without preincubation inhibited EET formation by <30%. Neither metyrapone nor SKF-525A inhibited the formation of (16–20)-OH-AA. α-NF (3×10^{-7} M) did not significantly inhibit the formation of EETs or (16–20)-OH-AA in microsomes from either untreated or β-NF-induced guinea pigs. In other experiments, this concentration of α-NF inhibited ERF activity by ~50% and ~75% in microsomes from untreated and β-NF-induced animals, respectively (data not shown).

The ability of ABT, a mechanism-based inhibitor of P450, and its *N*-aralkylated derivatives BBT and αMB to inhibit the formation of (16–20)-OH-AA and EETs was also examined (Table 4). The inhibitor concentrations were chosen based on earlier experiments; ABT was used at a high concentration to ensure complete inactivation of the three major P450 isozymes previously known to be in guinea pig lung (P450 2Bx, 4Bx, and 1A1), and BBT and αMB were used at concentrations where they are P450 2Bx-selective inactivators in lung (17). In pulmonary microsomes from β-NF-induced guinea pigs, ABT (1 mM), which inactivated PRF, ERF, and ABP activities by >95%, virtually abolished the formation of both (16–20)-OH-AA and EETs. BBT, which displayed some selectivity for P450 2Bx, inactivating PRF more than ERF and ABP activities at each concentration (>95% versus 66% and 55% inactivation, respectively, at 10 μM), inhibited the formation of both classes of P450 metabolites of AA; the inhibition of EET formation was equivalent to the inhibition of PRF activity (>95% at both

TABLE 4

Inhibition of P450-dependent AA metabolism and isozyme-selective monooxygenase activities in pulmonary microsomes from β-NF-induced guinea pigs, incubated *in vitro* with ABT, BBT, and αMB

Data were obtained from one set of microsomes pooled from at least eight animals. Control (100%) values were 0.09 [(16-20)-OH-AA], 0.14 (EETs), 0.013 (PRF), 0.022 (ERF), and 245 (ABP) nmol/min/mg of protein.

Inhibitor	Inhibition				
	(16-20)-OH-AA	EETs	PRF	ERF	ABP
	%				
1 mM ABT	>95	>95	>95	>95	>95
10 μM BBT	67	>95	>95	66	55
100 μM BBT	78	>95	>95	81	63
1 μM αMB	<10	>95	>95	16	21

10 and 100 μM BBT), whereas the inhibition of (16–20)-OH-AA formation was concentration dependent and paralleled more closely the loss in ERF and ABP activities. αMB, the most P450 2Bx-selective of these inhibitors, inactivated PRF activity by >95% with little loss of ERF and ABP activities (<25%) and abolished the formation of EETs (>95% loss) while having little effect on (16–20)-OH-AA formation (<10% loss).

ABT, BBT, and αMB administered intravenously (via the jugular vein) to PB-induced guinea pigs were also effective *in vivo* inhibitors of the pulmonary P450 monooxygenase (Table 5). ABT (75 μmol/kg) inactivated ERF, PRF, and ABP activities and the formation of EETs by ≥85%; BBT and αMB also inhibited EET formation. With BBT (0.075 and 7.5 μmol/kg) and αMB (75 nmol/kg), the inhibition of EET formation approximated the loss in PRF activity, which was about 30% greater than the loss in ERF activity. Neither BBT nor αMB significantly inhibited ABP activity even at the highest dose administered (7.5 and 75 μmol/kg, respectively). Peak 1 [(16–20)-OH-AA and/or a NADPH-independent metabolite] was not inhibited by ABT or its analogues, suggesting that its formation is not P450 mediated in PB-induced guinea pig (0.12 ± 0.01 versus 0.15 ± 0.02 nmol/min/mg of protein at 0 versus 75 μmol/kg ABT, respectively).

The ability of antibodies to rabbit P450 2B4 and P450 4B1 to inhibit the formation of (16–20)-OH-AA and EETs was determined in pulmonary microsomes from β-NF-induced guinea pigs (Table 6). Antibodies to P450 2B4 inhibited EET formation and PRF activity in a concentration-dependent manner, with >95% inhibition at 2 mg of IgG/mg of microsomal protein. ERF activity was also inhibited by P450 2B4 antibodies but to a much lesser degree than PRF activity and EET formation (28% at 2 mg of IgG/mg of microsomal protein). On the other hand, there was no inhibition of (16–20)-OH-AA formation or ABP activity by antibodies to P450 2B4. Antibodies to P450 4B1 (2 mg of IgG/mg of microsomal protein), which inhibited ABP activity by ~70% without affecting ERF or PRF activity, did not inhibit the formation of either class of P450 metabolites of AA.

Liver microsomes from guinea pig produced (16–20)-OH-AA (peak I), EETs (peak II), and two other classes of NADPH-dependent AA metabolites (peaks III and IV) (Fig. 2). Peak IV contains HETEs and peak III, which is more polar than the other classes of P450 metabolites of AA, is composed of secondary AA metabolites such as the DiHETEs and 20-COOH-AA. In hepatic microsomes from untreated guinea pigs the rates

TABLE 5

Inhibition of P450-dependent AA metabolism and isozyme-selective monooxygenase activities in pulmonary microsomes from PB-induced guinea pigs treated *in vivo* with ABT, BBT, and αMB

Values are mean ± standard error.

Inhibitor dose	EETs	PRF	ERF	ABP
μmol/kg	*nmol/min/mg*	*pmol/min/mg*	*pmol/min/mg*	*nmol/min/mg*
ABT				
0 ($n = 3$)[a]	0.29 ± 0.05	41.6 ± 3.0	13.1 ± 3.0	218 ± 26
7.5 ($n = 3$)	0.08 ± 0.02[b] (72)[c]	19.0 ± 6.6[b] (54)	6.4 ± 1.4[b] (52)	96 ± 9[b] (56)
75 ($n = 3$)	0.01 ± 0.03[b] (>95)	5.4 ± 0.3[b] (88)	2.2 ± 0.2[b] (85)	8 ± 2[b] (>95)
BBT				
0 ($n = 3$)	0.26 ± 0.05	62.6 ± 5.2	13.5 ± 1.0	232 ± 16
0.075 ($n = 3$)	0.15 ± 0.02 (42)	25.9 ± 2.0[b] (59)	10.1 ± 0.1[b] (25)	278 ± 9[b] (<10)
7.5 ($n = 3$)	0.03 ± 0.01[b] (88)	5.8 ± 1.5[b] (91)	5.2 ± 0.8[b] (61)	219 ± 10 (<10)
αMB				
0 ($n = 2$)	0.49, 0.28	56.6, 58.9	18.6, 19.8	209, 284
0.075 ($n = 2$)	0.10, 0.07 (77)	6.5, 9.4 (86)	8.2, 8.8 (54)	181, 307 (<10)
75 ($n = 2$)	0.02, 0.02 (>95)	0.7, 0.5 (>95)	1.6, 1.9 (91)	252, 190 (11)

[a] *n*, Number of individual animals.
[b] $p < 0.05$ (different from 0 μmol/kg control; analysis of variance followed by Newman-Keuls test).
[c] Values in parentheses, percentage.

TABLE 6

Inhibition of P450-dependent AA metabolism and isozyme-selective monooxygenase activities in pulmonary microsomes from β-NF-induced guinea pigs, incubated *in vitro* with antibodies to rabbit P450 2B4

Data were obtained from one set of microsomes pooled from at least eight animals. Control (100%) values were 0.11 [(16-20)-OH-AA], 0.22 (EETs), 0.015 (PRF), 0.014 (ERF), and 204 (ABP) nmol/min/mg of protein.

Antibody	Inhibition				
	(16-20)-OH-AA	EETs	PRF	ERF	ABP
mg of IgG/mg of microsomal protein		%			
Preimmune serum (0.2)	—[a]	—	—	—	—
Preimmune serum (2)	—	—	—	—	—
Anti-P450 4B1 (0.2)	—	—	—	—	17
Anti-P450 4B1 (2)	—	—	—	—	68
Anti-P450 2B4 (0.2)	—	55	73	15	—
Anti-P450 2B4 (2)	—	>95	>95	28	—

[a] —, <10% inhibition.

of formation of EETs and (16–20)-OH-AA, the two classes of P450-dependent metabolites formed in lung, were 1.96 and 1.74 nmol/min/mg of protein, respectively (Table 7). PB induction markedly increased the rate of EET formation in liver microsomes (4.6-fold), with little change in the rate of (16–20)-OH-AA formation (1.4-fold increase). Hepatic PRF activity, catalyzed primarily by an orthologue of P450 2B4 in mammalian liver (16, 24), was increased 7.5-fold by PB induction in guinea pigs.

In hepatic microsomes from PB-induced guinea pigs, metyrapone (10^{-5} M) and SKF-525A (10^{-5} M) inhibited the formation of EETs by 23% and 48%, respectively, whereas α-NF did not inhibit EET formation (Table 8). The highest concentration of α-NF studied, 3×10^{-7} M, inhibited ERF and ABP activities, catalyzed primarily by P450 1A1 and 1A2, respectively, (25, 26), by ~75% in hepatic microsomes from β-NF-induced guinea pigs (5).

Antibodies to rabbit P450 2B4 inhibited EET formation and PRF activity in hepatic microsomes from PB-induced guinea pigs in a concentration-dependent manner (Table 9). At each concentration EET formation was inhibited much less than PRF activity; 5 mg of IgG/mg of microsomal protein, which inhibited PRF activity by >90%, inhibited EET formation by only 36%.

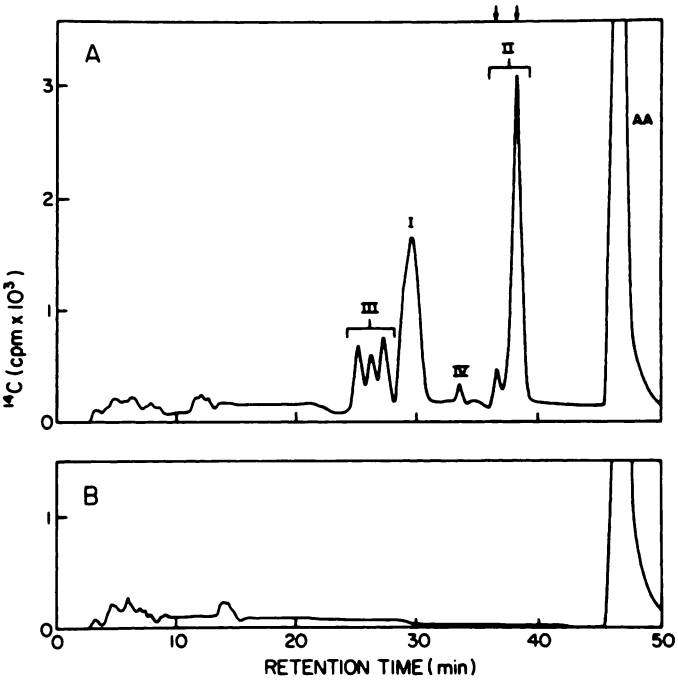

Fig. 2. HPLC chromatograms of the [^{14}C]AA metabolites formed by hepatic microsomes from untreated guinea pigs in the presence (A) and absence (B) of NADPH. *Arrows*, retention times of synthetic 14,15-EET (*left*) and 5,6-, 8,9-, and 11,12-EET (*right*).

At much higher doses (75 μmol/kg) than cause nearly complete inhibition of pulmonary P450 2Bx (75 nmol/kg), αMB was also an effective *in vivo* inactivator of hepatic P450 2Bx when administered intravenously (via the jugular vein) to PB-induced guinea pigs (Table 10). This high dose, which inactivated P450 2Bx-catalyzed PRF by >95%, inhibited the formation of EETs by only 49%; ERF and ABP activities were also inhibited by αMB (>80%) at 75 μmol/kg).

Discussion

In this study pulmonary microsomes from guinea pig lung were shown to convert AA to two classes of primary P450

TABLE 7

Comparison of P450-dependent AA metabolism and PRF activity in hepatic microsomes from untreated versus PB-induced guinea pigs

Values are mean ± standard error.

	Untreated (n = 5)[a]	PB-induced (n = 9)
EETs (nmol/min/mg)	1.96 ± 0.26	9.17 ± 0.59[b] (4.6)[c]
(16-20)-OH-AA (nmol/min/mg)	1.74 ± 0.18	2.35 ± 0.11[d] (1.4)
PRF (pmol/min/mg)	23 ± 4	171 ± 16[b] (7.5)

[a] n, number of individual animals.
[b] $p < 0.01$ (different from untreated; unpaired Student's t test).
[c] Values in parentheses, fold increase in activity, compared with microsomes from untreated animals.
[d] $p < 0.05$ (different from untreated; unpaired Student's t test).

TABLE 8

Inhibition of EET formation by various P450 inhibitors in hepatic microsomes from PB-induced guinea pigs *in vitro*

	Inhibition
	%
No preincubation[a]	
Metyrapone (10^{-5} M)	23
α-NF (1×10^{-7} M)	<5
α-NF (3×10^{-7} M)	<5
Preincubation[b]	
SKF-525A (10^{-5} M)	48

[a] Control (100%) was 8.99 nmol/min/mg of protein. Data were obtained from microsomes from one animal.
[b] Microsomes were incubated for 5 min at 37° with SKF-525A in the presence of 1 mM NADPH immediately before determination of AA metabolism. Control (100%) was 6.77 nmol/min/mg of protein. Data were obtained from microsomes from one animal.

TABLE 9

Inhibition of EET formation and PRF activity in hepatic microsomes from PB-induced guinea pigs incubated *in vitro* with antibodies to rabbit P450 2B4

Values are percentage inhibition, compared with determination in the presence of the same amount of preimmune serum. Control (100%) values were 8.01 (EETs) and 0.124 (PRF) nmol/min/mg of protein for 2 mg of IgG/mg of microsomal protein and 8.01 (EETs) and 0.101 (PRF) nmol/min/mg of protein for 5 mg of IgG/mg of microsomal protein. Data were obtained from one individual liver.

Antibody	Inhibition	
	EETs	PRF
mg of IgG/mg of microsomal protein	%	
2	14	59
5	36	93

metabolites, EETs and (16–20)-OH-AA. These metabolites were formed only in the presence of NADPH, a required cofactor for P450 monooxygenases. ABT (1 mM), a mechanism-based inhibitor of P450 with relatively low isozyme selectively, virtually abolished the formation of these two metabolite classes (>95% inhibition). In lung microsomes from untreated animals the rates of formation of EETs and (16–20)-OH-AA were 0.18 and 0.04 nmol/min/mg of protein, respectively, about 10% and 2%, respectively, of the rates obtained in hepatic microsomes from untreated guinea pigs.

EETs, the major class of P450 metabolites of AA in guinea pig lung, were formed in a regioselective manner. In lung microsomes from untreated or β-NF-induced guinea pigs 8,9-EET was the most abundant regioisomer (44–45% of total EETs), with 11,12- and 14,15-EET each accounting for about 25% of total EETs. Smaller amounts of 5,6-EET were found

TABLE 10

Inhibition of P450-dependent AA metabolism and isozyme-selective monooxygenase activities in hepatic microsomes from PB-induced guinea pigs treated *in vivo* with αMB

Values are mean ± standard error.

	Activity		
	0 μmol/kg αMB (n = 4)[a]	7.5 μmol/kg αMB (n = 4)	75 μmol/kg αMB (n = 5)
	nmol/min/mg		
EETs	7.80 ± 0.36	5.89 ± 0.88[b] (24)[c]	3.98 ± 0.27[b] (49)
PRF	0.193 ± 0.024	0.105 ± 0.023[b] (46)	0.006 ± 0.001[b] (>95)
ERF	0.252 ± 0.02	0.228 ± 0.009	0.043 ± 0.007[b] (83)
ABP	373 ± 32	197 ± 26[b] (47)	20 ± 2[b] (94)
END[d]	5.71 ± 0.65	5.51 ± 0.60	5.86 ± 0.22

[a] n, number of individual animals.
[b] $p < 0.05$ (different from 0 μmol/kg control; analysis of variance followed by Newman-Keuls test).
[c] Values in parentheses, percentage inhibition (compared with 0 μmol/kg control).
[d] END, erythromycin N-demethylation.

(about 10% of EETs) but this may be due to its ability to serve as a cyclooxygenase substrate (27), given the abundance of this latter enzyme in lung.

Experiments examining the role of individual pulmonary P450 isozymes were primarily carried out using microsomes from β-NF-induced guinea pigs. Polycyclic aromatic hydrocarbons, including β-NF, are known to induce P450 1A1 in guinea pig lung (14), allowing better assessment of the ability of this pulmonary isozyme to metabolize AA. Also, the rate of (16–20)-OH-AA formation was greater (~3-fold) in pulmonary microsomes from β-NF-induced versus untreated guinea pigs.

EETs were formed solely by P450 2Bx in guinea pig lung. This is supported by several experimental findings. 1) Metyrapone and SKF-525A, both P450 2B-selective inhibitors (24, 28), strongly inhibited the formation of EETs in pulmonary microsomes. The majority of the inhibition by SKF-525A required preincubation with the microsomal protein and NADPH, because SKF-525A is converted by P450 to a metabolite that forms a stable inhibitory P450-metabolite complex (28). 2) αMB, a highly selective, mechanism-based, P450 2Bx inhibitor in guinea pig lung, inactivated P450 2Bx-catalyzed PRF activity by >95%, with little effect on ERF and ABP activities, and virtually abolished the formation of EETs *in vitro*. 3) Antibodies to P450 2B4 inhibited the formation of EETs by >95% in guinea pig lung microsomes. These antibodies also inhibited ERF activity to a small extent (28% at 2 mg of IgG/mg of microsomal protein), likely due to P450 2Bx possessing a small amount of ERF activity. 4) Neither α-NF, a potent and highly selective inhibitor of P450 1A1 in lung (29), nor antibodies to rabbit P450 4B1 inhibited the formation of EETs, demonstrating that P450 1A1 and P450 4Bx do not metabolize AA to EETs in guinea pig lung.

P450 2Bx also contributes to the formation of EETs in guinea pig liver. PB, an inducer of this isozyme in liver, increased EET formation ~4-fold. Also metyrapone, SKF-525A, and antibodies to P450 2B4 inhibited their formation in microsomes from PB-induced guinea pigs. However, P450 2Bx accounts for <50% of total EET formation in liver of PB-induced guinea pigs. *In vitro*, antibodies to P450 2B4 that inhibited PRF activity by >90% inhibited EET formation by only 36%. *In vivo*, αMB (75 μmol/kg, intravenously) inhibited PRF activity by >95%, with <50% loss of EET formation.

The ability of guinea pig P450 2Bx, in both lung and liver,

to convert AA to EETs is consistent with the oxidation of AA to EETs by purified P450 2B4 and 2B1 in reconstituted monooxygenase systems (6, 30). Purified rat P450 2C2, which is expressed constitutively in liver, also converts AA to EETs in reconstituted systems (6), and an orthologue may be contributing to EET formation in guinea pig liver.

Guinea pig P450 2Bx is not responsible for the conversion of AA to (16–20)-OH-AA in lung microsomes, as demonstrated by the inability of metyrapone, SKF-525A, αMB, or anti-P450 2B4 antibodies to inhibit its formation. P450 1A1 and 4Bx also do not contribute, because neither α-NF nor antibodies to rabbit P450 4B1 inhibited the formation of (16–20)-OH-AA. These data suggest that a P450 isozyme other than 2Bx, 4Bx, or 1A1 must be involved.

It is not surprising that P450 2Bx does not convert AA to (16–20)-OH-AA in guinea pig lung, because >99% of the AA metabolites produced by purified P450 2B1 were identified as EETs (6). In contrast, the finding that P450 1A1 does not contribute to the formation of (16–20)-OH-AA in guinea pig lung was unexpected. β-NF, a known inducer of P450 1A1 in lung, increased the rate of formation of (16–20)-OH-AA ~3-fold in guinea pig lung. Also, purified P450 1A1 from rat and rabbit liver converts AA to (16–20)-OH-AA in reconstituted systems (6, 30, 31). However, we have also recently shown that P450 1A1 does not contribute to the formation of (16–20)-OH-AA or EETs in kidney microsomes from β-NF-induced guinea pigs (5). The apparent discrepancy in the ability of P450 1A1 from different species to metabolize AA may be due to differences in protein structure. Guinea pig P450 1A1 is about 3 kDa shorter than either rabbit or rat P450 1A1 (13, 32) and is likely missing an amino acid sequence critical for AA binding and/or oxidation. This is the first report of the inability of a P450 4B isozyme from any species to oxidize AA.

Other forms of P450 have not been identified in guinea pig lung, but several additional isozymes have been found in lung of other species, including P450 2A3 in rats (33), P450 2E1 in rats (34) and hamsters (35), P450 2F1 in humans (36), P450 2F2 in mice (37), and P450 4A4 in pregnant (38) and progesterone-treated (39) rabbits. Purified rat and rabbit hepatic P450 2E1 convert AA to (16–20)-OH-AA in reconstituted systems (31, 40) but the ability of the other isozymes to metabolize AA is currently unknown. Of these isozymes only P450 2A3 has been reported to be induced by polycyclic aromatic hydrocarbons in lung (33). Western blot data obtained in our laboratory using antibodies to mouse P450 2A4/2A5 (kindly provided by Dr. Masa Negishi, National Institute of Environmental Health Sciences, Research Triangle Park, NC) indicate that a β-NF-inducible P450 2A isozyme is present in guinea pig lung.[2] Perhaps this P450 2A isozyme is the β-NF-inducible form of P450 that catalyzes the formation of (16–20)-OH-AA in guinea pig pulmonary microsomes.

A variety of peaks were present in the HPLC chromatograms obtained by incubating pulmonary microsomes with AA in the absence of NADPH. These peaks were virtually abolished by boiling of the microsomes before the incubation, indicating that they are formed enzymatically. They are apparently not products of the cyclooxygenase or lipooxygenase pathways of AA metabolism, because inhibitors of these enzymes (indomethacin and nordihydroguaiaretic acid, respectively) did not block their formation. P450 can metabolize AA independently of NADPH if AA hydroperoxides or other lipid hydroperoxides are present (21, 41), and this may account for these products.

In this study we have shown that the pulmonary P450 system of guinea pig oxidizes AA, an endogenous compound, to (16–20)-OH-AA and EETs (primarily the 8,9-, 11,12-, and 14,15-regioisomers). P450-dependent AA metabolites have a wide variety of biological effects and have been implicated as being important in physiology and pathophysiology (reviewed in Refs. 1 and 2). Currently, the effects of these metabolites on the pulmonary system are unknown. Experiments utilizing P450 inhibitors have suggested that P450-dependent AA metabolites may play a role in the vasodilation effects of AA on the pulmonary artery (42) and in the isolated rat lung (43). Because BBT and α-MB inactivate P450 2Bx, the isozyme catalyzing EET formation, in systems with intact cellular structure, they may be useful probes for determining the physiological and/or pathobiological roles of EETs in lung, especially under conditions (such as oxidant stress) that result in AA release. *In vivo*, it is possible that EETs produced in the lung may enter the bloodstream and reach the heart. In this respect, collaborative research from this laboratory (18) has shown that 11,12-EET and 5,6-EET delay the recovery of contractile force in reperfused isolated guinea pig heart after low-flow ischemia and that these EETs also increase the intracellular Ca^{2+} concentration and cell shortening in isolated guinea pig myocytes. All of the EET regioisomers have been reported by others to dilate preconstricted canine coronary arteries *in vitro* (44).

In summary, we have shown that guinea pig pulmonary microsomal P450 monooxygenases convert AA to two classes of primary metabolites, EETs and (16–20)-OH-AA. P450 2Bx is solely responsible for the bioactivation of AA to EETs. A P450 isozyme other than 2Bx, 4Bx, or 1A1, which is β-NF inducible, is responsible for (16–20)-OH-AA formation.

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