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Isozyme-selective, Lung-selective Mechanism-based Inhibitors Of Cytochrome P-450 And Determination Of P450 Isozyme Specificity For Arachidonic Acid Metabolism

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ISOZYME-SELECTIVE, LUNG-SELECTIVE MECHANISM-BASED INHIBITORS OF CYTOCHROME P450 AND DETERMINATION OF P450 ISOZYME SPECIFICITY FOR ARACHIDONIC ACID METABOLISM

by

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ABSTRACT

1-Aminobenzotriazole (ABT) and its N-benzyl (BBT) and N-α-methylbenzyl (αMB) derivatives were compared as isozyme-selective, lung-selective (vs liver) mechanism-based inhibitors of P450 in guinea pigs 4 hr following i.v. administration. Monooxygenase activities selective for guinea pig orthologues of rabbit P450 1A1, 2B4 and 4B1 (1A1, 2Bx and 4Bx, respectively) were determined in pulmonary and hepatic microsomes. BBT and αMB inactivated pulmonary P450 in an isozyme-selective manner. In non-induced and phenobarbital-induced animals the order of inactivation was 2Bx > 1A1 > > > > 4Bx whereas in β-naphthoflavone-induced animals αMB specifically inhibited 2Bx. BBT and αMB were also highly selective for the inactivation of pulmonary vs hepatic P450. In non-induced and induced animals at least one of the doses examined caused marked inactivation of pulmonary 2Bx (>80% with αMB and 50-70% with BBT) without inhibiting any of the hepatic monooxygenase activities. In contrast, ABT displayed little isozyme-selectivity and little tissue-selectivity.

Guinea pig lung microsomes were found to convert arachidonic acid to two classes of P450-dependent metabolites, 16- through 20-hydroxyeicosatetraenoic acids ((16-20)-OH-AA) and epoxyeicosatrienoic acids (EETs). (16-20)-OH-AA formation was increased by β-naphthoflavone treatment. P450 2B selective inhibitors (metyrapone, SKF-525A and αMB) as well as antibodies to rabbit P450 2B4 inhibited EETs formation by >85%-

>95% with little effect on (16-20)-OH-AA formation. Neither a P450 1A selective inhibitor (*α*-naphthoflavone) nor antibodies to rabbit P450 4B1 inhibited the formation of either class of metabolites. These data demonstrate that P450 2Bx is solely responsible for the metabolism of arachidonic acid to EETs in guinea pig lung and that a form of P450 other than 2Bx, 4Bx or 1A1, which is inducible by *β*-naphthoflavone, forms (16-20)-OH-AA.

Guinea pig kidney microsomes also formed (16-20)-OH-AA and EETs. The formation of these metabolites was not affected by *β*-naphthoflavone induction or P450 1A1 inhibitors demonstrating that P450 1A1 also does not contribute to arachidonic acid metabolism in guinea pig kidney.

In summary, BBT and *α*MB, at appropriate doses, are isozyme-selective/specific (P450 2Bx), lung-specific inhibitors of P450 in guinea pig *in vivo*. In guinea pig lung, P450 2Bx is responsible for the metabolism of arachidonic acid to EETs. Guinea pig P450 4Bx and 1A1 do not metabolize arachidonic acid.

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α-NF	*α*-naphthoflavone
*α*MB	N-*α*-methylbenzyl-1-aminobenzotriazole
AA	arachidonic acid (5,8,11,14-*cis*-eicosatetraenoic acid)
ABP	4-aminobiphenyl N-hydroxylation
ABT	1-aminobenzotriazole
AIA	2-isopropyl-4-pentamide (allylisopropylacetamide)
ANOVA	analysis of variance
A.U.	arbitrary unit
β-NF	*β*-naphthoflavone
BBT	N-benzyl-1-aminobenzotriazole
BND	benzphetamine N-demethylation
BSA	bovine serum albumin
20-COOH-AA	1,20-eicosatetraenedoic acid
cDNA	complementary deoxyribonucleic acid
DETAPAC	diethylenetriaminepentaacetic acid
DiHETE	dihydroxyeicosatrienoic acids
DMSO	dimethyl sulfoxide
EET	epoxyeicosatrienoic acid
END	erythromycin N-demethylation
ERF	7-ethoxyresorufin O-deethylation
HETE	hydroxyeicosatetraenoic acid

HPLC	high pressure liquid chromatography
i.p.	intraperitoneal
i.v.	intravenous
3-MC	3-methylcholanthrene
MI-complex	metabolic intermediate complex
mRNA	messenger ribonucleic acid
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced form
NADH	β-nicotinamide adenine dinucleotide, reduced form
NDGA	nordihydroguaiaretic acid
(16-20)-OH-AA	16-, 17-, 18-, 19- and 20-hydroxyeicosatetraenoic acid
P450	cytochrome P450
PAH	polycyclic aromatic hydrocarbon
PB	phenobarbital
PRF	7-pentoxyresorufin O-depentylation
SDS	sodium dodecyl sulfate
SKF-525A	diethylaminoethyl-2,2-diphenylvalerate HCl
TBS	Tris buffered saline (20 mM Tris-HCl, pH 7.4, 0.9% NaCl)
TBS-Tween	Tris buffered saline containing 0.05% Tween-20
TCDD	2,3,7,8-tetrachlorodibenzoparadioxin
TEMED	tetramethylethylenediamine

CHAPTER 1

INTRODUCTION

1.1 CYTOCHROME P450-DEPENDENT MONOOXYGENASE SYSTEM

1.1.1 Overview

The hemoprotein cytochrome P450 (P450), which exists as multiple isozymes, is the terminal component of the P450-dependent monooxygenase, or mixed-function oxidase, system. This system catalyzes the oxidation of a wide variety of lipophilic substrates of both exogenous and endogenous origin including drugs, carcinogens, antioxidants, solvents, anesthetics, dyes, pesticides, petroleum products, alcohols, eicosanoids, fatty acids, vitamins and steroids (Guengerich, 1988; Porter and Coon, 1991). P450 usually converts exogenous compounds to metabolites that are less biologically active and are excreted either unchanged or after conjugation with endogenous compounds such as glucuronic acid and glutathione. But, this system also transforms some xenobiotics into more biologically active and/or toxic metabolites. For example, some prodrugs, such as cyclophosphamide, must be metabolized by P450 for their therapeutic activity (Brock, 1967; Calabresi and Chabner, 1990), and compounds, such as procarcinogens, are converted by P450 to products with much greater cytotoxicity, mutagenicity or carcinogenicity (Guengerich and Shimada, 1991; Soucek and Gut, 1992). Some of the biotransformations of endogenous compounds carried out by P450 are essential for life such as the conversion of cholesterol to sex steroids and

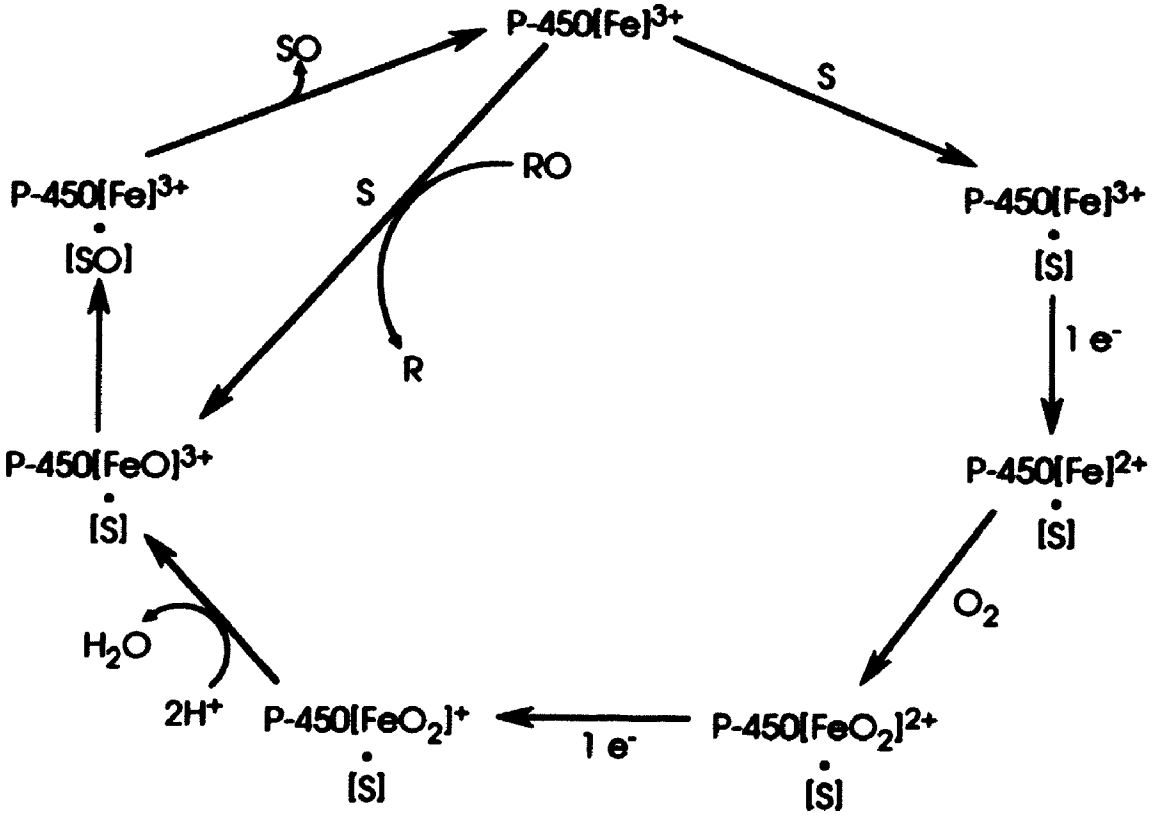
cortisol (Nebert and Gonzalez, 1987; Porter and Coon, 1991). Hence, the P450 system is of importance in pharmacology, toxicology and physiology.

In mammals P450 is localized primarily in liver although it is present at lower levels in almost every other tissue except striated muscle and erythrocytes (Guengerich, 1988). Within the cell, it is found predominantly in the endoplasmic reticulum (i.e. microsomes) and mitochondria. In addition to the hemoprotein P450, the mammalian microsomal P450 monooxygenase system consists of the flavoprotein NADPH-cytochrome P450 reductase which transfers electrons from NADPH to P450 (Black and Coon, 1987). In mammalian liver the stoichiometry of P450 to reductase molecules is about 20:1 (Estabrook *et al.*, 1971). In the mitochondrial system the electron transport chain consists of NADPH, a ferredoxin reductase, an iron-sulfur protein (ferredoxin) and P450 (Black and Coon, 1987).

Most of the reactions catalyzed by the P450 system are oxidative and result in the insertion of a single atom of oxygen, derived from O_2, into the substrate (Black and Coon, 1987; Guengerich *et al.*, 1990a; Porter and Coon, 1991). A generalized reaction scheme is illustrated in Fig. 1.1. Ferric P450 binds the substrate and then accepts an electron. Molecular oxygen binds to the reduced complex and a second electron is transferred. In the microsomal system in some instances cytochrome b_5 (via NADH cytochrome b_5 reductase) can contribute the second electron. The oxygen bonds splits and one oxygen atom is reduced to water and the other forms the putative $(FeO)^{3+}$ complex. Insertion of the oxygen into the substrate is believed to involve abstraction of

Figure 1.1

Scheme for the mechanism of action of P450. Fe represents the heme iron at the active site, S the substrate, SO a monooxygenation product, and RO an alternate oxygen donor (eg. iodosylbenzene or alkyl hydroperoxides). Adapted from Guengerich, 1990a.


P-450[Fe]3+
SO
S
P-450[Fe]3+
[SO]
S
RO
R
P-450[Fe]3+
[S]
1 e-
P-450[FeO]3+
[S]
P-450[Fe]2+
[S]
O2
H2O
2H+
P-450[FeO2]+
[S]
1 e-
P-450[FeO2]2+
[S]

a hydrogen atom or an electron from the substrate by $(FeO)^{3+}$ followed by "oxygen rebound" to an incipient radical site on the substrate (Guengerich, 1990a). The oxidized substrate then dissociates from the P450 active site regenerating the ferric P450 and completing the cycle. The oxidized substrate may be unstable and rearrange/breakdown to form the final product(s). The overall reactions include such processes as aliphatic and aromatic hydroxylation, N-,O-, and S-dealkylation, deamination, N-oxidation, N-hydroxylation, sulfoxide formation, dehalogenation and epoxidation (Nebert and Gonzalez, 1987; Guengerich, 1991). In some cases P450 can oxidize a substrate at the expense of a peroxy compound such as an alkyl hydroperoxide or peracid, or other oxidants such as iodosylbenzene, with no requirement for O_2, NADPH or the reductase (Guengerich, 1991; Porter and Coon, 1991).

The P450 system also catalyzes reduction reactions. Substrates that undergo reduction such as epoxides, N-oxides, nitro and azo compounds, and lipid hydroperoxides, bind to the ferric P450 and then accept two electrons in a stepwise fashion (Porter and Coon, 1991). An atypical reaction catalyzed by P450, requiring neither O_2, NADPH or the reductase, is the rearrangement of prostaglandin H_2 catalyzed by two specific P450 isozymes, known as thromboxane synthase and prostacyclin synthase (Hecker and Ullrich, 1989).

P450 exists as multiple isozymes encoded by a gene superfamily. Greater than 30 P450 genes may be expressed in a single organism (Guengerich, 1991). The P450 isozymes have been classified into families and subfamilies based on their amino acid sequences deduced directly from protein

sequencing or indirectly from cDNA sequencing (Nelson *et al.*, 1993). P450 proteins with >40% sequence similarity are included in the same family and those with >55% similarity are included in the same subfamily. P450s are named by a number designating the family, followed by a letter for the subfamily and another number denoting the individual P450 form.[1] Currently, 12 families, comprising 22 subfamilies have been identified in mammals (Nelson *et al.*, 1993). A hemoprotein is classified as a P450 based on its absorption spectrum; the reduced-carbon monoxide complex has a characteristic absorption maximum near 450 nm due to axial ligation with a cysteine thiolate of the protein (Black and Coon, 1987; Guengerich, 1991).

Mammalian P450 isozymes in families 1, 2, 3 and 4, which include at least thirteen subfamilies, are localized in the endoplasmic reticulum and have unusually broad and overlapping substrate specificities. These P450s primarily metabolize exogenous compounds and are referred to as "xenobiotic-metabolizing P450s" (Nebert and Gonzalez, 1987; Gonzalez, 1992). It has been proposed that most, if not all, of the isozymes in these four families have evolved in animals initially in response to plant metabolites and later in evolution in response to combustion products (Nebert and Gonzalez, 1987). P450 isozymes in the remaining eight mammalian families, which include nine subfamilies, metabolize endogenous compounds and display rather rigid

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[1]This nomenclature system is used to refer to specific P450 isozymes throughout this thesis. In guinea pig only P450 1A1 has been sequenced and assigned to this nomenclature family, therefore, the guinea pig orthologues of rabbit 2B4 and 4B1 are referred to as 2Bx and 4Bx, respectively, throughout this thesis.

substrate and product specificities (Gonzalez, 1992; Nelson *et al.*, 1993). These include the P450s involved in the synthesis of steroids and bile acids as well as thromboxane synthase and 25-hydroxyvitamin D_3 24-hydroxylase. It should be noted that some P450s, such as prostacyclin synthase, have not as of yet been assigned to a P450 family/subfamily because their amino acid sequences have not been determined.

Cytochrome P450 isozymes are differentially expressed. Some P450s are expressed "constitutively" but most exhibit developmental, sex and/or tissue specific regulation (Nebert and Gonzalez, 1987; Gonzalez 1989). Some of the "xenobiotic-metabolizing P450s" are induced (ie. the amount is increased) by foreign chemicals. Although the number of chemicals that induce P450 is very large, most can be classified into one of five basic categories based on the P450 subfamily which they selectively induce. Polycyclic aromatic hydrocarbons (PAH)-type inducers, including 2,3,7,8-tetrachlorodibenzoparadioxin (TCDD), 3-methylcholanthrene (3-MC), and β-naphthoflavone (β-NF), induce P450 1A; phenobarbital (PB) and a variety of structurally diverse chemicals preferentially induce P450 2B; ethanol, as well as acetone, isopropanol, isoniazid, imidazole and pyrazole induce P450 2E; pregnenolone-16α-carbonitrile, glucocorticoids (eg. dexamethasone), macrolide antibiotics (eg. triacetyloleandomycin and erythromycin), and antifungal agents (eg. ketaconazole, clotrimazole) are inducers of P450 3A; and hypolipidemic drugs such as clofibrate induce P450 4A (Okey, 1990).

Although the liver contains the greatest total amount of P450 and the

greatest number of individual isozymes, P450 expressed in other tissues is also of significance. In terms of xenobiotic metabolism, extrahepatic P450 generally contributes little quantitatively but is involved in chemical-mediated organ toxicity. Extrahepatic P450 also plays an important role in the metabolism of endogenous compounds.

1.1.2 Lung

The lung is exposed to blood-borne compounds of both endogenous and exogenous origin, as well as air-borne xenobiotics, because of its intimate contact with both blood and the external environment. It receives the entire output of the right heart and is the major portal of entry for airborne xenobiotics. The pulmonary P450 system is of importance in chemical-mediated toxicity. Many xenobiotics that cause lung toxicity including 4-ipomeanol, a lung specific toxin, and benzo(*a*)pyrene, a pulmonary carcinogen, are bioactivated by pulmonary P450 to their toxic metabolites (Boyd, 1980; Yost *et al.*, 1989; Cohen, 1990; Shimada *et al.*, 1992). Pulmonary P450 also metabolizes endogenous compounds such as laurate, palmitate, arachidonate and prostaglandins (Oliw and Moldeus, 1982; Williams *et al.*, 1984; Yamamoto *et al.*, 1984) although the physiological/pathophysiological significance of these biotransformations in lung is not known.

The lung is a heterogenous organ containing greater than 40 different cell types (Sorokin, 1970). Clara and alveolar type II cells generally contain the highest concentrations of P450 although lower amounts occur in ciliated,

goblet and endothelial cells as well as alveolar macrophages (Domin *et al.*, 1986; Keith *et al.*, 1987; Serabjit-Singh *et al.*, 1988; Overby *et al.*, 1992). The high concentration of P450 in Clara cells, and relative lack of detoxication enzymes, makes these cells especially sensitive to toxicity mediated by compounds activated by pulmonary P450. As mentioned above, 4-ipomeanol induced necrosis is localized primarily in Clara cells (Boyd, 1980).

The pulmonary microsomal P450 monooxygenase system was first characterized in the rabbit and was found to consist largely of single isozymes from three subfamilies: P450 1A1, 2B4 and 4B1 (Domin *et al.*, 1984; Philpot *et al.*, 1985). Subsequently, orthologues of these isozymes have been found in the lung of many other species.

P450 1A1, but not the closely related 1A2, is expressed in lung of rabbit, rat, mouse, hamster, guinea pig, baboon and human (Liem *et al.*, 1980; Domin *et al.*, 1984; Goldstein and Linko, 1984; Philpot *et al.*, 1985; Watanabe *et al.*, 1987; Wheeler *et al.*, 1990; Sagami *et al.*, 1991; Beebe *et al.*, 1992). The amount of pulmonary 1A1 is very low, and sometimes not immunodetectable, in untreated animals but is increased by PAH-type inducers. P450 1A1 comprises 1-3% of the pulmonary P450 in untreated rabbits whereas in rabbits induced with TCDD 15-20% of the pulmonary P450 is 1A1 (Domin *et al.*, 1984). In humans the expression of pulmonary 1A1 is higher in smokers compared to non-smokers (McLemore *et al.*, 1990; Shimada *et al.*, 1992). P450 1A1 activates PAH procarcinogens, such as benzo(a)pyrene, Shimada *et al.*, 1992), and has been implicated in the development of lung

cancer in smokers (Anttila *et al.*, 1991; reviewed in Kawajiri and Fujii-Kuriyama, 1991).

Orthologues of rabbit 2B4 (rat 2B1) have been found in the lung of untreated rabbit, rat, hamster, mouse, guinea pig, monkey and sheep (Domin *et al.*, 1984; Vanderslice *et al.*, 1987; Adali and Arinc, 1990; Williams *et al.*, 1991; Yamada *et al.*, 1992). In rabbit, this isozyme comprises 30-40% of the pulmonary P450 (Domin *et al.*, 1984). In all species examined, 2B isozymes are induced by PB in the liver but not in the lung (Domin *et al.*, 1984; Imaoka *et al.*, 1989a; Yamada *et al.*, 1992). Pulmonary 2B4/2B1 is also not induced by PAH-type inducers (Serabjit-Singh *et al.*, 1983; Domin *et al.*, 1984; Keith *et al.*, 1987). Recently it was reported that two mRNAs are present in rabbit lung coding for proteins which differ only by 6 amino acids suggesting two closely related 2B4 proteins may be present in rabbit lung (Gasser *et al.*, 1988). Rat 2B2, which is 97% similar in amino acid sequence to rat 2B1, is not expressed in lung (Domin *et al.*, 1984; Imaoka *et al.*, 1989a).

P450 4B1 is present in lung of untreated rabbit, rat, mouse, hamster, guinea pig and monkey (Vanderslice *et al.*, 1987). In rabbit, it comprises 30-40% of the total pulmonary P450 (Domin *et al.*, 1984). mRNA coding for P450 4B1 has also been detected in human lung (Nhamburo *et al.*, 1989). Pulmonary P450 4B1 is not induced by either PB or PAH-type inducers (Serabjit-Singh *et al.*, 1983; Vanderslice *et al.*, 1987; Gasser and Philpot, 1989). In liver, the expression of P450 4B1 is regula :d in a species-dependent manner. This isozyme is expressed in liver of rabbit and hamster

but not mouse, guinea pig, rat and monkey, and is inducible by PB only in rabbit (Vanderslice *et al.*, 1987; Gasser and Philpot, 1989). P450 4B1 activates aromatic amines such as 2-aminoanthracene, 2-aminofluorene, and 2-acetylaminofluorene (Robertson *et al.*, 1981; Vanderslice *et al.*, 1987) although human 4B1 appears to be an exception (Nhamburo *et al.*, 1989). Rabbit 4B1 also hydroxylates lauric acid in the ω and ω-1 positions (Williams *et al.*, 1984).

Other forms of P450 are expressed in lung but they are present in very low amounts, and/or their expression appears to be limited to certain species and/or conditions. These include isozymes from the 2A, 2E, 2F and 4A subfamilies.

mRNA coding for P450 2A3 is present in rat lung and its levels are increased approximately 3-fold by pretreatment with 3-MC but are not affected by PB pretreatment (Kimura *et al.*, 1989a). P450 2A3 mRNA is not expressed in liver, kidney or intestine of the rat (Kimura *et al.*, 1989a). The catalytic function of rat 2A3 has not been examined.

Immunoreactive 2E protein is present in low amounts in lung of untreated rat and hamster, and its levels are increased, as in the liver, by treatment with acetone, isoniazid or streptozotocin (Song *et al.*, 1987; Thomas *et al.*, 1987; Ueng *et al.*, 1991). Interestingly, mRNAs coding for 2E1 and 2E2 are present in rabbit lung (Porter *et al.*, 1989) but no immunoreactive 2E protein has been detected (Ding *et al.*, 1986; Ueng *et al.*, 1987).

P-50 2F2 is expressed in mouse lung (Ritter *et al.*, 1991). This isozyme

hydroxylates naphthalene and is postulated to be responsible for the naphthalene-induced necrosis of Clara cells in this species (Ritter *et al.*, 1991). P450 2F2 mRNA is not induced in mouse lung by PB, pyrazole, pregnenolone 16α-carbonitrile, or 3-MC (Ritter *et al.*, 1991). mRNA coding for another 2F isozyme, P450 2F1, is present in very low levels in human lung (Nhamburo *et al.*, 1990). Vaccinia-expressed human 2F1 bioactivates 3-methylindole, a pulmonary toxin (Thornton-Manning, 1991). It is likely that a 2F isozyme closely related to human 2F1 is expressed in rat lung as cDNA probes to human P450 2F1 (Nhamburo *et al.*, 1990), but not mouse 2F2 (Ritter *et al.*, 1991), recognize an mRNA species in rat lung that comigrates on Northern blots with human 2F1 mRNA.

P450 4A4 has been purified from lung of pregnant (Williams *et al.*, 1984) or progesterone-treated (Yamamoto *et al.*, 1984) rabbits. In pregnant rabbits, this isozyme constitutes about 25% of the pulmonary P450 but in control animals (ie. nonpregnant, untreated) the levels of this isozyme are very low, or undetectable (Williams *et al.*, 1984). Purified P450 4A4 ω-hydroxylates a number of prostaglandins, including PGE_1, PGE_2, and PGA_1, and $\omega/(\omega-1)$-hydroxylates palmitate and myristate (Yamamoto *et al.*, 1984). Changes in 4A4 expression, assessed at both the protein and mRNA levels, account for the marked increase (>100-fold) in prostaglandin ω-hydroxylation in lung microsomes during pregnancy (Powell, 1978; Matsubara *et al.*, 1987; Muerhoff *et al.*, 1987). Orthologues of this isozyme have not been described in other species. Hamster and rat do not demonstrate increased ω-

hydroxylation of prostaglandins during pregnancy (Powell, 1978) and in pregnant rat, no lung 4A mRNA was detected on Northern blots (Nhamburi *et al.*, 1989).

1.1.3 Kidney

The kidney P450 monooxygenase system is of significance. The kidney receives 25% of the resting cardiac output and due to the inherent concentrative nature of this organ, extremely high concentrations of xenobiotics result in the proximal tubule. The microsomal P450 system in the kidney is of particular importance in renal toxicity. Some compounds that cause renal toxicity and/or cancer, such as acetaminophen and nitrosamines, are bioactivated by renal P450 (Bend and Serabjit-Singh, 1984; Hong *et al.*, 1989). The microsomal P450 system of kidney also efficiently metabolizes a variety of endogenous fatty acids including laurate, palmitate and arachidonate (Ellin *et al.*, 1973; Schwartzman *et al.*, 1986; Lapuerta *et al.*, 1988). P450-dependent metabolites of arachidonic acid (5,8,11,14-cis-eicosatetraenoic acid; AA) are believed to be important in the regulation of renal function and the development of hypertension (Section 1.2.2). The mitochondrial P450 system in the kidney is important in vitamin D metabolism and consequently in maintaining Ca^{2+} homeostasis. It hydroxylates 25-hydroxy-vitamin D_3 forming 1α,25-dihydroxy-vitamin D_3, the most biologically active form of this vitamin, and 24,25-dihydroxy-vitamin D_3 (DeLuca and Schnoes, 1983).

In the kidney nephron P450 is localized primarily in the proximal tubule

(Endou, 1983) although other parts including the glomerulus, medullary ascending limb of Henle, distal convoluted tubule, cortical collecting tubule and the medullary collecting tubule also contain a functional P450 monooxygenase system (Omata *et al.*, 1992a). Several subfamilies of microsomal P450 are found in the kidney of mammals including 1A, 2A, 2B, 2C, 2E, 3A and 4A, although it is not known if all of these subfamilies are expressed in a given species.

P450 1A1, but not the closely related 1A2, is expressed in rat (Goldstein and Linko, 1984; Christou *et al.*, 1987; Pasco *et al.*, 1988) and rabbit (Liem *et al.*, 1980) kidney. Both the amount of 1A1 protein and an associated activity, 7-ethoxyresorufin O-deethylation (ERF), are very low in untreated animals but are increased 50-100-fold by PAH inducers (Liem *et al.*, 1980; Goldstein and Linko, 1984; Iwasaki *et al.*, 1986; Christou *et al.*, 1987). ERF activity is also markedly greater in renal microsomes from β-NF-treated compared to untreated guinea pigs indicating that 1A1 is also expressed and induced in guinea pig kidney (Falkner *et al.*, 1993).

Isozymes of the 2A subfamily are expressed in mouse kidney in a sex-dependent manner (Squires and Negishi, 1988, 1990; Henderson *et al.*, 1990). P450 2A4 mRNA is present in the kidney of male mice whereas P450 2A5 mRNA is found in female mice (Squires and Negishi, 1988, 1990). No evidence has indicated that the 2A subfamily is expressed in kidney of other species. P450 2A3 mRNA is not present in rat kidney (Kimura *et al.*, 1989a) nor does this tissue contain detectable anti-2A1 immunoreactive protein

(Yeowell *et al.*, 1988; Sonderfan *et al.*, 1989).

The P450 2C subfamily is expressed in rabbit and rat kidney. In rabbit kidney 2C2, but not 2C1, 2C3 or 2C5, is expressed constitutively and is induced by PB treatment (Leighton and Kemper, 1984; Finlayson *et al.*, 1987). Reconstituted P450 2C2 purified from rabbit renal cortex catalyzes the oxidation of AA and ω/(ω-1)-hydroxylates lauric acid (Laethem *et al.*, 1992). P450 2C2 expressed in COS-1 cells also catalyzes these reactions (Laethem and Koop, 1992). Rats express a protein that is immunoreactive with 2C11 antibodies and like 2C11 catalyzes 2α- and 16α-hydroxylation of testosterone (Sonderfan *et al.*, 1989). This protein may not be 2C11 but a closely related protein because 2C11 mRNA, as well as 2C6, 2C7 and 2C12 mRNA, are not detectable in rat kidney (Sundseth and Waxman, 1992). 2C24 mRNA has been found in the kidney of male rats (Zaphiropoulos, 1991) but the catalytical activities of the 2C24 protein are not known.

P450 2B1 protein is not detectable immunochemically in kidney of untreated rats but has been detected, in extremely low levels in some, but not all, rats treated with PB (Guengerich *et al.*, 1982a; Christou *et al.*, 1987; Imaoka *et al.*, 1989a). Interestingly, cDNA probes for 2B1/2B2 but not oligonucleotide probes specific for 2B1 or 2B2, hybridized with a PB inducible mRNA suggesting that a mRNA similar to 2B1 and 2B2, but possibly neither of these, is expressed in rat kidney. In mice a 2B1 immunoreactive protein, which is present in males but not females and is induced by dexamethasone but not PB, has been detected (Henderson *et al.*, 1990).

P450 2E1 is present in kidney of untreated rats (Hong *et al.*, 1987; Thomas *et al.*, 1987; Yun *et al.*, 1992), rabbit (Ding *et al.*, 1986; Porter *et al.*, 1989), mouse (Hong *et al.*, 1989) and hamster (Ueng *et al.*, 1991) and its concentration is increased by fasting or treatment with acetone, ethanol, imidazole or streptozotocin. 2E2, a closely related form, is expressed in liver but not the kidney of rabbit (Khani *et al.*, 1988). In certain mouse strains 2E1 expression is higher in males than females (Hong *et al.*, 1987, 1989). This may account for the higher susceptibility of male mice, compared to female mice, to N-nitrosodimethylamine renal toxicity as P450 2E1 converts this chemical to a carcinogenic metabolite.

P450 3A isozymes are also expressed in the kidney of rat (Schuetz *et al.*, 1992), mouse (Henderson *et al.*, 1990) and human (Schuetz *et al.*, 1992). Also, a toad kidney epithelial cell line has been shown to contain 3A proteins and mRNAs, and to catalyze associated monooxygenase activities (Schuetz *et al.*, 1992). Dexamethasone and corticosterone increased the amount of the 3A immunoreactive proteins in this cell line (Schuetz *et al.*, 1992).

The P450 4A subfamily is expressed in kidney of rabbit, rat and human. P450 4A5, 4A6 and 4A7 proteins and cDNA clones have been isolated from the rabbit kidney (Kusunose *et al.*, 1989; Yokotani *et al.*, 1989; Johnson *et al.*, 1990; Yoshimura *et al.*, 1990; Sawamura *et al.*, 1993). Rat kidney expresses several 4A mRNAs and proteins of which only 4A2, the major form expressed constitutively, has been purified (Imaoka and Funae, 1986; Kimura *et al.*, 1989b; Sharma *et al.*, 1989a). In both species 4A isozymes are induced in

kidney by peroxisome proliferators such as clofibrate (Kimura *et al.*, 1989b; Sharma *et al.*, 1989a; Yokotani *et al.*, 1989; Muerhoff *et al.*, 1992). P450 4A11 has been purified from human kidney cortex (Imaoka *et al.*, 1990; Kawashima *et al.*, 1992). 4A isozymes catalyze the $\omega/(\omega\text{-}1)$-hydroxylation of fatty acids including laurate, myristate, palmitate and arachidonate (Imaoka *et al.*, 1989b; Kusunose *et al.*, 1989; Johnson *et al.*, 1990; Yoshimura *et al.*, 1990; Kawashima *et al.*, 1992). Some 4A isozymes also ω-hydroxylate prostaglandins (Kusunose *et al.*, 1989; Johnson *et al.*, 1990; Sawamura *et al.*, 1992).

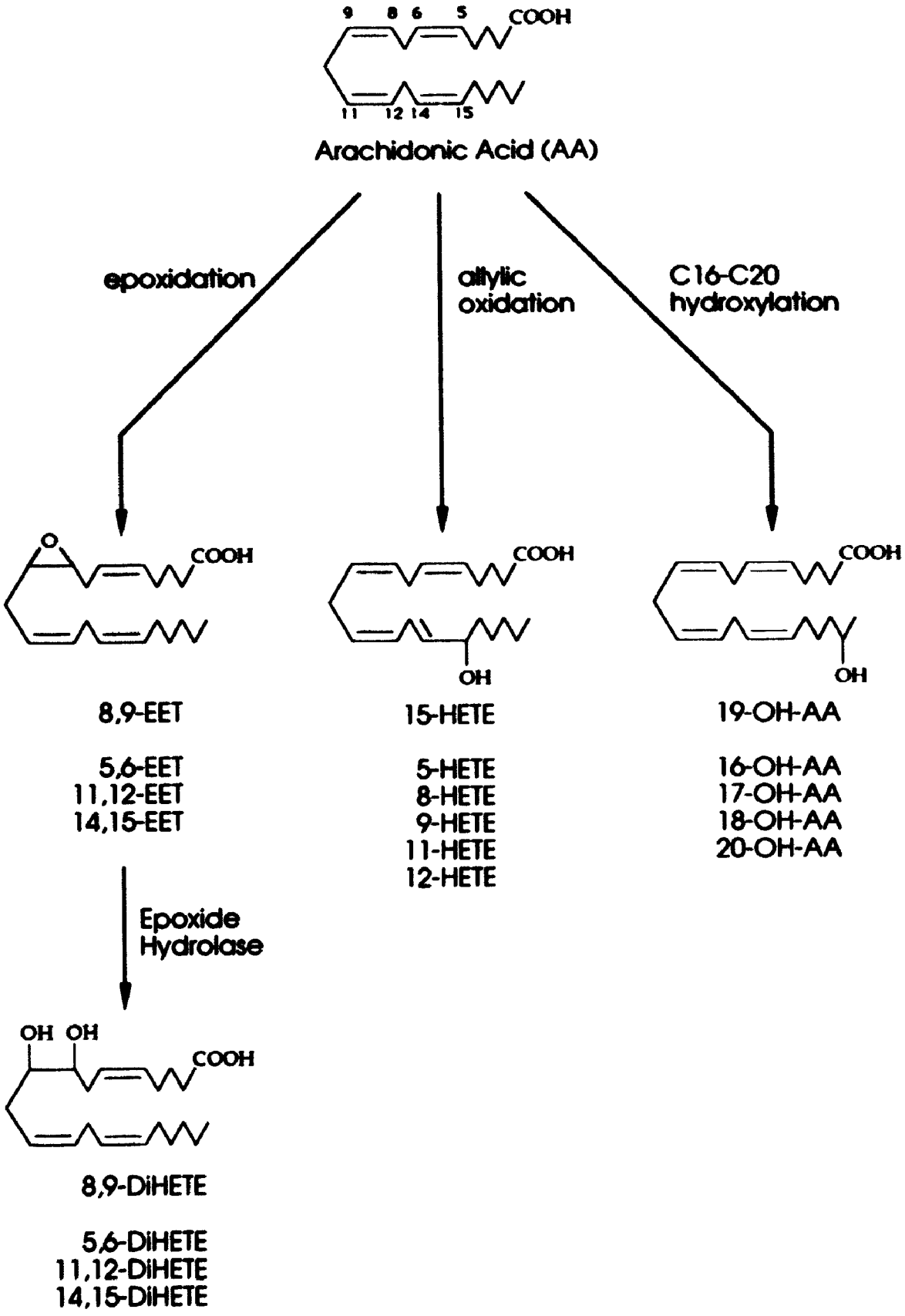
1.2 CYTOCHROME P450-DEPENDENT ARACHIDONIC ACID METABOLISM

1.2.1 Overview

In 1981, three laboratories reported that in addition to the cyclooxygenanse and lipoxygenase pathways for AA metabolism, there exists a microsomal NADPH-dependent pathway (Capdevila *et al.*, 1981; Morrison and Pascoe, 1981; Oliw *et al.*, 1981). It is now well recognized that the microsomal P450 monooxygenase system converts AA to three classes of primary metabolites (Fig. 1.2; reviewed in Fitzpatrick and Murphy, 1989; McGiff, 1991; Capdevila *et al.*, 1992a): 1/ four regioisomeric *cis*-epoxyeicosatrienoic acids (EETs), 5,6-EET, 8,9-EET, 11,12-EET and 14,15-EET are formed by olefin epoxidation; 2/ six regioisomeric *cis, trans*-conjugated hydroxyeicosatetraenoic acids (HETEs), 5-, 8-, 9-, 11-, 12- and 15-HETE are formed by allylic oxidation; 3/ 16-, 17-, 18-, 19- and 20-hydroxy-

Figure 1.2

AA metabolites formed by P450-dependent monooxygenases.


9 8 6 5 COOH
11 12 14 15
Arachidonic Acid (AA)
epoxidation
allylic oxidation
C16-C20 hydroxylation
COOH
OH
8,9-EET
5,6-EET
11,12-EET
14,15-EET
15-HETE
5-HETE
8-HETE
9-HETE
11-HETE
12-HETE
19-OH-AA
16-OH-AA
17-OH-AA
18-OH-AA
20-OH-AA
Epoxide Hydrolase
OH OH
8,9-DiHETE
5,6-DiHETE
11,12-DiHETE
14,15-DiHETE

eicosatetraenoic acid [(16-20)-OH-AA] are formed by C16 through C20 hydroxylation. These primary metabolites may also undergo further metabolism. For example, EETs can be hydrolyzed by cytosolic epoxide hydrolase to form the corresponding vicinal diols (dihydroxyeicosatrienoic acids; DiHETEs; Chacos *et al.*, 1983); 5,6-EET and 20-OH-AA can be metabolized by cyclooxygenase to form novel prostaglandins (Oliw, 1984a, 1984b; Oliw and Bunthin, 1985; Schwartzman *et al.*, 1989); and 20-OH-AA can be oxidized to 1,20-eicosatetraenedioic acid (20-COOH-AA; Oliw *et al.*, 1981; Carroll *et al.*, 1991).

P450-dependent AA metabolites are produced by microsomal fractions, or isolated cell preparations from numerous tissues including kidney (Lapuerta *et al.*, 1988; Schwartzman *et al.*, 1986, 1990), liver (Oliw *et al.*, 1981; Capdevila *et al.*, 1990a), lung (Oliw and Moldeus, 1982), hypothalamus (Capdevila *et al.*, 1983), cornea (Schwartzman *et al.*, 1985a), pituitary gland (Capdevila *et al.*, 1984a) and adrenal gland (Nishimura *et al.*, 1989; Campbell *et al.*, 1991). Many purified P450 isozymes have been reported to metabolize AA in reconstituted systems including rat hepatic 1A1, 1A2, 2B1, 2B2, 2C6, 2C11, 2E1 and 4A1 (Oliw *et al.*, 1982; Bains *et al.*, 1985; Capdevila *et al.*, 1990a; Tanaka *et al.*, 1990); rabbit hepatic 1A1, 2B4 (Oliw *et al.*, 1982) and 2E1 (Laethem *et al.*, 1993); human hepatic P450-AA (Laniado-Schwartzman *et al.*, 1988); and rabbit renal P450 2C2 (Laethem *et al.*, 1992) and 4A7 (Sawamura *et al.*, 1993). Other purified isozymes, such as rat hepatic P450 2A1 and 2C12, do not catalyze AA metabolism to a significant extent

(Capdevila *et al.*, 1990a). Rabbit P450 2C1 and 2C2 expressed in COS-1 cells also metabolize AA (Laethem and Koop, 1992).

The profile of P450 dependent metabolites of AA produced by a tissue is dependent on its P450 isozyme composition as individual isozymes vary not only in their ability to turn over AA, but also in the type of oxidation (ie. epoxidation, allylic oxidation, or C16-C20 hydroxylation) and the regio- and stereoselectivity of attack. For example, purified rat P450 1A1 primarily forms (16-20)-OH-AA (~87% of P450-dependent metabolites), 2B1 primarily forms EETs (>99%), whereas with 1A2 not more than 44% of the metabolites belong to the same metabolite class (Capdevila *et al.*, 1990a). Of the EETs rmed by purified rat 1A1, 1A2 and 2B1, 22%, 58% and 37%, respectively, are the 11,12 regioisomer with 13%, 95% and 54% of this regioisomer, respectively, as the [R,S]- enantiomer (Capdevila *et al.*, 1990a). In respect to (16-20)-OH-AA formation, 1A1 primarily forms 19-OH-AA whereas 1A2 primarily forms 16-OH-AA (Falck *et al.*, 1990).

P450-dependent AA metabolites are formed not only *in vitro*, but also *in vivo*. EETs are endogenous constituents of several tissues including kidney (Falck *et al.*, 1987; Karara *et al.*, 1990; Takahashi *et al.*, 1990), liver (Capdevila *et al.*, 1984b; Karara *et al.*, 1991) and brain (Junier *et al.*, 1990), and occur in plasma (Karara *et al.*, 1992) and urine (Toto *et al.*, 1987; Catella *et al.*, 1990). The regio- and stereoselectivity of endogenous EETs formation in the liver can be altered by treating animals with isozyme-selective inducers (Capdevila *et al.*, 1990a). In both liver and plasma >90% of the EETs are

esterified to the sn-2 position of phospholipids suggesting that EETs can be released from phospholipid membranes independent of AA oxidation (Karara *et al.*, 1991, 1992). In urine, 20-OH-AA has also been found (Schwartzman *et al.*, 1991).

During the past decade, determining the physiological relevance of AA metabolites formed by P450 monooxygenases has become an area of intense research. These metabolites have been shown to have a wide range of biological effects. They stimulate peptide hormone release, have vasoactivity, alter ion transport/movement in particular Na^+/K^+ ATPase, inhibit platelet aggregation, and inhibit cyclooxygenase activity (Table 1.1). In some instances the primary P450-dependent AA metabolite requires further metabolism for manifestation of its biological activity. For example, 5,6-EET and 20-OH-AA are metabolized by cyclooxygenase to a potent vasodilator and vasoconstrictor, respectively (Escalante *et al.*, 1989; Schwartzman *et al.*, 1989; Carroll *et al.*, 1990, 1993). In terms of physiological/pathophysiological relevance, these metabolites are implicated in mediation of release of peptide hormones such as dopamine stimulated release of somatostatin from hypothalamic neurons (Junier *et al.*, 1990), increased corneal thickness and conjunctival inflammation association with contact lens complications (Davis *et al.*, 1992), regulation of renal function (Section 1.2.2), and development of hypertension (Section 1.2.2).

Table 1.1 **Biological actions of P450-dependent arachidonic acid metabolites.**

Metabolite	Biological Action	Reference
PEPTIDE HORMONE RELEASE		
5,6-, 8,9-, 11,12- EET	↑ somatostatin release from rat hypothalamic median eminence *in vitro*	Capdevila *et al.*, 1983; Junier *et al.*, 1990
5,6-, 8,9-, 11,12-, 14,15-EET	↑ luteinizing hormone release from isolated rat anterior pituitary cells	Snyder *et al.*, 1983
5,6-EET	↑ luteinizing hormone releasing hormone from rat hypothalmic median eminence *in vitro*	Capdevila *et al.*, 1983
5,6-EET	↑ insulin release from isolated rat pancreatic islets	Falck *et al.*, 1983
8,9-, 11,12-, 14,15-EET; 5-, 12-HETE	↑ glucagon release from isolated rat pancreatic islets	Falck *et al.*, 1983
11,12-, 14,15-EET	↑ cortisol formation in isolated bovine adrenal fasciculata cells	Nishimura *et al.*, 1989
ION TRANSPORT/MOVEMENT		
5,6-EET	↓ Na^+ absorption and ↑ K^+ secretion in isolated rabbit cortical collecting tubules	Jacobson *et al.*, 1984
19[S]-OH-AA	↑ rat renal cortical Na^+/K^+ ATPase activity (ATPase partially purified)	Escalante *et al.*, 1988
12[R]-HETE	↓ rat renal cortical Na^+/K^+ ATPase activity (ATPase partially purified)	Escalante *et al.*, 1988; Masferrer *et al.* 1990
20-OH-AA, 20-COOH-AA	↓ rabbit renal medullary Na^+/K^+ ATPase activity (ATPase partially purified)	Carroll *et al.*, 1991
20-OH-AA, 20-COOH-AA	↓ ^{86}Rb uptake in isolated rabbit medullary thick ascending loop of Henle cells	Escalante *et al.*, 1991
20-OH-AA	↑ in urinary Na^+ excretion in anesthetized rats	Takahashi *et al.*, 1990
12[R]-HETE	↑ urine volume, Na^+ excretion and K^+ excretion in isolated rat kidney	Quilley and McGiff, 1990

Table 1.1 - Continued

Metabolite	Biological Action	Reference
ION TRANSPORT/MOVEMENT - continued		
5,6-, 8,9-, 14,15-EET	↑ cytosolic Ca^{2+} in isolated rabbit proximal tubule epithelial cells	Madhun *et al.*, 1991
12[R]-HETE	↓ bovine corneal epithelium Na^+/K^+ ATPase activity (ATPase partially purified)	Schwartzman *et al.*, 1987; Masferrer *et al.*, 1990
12[R]-HETE	↓ rat heart ventricular Na^+/K^+ ATPase activity (ATPase partially purified)	Masferrer *et al.*, 1990
20-OH-AA	↑ ^{86}Rb uptake in thoracic rat aortic rings *in vitro*	Escalante *et al.*, 1990
5,6-EET	↑ cytosolic Ca^{2+} in isolated rat anterior pituitary cells	Snyder *et al.*, 1986
5,6-, 11,12-EET	↑ intracellular Ca^{2+} in isolated guinea pig ventricular myocytes	Moffat *et al.*, 1993
5,6-, 8,9-, 11,12-, 14,15-EET	activation of K^+ channel in rabbit portal vein, rat caudal artery, guinea pig aorta and porcine coronary artery *in vitro*	Hu and Kim, 1993
VASOACTIVITY		
19[R]-, 19[S]-, 20-OH-AA; 5,6-, 8,9-, 11,12-EET	↓ renal perfusion pressure in phenylephrine-treated isolated rabbit kidney	Carroll *et al.*, 1992, 1993
14,15-EET	↑ renal perfusion pressure in phenylephrine-treated isolated rabbit kidney	Carroll *et al.*, 1992
5,6-EET	dilate phenylephrine preconstricted rat caudal tail artery *in vitro*	Carroll *et al.*, 1987, 1990
5,6-, 14,15-EET	dilate fetal lamb ductus arteriosus *in vitro*	Coceani *et al.*, 1988
5,6-, 8,9-, 11,12-, 14,15-EET	dilate U46619 (thromboxane-mimetic) preconstricted canine coronary arteries *in vitro*	Rosolowsky *et al.*, 1990
5,6-, 8,9-EET	dilate submucosal intestinal arterioles in anesthetized rat	Proctor *et al.*, 1987

Table 1.1 - Continued

Metabolite	Biological Action	Reference
VASOACTIVITY - continued		
5,6-, 8,9-EET	dilate cerebral arterioles in anesthetized rabbit and cat	Ellis *et al.*, 1990
20-OH-AA	contract rabbit arteries (aorta, mesenteric, carotid and renal) *in vitro*	Escalante *et al.*, 1993
19[R]-, 19[S]-, 20-OH AA; 20-COOH-AA	dilate phenylephrine precontracted rabbit mesentric arterial rings *in vitro*	Carroll *et al.*, 1991
19[R]-, 19[S]-, 20-OH-AA	contract rat aortic rings *in vitro*	Escalante *et al.*, 1989
19[R]-, 19[S]-, 20-OH-AA	↑ magnitude of K^+-induced relaxation of rat thoracic aorta rings *in vitro*	Escalante *et al.*, 1990
12[R]-HETE	↓ K^+-induced relaxation and ↑ phenylephrine-induced contraction of rabbit thoracic aortic rings *in vitro*	Masferrer and Mullane, 1988
OTHER		
12[R]-HETE	↓ renin activity in venous effluent from isolated rat kidney	Quilley and McGiff, 1990
8,9-, 11,12-14,15-EET	↓ cyclooxygenase activity *in vitro*	Fitzpatrick *et al.*, 1986
11,12-, 14,15-EET	↓ platelet aggregation *in vitro*	Fitzpatrick *et al.*, 1986
20-OH-AA	↓ platelet aggregation *in vitro*	Hill *et al.*, 1992

1.2.2 Kidney

Renal P450 from rat, rabbit and human metabolizes AA to EETs (and DiHETEs) and (16-20)-OH-AA (and 20-COOH-AA). The ability to form P450-dependent AA metabolites is differentially distributed throughout the nephron. P450-derived AA metabolites are formed by medullary and cortical microsomes (Morrison and Pascoe 1981; Oliw *et al.*, 1981, Lapuerta *et al.*, 1988; Schwartzman *et al.*, 1986, 1990); cells from the medullary thick ascending limb of Henle's loop (Schwartzman *et al.*, 1985b; Carroll *et al.*, 1991); and tubule segments including the glomerulus, proximal convoluted tubule, thick ascending limb of Henle's loop and collecting tubule (Omata *et al.*, 1992a). The highest rate of AA metabolite formation is generally associated with the proximal tubule, the section of the nephron containing the highest concentration of P450.

P450 derived metabolites of AA are believed to be important in the physiological regulation of renal function, in particular Na^+ and water excretion, due to their effects on renal vasculature and renal Na^+/K^+ movement (Table 1.1). The urinary excretion of EETs is increased in animals loaded with dietary Na^+ for several days (Capdevila *et al.*, 1992b). Also, renal microsomes, but not hepatic microsomes, isolated from these animals display increased rates of EETs formation (Capdevila *et al.*, 1992b). This increase in EETs formation in the kidney may be an adaptive response to increased dietary Na^+ and be important in preventing salt retention. P450-dependent AA metabolites may mediate some of the renal effects of peptide hormones as several peptide

hormones alter their rate of formation. Vasopressin and calcitonin increase P450-dependent AA metabolism in isolated medullary thick ascending loop of Henle cells (Schwartzman *et al.*, 1985b). Vasopressin also increases the amount of P450-dependent AA metabolites released into the effluent of isolated perfused kidneys (Omata *et al.*, 1992b). In isolated proximal tubule segments parathyroid hormone and epidermal growth factor increase the rates of 19-OH-AA and 20-OH-AA formation whereas angiotensin II increases EETs formation (Schwartzman *et al.*, 1985b; Omata *et al.*, 1992a). 5,6-EET has been proposed to mediate angiotensin II induced natriuresis and inhibition of renin secretion (Omata *et al.*, 1992a).

Evidence has been accumulated in support of a role for renal P450-dependent AA metabolism in the development of hypertension. The amount of renal P450 AA metabolites formed is increased in experimental, pregnancy-induced and genetic forms of hypertension. Suprarenal aortic coarctation, an experimental model of hypertension, increases the formation of metabolites P1 and P2, identified as 19/20-OH-AA and 20-COOH-AA, respectively, in medullary thick ascending limb of Henle's loop cells (Carroll *et al.*, 1988, 1991). In humans, the urinary excretion of EETs is higher in patients with pregnancy-induced hypertension than in normotensive pregnant women (Catella *et al.*, 1990). In spontaneously hypertensive rats, the formation of 19-OH-AA, 20-OH-AA and 20-COOH-AA is increased, compared to normotensive rats, during the development of hypertension (Omata *et al.*, 1992b). Selective depletion of renal P450 with stannous chloride returned

blood pressure to normal during the developmental phase of hypertension in spontaneously hypertensive rats further implicating a role for P450 metabolites (Sacerdoti *et al.*, 1988).

Some of the P450 isozymes contributing to renal AA metabolism have been identified. P450 2C2 purified from rabbit renal cortex forms EETs and much less 19-OH-AA (Laethem *et al.*, 1992). Antibodies to 2C2 inhibited the formation of EETs in cortical microsomes from PB-induced rabbits by 63% (Laethem *et al.*, 1992). In renal microsomes from untreated rat antibodies to 2C11 virtually abolished the formation of EETs (Capdevila *et al.*, 1992b). Isozymes of the 4A subfamily, including several isozymes purified from the kidney, hydroxylate fatty acids including AA in the ω and (ω-1) positions (Bains *et al.*, 1985; Imaoka *et al.*, 1989b; Capdevila *et al.*, 1990b; Sawamura *et al.*, 1993). Formation of 19/20-OH-AA is induced in kidney by treatment with clofibrate, which selectively induces renal 4A, (Sharma *et al.*, 1989b) and antibodies to lauric acid ω-hydroxylase (P450 4A) inhibit the formation of 19/20-OH-AA by 40% in human renal microsomes (Schwartzman *et al.*, 1990). Hepatic P450 2E1 purified from rat (Tanaka *et al.*, 1990) and rabbit (Laethem *et al.*, 1993) metabolize AA to (16-20)-OH-AA and antibodies to P450 2E1 inhibit the formation of 18-OH-AA and 19-OH-AA by 65% and 25%, respectively, in renal cortical microsomes from acetone-pretreated rabbits (Laethem *et al.*, 1993).

It is also possible that P450 1A1 contributes to renal AA metabolism. Purified hepatic rat 1A1 (Tanaka *et al.*, 1990; Capdevila *et al.*, 1990a) and

rabbit 1A1 (Oliw *et al.*, 1982) metabolize AA. Also, the rate of formation of P450 derived AA metabolites is greater in microsomes (cortical and medullary; Schwartzman *et al.*, 1986) and medullary ascending limb of Henle's loop cells (Schwartzman *et al.*, 1985b) from rabbits treated with 3MC and β-NF, inducers of renal 1A1, compared to untreated animals.

1.2.3 Lung

Pulmonary P450 from rabbit metabolizes AA to 19/20-OH-AA with a small amount of DiHETEs also being formed (Oliw and Moldeus, 1982). This is the only species in which P450-dependent AA metabolism has previously been examined in the lung.

Although the effects of P450 derived AA metabolites on the lung have not been directly examined, experiments utilizing inhibitors of P450 have suggested that these products may play a role in the vasodilation effects of AA on the pulmonary artery (Pinto *et al.*, 1986) and in the isolated rat lung (Feddersen *et al.*, 1990).

1.3. MECHANISM-BASED INACTIVATION OF CYTOCHROME P450

1.3.1 Overview

A mechanism-based inactivator is a substrate for the target enzyme which during the process of catalytic conversion is changed into an intermediate(s) or product(s) that inactivates the enzyme (Rando, 1984). Mechanism-based inactivators of P450 can be classified into two broad

categories: 1) chemicals that cause reversible inactivation and 2) chemicals that irreversibly inactivate the enzyme, also known as suicide substrates (Testa, 1990).

Reversible mechanism-based inhibitors of P450 are metabolized by P450 to metabolites that bind the heme iron in P450 (Testa, 1990). This type of inactivation is limited to a few chemical structures including methylenedioxybenzene containing compounds such as dihydrosafrole, isosafrole and piperonyl butoxide (Franklin, 1977), certain hydrazines (Hines and Prough, 1980; Moloney *et al.*, 1984), and alkylamine derivatives such as amphetamine (Lindeke *et al.*, 1982), desipramine (Murray and Field, 1992), diethylaminoethyl-2,2-diphenylvalerate HCl (SKF-525A; Schenkman *et al.*, 1972), erythromycin (Danan *et al.*, 1981), methamphetamine (Lindeke *et al.*, 1979), nortripyline (Murray and Field, 1992), orphenadrine (Bast and Noordhoek, 1982) and triacetyloleandomycin (Pessayre *et al.*, 1981). The inactivation caused by these agents is reversible because the complexes can be dissociated *in vitro* reactivating the enzyme. Complexes derived from alkylamine derivatives are stable in the ferrous form but are readily dissociated by oxidation of the heme to the ferric form (Franklin, 1977). Complexes derived from dioxole compounds are stable in the ferrous form but can be displaced by lipophilic substrates following oxidation to the ferric form (Franklin, 1977). Although catalytic function can be restored *in vitro*, it is likely that *in vivo* the complexed P450 would be subject to normal catabolic processes involving heme and apoprotein turnover (Murray and Reidy, 1990).

Many compounds cause irreversible mechanism-based inactivation of P450 including terminal olefins such as 2-isopropyl-4-pentamide (allylisopropylacetamide; AIA) and secobarbital (Ortiz de Montellano and Correia, 1983); acetylenes such as synthetic steroids and fatty acid analogues (Ortiz de Montellano and Correia, 1983; Ortiz de Montellano and Reich, 1984); cycloalkylamines such as benzylcyclopropylamine (Bondon *et al.*, 1989); chloramphenicol and its analogues (Miller and Halpert, 1986; Stevens and Halpert, 1988); 1-aminobenzotriazole (ABT) and its derivatives (Ortiz de Montellano and Mathews, 1981; Mathews and Bend, 1986); phencyclidine (Hoag *et al.*, 1984); and 4-alkyl analogues of 3,5-diethoxy-2,4,6-trimethyl-1,4-dihydropyridines (Augusto *et al.*, 1982; Riddick *et al.*, 1989, 1990). Suicidal mechanism-based inhibitors of P450 inactivate the enzyme by three mechanisms. 1) A reactive metabolite of the inhibitor covalently binds to the P450 prosthetic heme moiety. For example, a number of acetylenic compounds (Ortiz de Montellano and Correia, 1983) and 4-alkyl analogues of 3,5-dicarbethoxy-2,4,6-trimethyl-1,4-dihydropyridines (Ortiz de Montellano *et al.*, 1981a; Augusto et al., 1982) N-alkylate the heme. 2) A reactive metabolite of the inhibitor covalently binds to the P450 apoprotein. For example, chloramphenicol undergoes oxidative dechlorination forming a reactive oxamyl metabolite that covalently modifies one, or more, lysine residues in the apoprotein impairing electron transport from NADPH-P450 reductase to P450 (Halpert, 1981; Halpert *et al.*, 1982, 1985). 10-Undecynoic acid also inactivates P450 via protein alkylation (CaJacob *et al.*,

1988). 3) The third pathway involves covalent binding of the heme, or heme fragments, to the apoprotein. 3,5-Diethoxycarbonyl-2,6-dimethyl-4-ethyl-1,4-dihydropyridine inactivates rat P450 3A by this pathway (Correia *et al.*, 1987). A particular inhibitor may inactivate P450 by one, or more of these pathways; chloramphenicol inactivates P450 solely by protein alkylation (Halpert, 1981; Halpert *et al.*, 1982; 1985) whereas AIA inactivates P450 by all three of these pathways (Davies *et al.*, 1986; Bornheim *et al.*, 1987).

With most mechanism-based inhibitors inactivation of the enzyme is not observed with every catalytic cycle; other metabolites which do not inactivate the enzyme are also formed. The partition ratio is an estimate of the number of molecules of inhibitor metabolized per molecule of P450 destroyed/inactivated and is a measure of the efficiency of the inactivator (Ortiz de Montellano *et al.*, 1984). Partition ratios of mechanism-based inhibitors of P450 vary widely; values from as low as 3 to as high as 500 have been reported (CaJacob *et al.*, 1988; Guengerich *et al.*, 1984).

Mechanism-based inhibitors of P450 can be employed as probes to obtain information regarding the role of P450 in the metabolism (bioactivation or detoxication) of endogenous and exogenous compounds. They can inactivate enzymes in systems with intact cellular structure (ie. cells, organs) and therefore are useful not only *in vitro*, like antibodies, but also *in vivo*. Recently, attention has focused on the identification of mechanism-based inhibitors of P450 that are isozyme-selective or -specific. Such inhibitors can be used to delineate the role of specific P450 isozymes in the metabolism of

compounds. In addition, they are potentially useful *in vivo* for therapeutic purposes. It should be noted that an inhibitor that inactivates P450 *in vitro* may not be effective *in vivo* due to pharmacokinetic limitations (CaJacob and Ortiz de Montellano, 1986). Several irreversible mechanism-based inhibitors of P450 reported to be highly selective, or specific, for a particular P450 isozyme(s) are listed in Table 1.2.

The potential exists, using mechanism-based inhibitors, to modulate P450 *in vivo* in a tissue-specific manner. Attempts have been made in our laboratory to design inhibitors which specifically inactivate pulmonary P450. Isozyme-specific, lung-specific mechanism-based inhibitors of P450 would be useful for determining the role of particular pulmonary P450 isozymes in the *in vivo* metabolism of endogenous and exogenous compounds. Also, such inhibitors have potential as therapeutic agents both as prophylactic treatment against lung specific toxins, and to prolong the duration of action of other therapeutic agents metabolized by pulmonary P450.

Mechanism-based inhibitors of P450 can also be employed to gain information regarding P450 structure, function and regulation. Compounds that alkylate the heme and/or protein offer information regarding active site topology (Ortiz de Montellano *et al.*, 1992). Isozyme selective/specific inhibitors can be used in isolated cells, organs, or *in vivo* to explore the endogenous role of particular P450 isozymes; what is the consequence of blocking the metabolism of endogenous compounds (Ortiz de Montellano *et al.*, 1992)? Regulation of heme biosynthesis (Marks *et al.*, 1988) and P450

Table 1.2 **Isozyme-selective/specific mechanism-based inhibitors of cytochrome P450.**

Compound(s)	Isozyme(s) Inactivated	Isozyme(s) Not Inactivated	Species	Reference
1-ethynylpyrene and 3-ethynylphenanthrene	1A	2B	rat	Hopkins *et al.*, 1992
9-ethynylphenanthrene and 1-ethylanthracene	2B	1A	rat	Hopkins *et al.*, 1992
secobarbital	2B	2C6, 2C11, 3A	rat	Lunetta *et al.*, 1989
N-(2-nitrophenethyl)-chlorofluoroacetamide	2B1/2	1A1, 2A1, 2C6, 2C11, 3A1/2	rat	Halpert *et al.*, 1990
phencyclidine	2B4	1A1, 1A2, 2E1, 2C3	rabbit	Osawa and Coon, 1989
21-chloro-21-fluoropregnenolone	2C5	2C3, 3A6	rabbit	Halpert *et al.*, 1989a
preg-4,20-diene-3-one	2C6>2B1	2A1/2, 2C11, 3A	rat	Halpert *et al.*, 1989a
diallyl sulfone	2E1	2B1	rat	Brady *et al.*, 1991a
21,21-dichloropregnenolone	3A>2C6	2C11, 2A1/2	rat	Halpert *et al.*, 1989b
gestodene	3A4/5	1A2, 2D6, 2E1, 2C	human	Guengerich, 1990b
12-hydroxy-16-heptadecynoic acid	4A4	2B4, 4B1	rabbit	Muerhoff *et al.*, 1989

proteolysis (Correia *et al.*, 1992a; Tierney *et al.*, 1992) have also been investigated using mechanism-based inhibitors of P450.

1.3.2 1-Aminobenzotriazole

ABT is an irreversible mechanism-based inhibitor of P450. *In vitro* it inactivates P450 in hepatic (Ortiz de Montellano and Mathews, 1981; Ortiz de Montellano *et al.*, 1981b; Woodcroft and Bend, 1990), pulmonary (Mathews *et al.*, 1985; Mathews and Bend, 1986; Woodcroft *et al.*, 1990) and renal (Mugford *et al.*, 1992) microsomes as well as in perfused lung (Mathews *et al.*, 1985). ABT is also an effective suicide substrate of hepatic, renal and pulmonary P450 *in vivo* (Ortiz de Montellano *et al.*, 1981; Ortiz de Montellano and Costa, 1986; Capello *et al.*, 1990; Mugford *et al.*, 1992).

ABT inactivates a broad spectrum of P450 isozymes. In liver, lung and kidney most of the spectrally detected microsomal P450 (>75%) is destroyed by high concentrations (*in vitro*), or doses (*in vivo*), of ABT (Ortiz de Montellano and Mathews, 1981; Ortiz de Montellano *et al.*, 1981b; Mathews *et al.*, 1985; Mugford *et al.*, 1992). To date, all P450-dependent monooxygenase activities examined, including activities highly selective for 1A, 2B, 2E, 4A and 4B isozymes, are inactivated by ABT and the identity of isozymes not affected, if any, remains unknown (Mathews *et al.*, 1985; Ortiz de Montellano and Costa, 1986; Reich and Ortiz de Montellano, 1986; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990; Tierney *et al.*, 1992). Although many isozymes are inactivated by ABT, in guinea pig pulmonary

microsomes some isozyme selectivity is apparent at low inhibitor concentrations; 10 μM inhibited P450 4Bx about 25-30% more than 1A1 and 2Bx (Woodcroft *et al.*, 1990). The partition ratio for ABT has been estimated to be around 12 (Ortiz de Montellano *et al.*, 1984).

Of significance, ABT inactivates P450 without affecting other enzymes involved in drug metabolism; NADPH-P450 reductase, flavin dependent monooxygenase, glucuronyltransferase and glutathione-S-transferase activities are unaltered by ABT (Mathews *et al.*, 1985; Mugford *et al.*, 1992). Also, cytochrome b_5 and glutathione levels are not affected by ABT (Ortiz de Montellano and Mathews, 1981; Mugford *et al.*, 1992). Due to the ability of ABT to inactivate only P450 and its low apparent toxicity (Mico *et al.*, 1988), it is a useful probe for screening for the involvement of P450 in the metabolism of compounds both *in vitro* and *in vivo*. For example, it was recently used to show that the *in vivo* metabolism of flurazepam is P450-dependent and that the parent compound (and not just its metabolites) has anticonvulsant activity (Capello *et al.*, 1990).

ABT inactivates P450 via arylation of the prosthetic heme moiety by the reactive metabolite benzyne. Following oxidation of its amino group, ABT undergoes rearrangement releasing benzyne and two molecules of N_2. The benzyne adds to two vicinal nitrogens of the heme moiety as demonstrated by the isolation of N,N'-bridged phenylene-protoporphyrin IX adducts from the livers of rats treated with ABT (Ortiz de Montellano and Mathews, 1981; Ortiz de Montellano *et al.*, 1984). ABT may also inactivate a small amount of total

P450-dependent monooxygenase activity by a related but different mechanism. In rabbit lung perfused with 1-amino-[^{14}C]2,3-benzotriazole (^{14}C-ABT; Mathews *et al.*, 1985) and guinea pig hepatic microsomes incubated with ^{14}C-ABT in the presence of NADPH (K.J. Woodcroft and J.R. Bend, unpublished data) radioactivity was tightly bound to microsomal protein suggesting that ABT may also inactivate P450 via covalent modification of protein by benzyne.

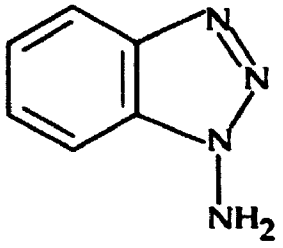
1.3.3 N-Aralkylated Derivatives of 1-Aminobenzotriazole

N-Benzyl-1-aminobenzotriazole (BBT) and N-α-methylbenzyl-1-aminobenzotriazole (αMB) are novel derivatives of ABT designed in our laboratory as potential isozyme-selective (P450 2B), lung-selective mechanism-based inhibitors of P450 *in vivo* (Mathews and Bend, 1986; Figure 1.2). These compounds contain the ABT nucleus, for mechanism-based inactivation, and N-aralkylated substituents to structurally mimic benzphetamine, a substrate that is specifically oxidized by P450 2B4 in lung (Serabjit-Singh *et al.*, 1979, 1983). These derivatives should also be concentrated in the lung *in vivo* via the pulmonary facilitated uptake system for basic (pKa>8.5) lipophilic amines (Orton *et al.*, 1973; reviewed in Bend *et al.*, 1985) whereas ABT is not (Mathews *et al.*, 1985).

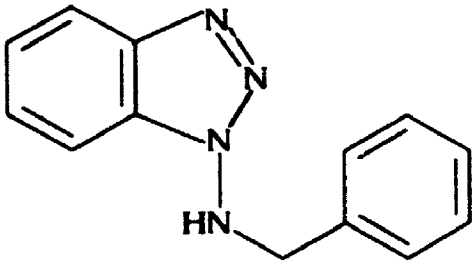
In vitro, both BBT and αMB were found to be potent, isozyme-selective mechanism-based inhibitors of P450 in pulmonary microsomes from rabbit (Mathews and Bend, 1986) and guinea pig (Woodcroft *et al.*, 1990), with αMB displaying greater selectivity and potency than BBT. In each species P450 2B4

Figure 1.3

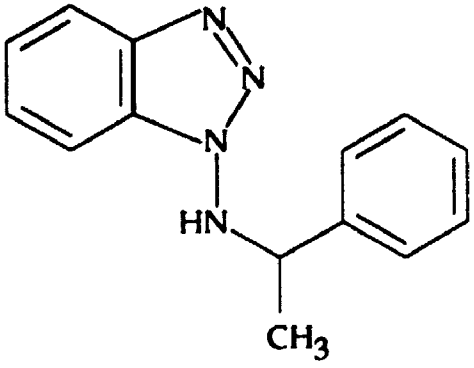
Structures of ABT, BBT and *o*MB.



1-aminobenzotriazole
(ABT)



N-benzyl-1-aminobenzotriazole
(BBT)



N-α-methylbenzyl-1-aminobenzotriazole
(αMB)

was inactivated more than 1A1 and 4B1; 1 μM αMB inhibited 2Bx by >90% in microsomes from β-NF-induced guinea pigs with <15% loss in 1A1 and 4Bx catalyzed activities. BBT and αMB also inactivated 2B and 1A isozymes in rabbit (Mathews and Bend, 1993) and guinea pig (Woodcroft and Bend, 1990) hepatic microsomes *in vitro*. The partition ratios for BBT and αMB have been estimated to be 14 and 11, respectively (Mathews and Bend, 1986).

The ability of these compounds to inactivate pulmonary and hepatic P450 *in vivo* was examined in β-NF-induced or PB-induced rabbits one hour following intravenous (i.v.) administration (Mathews and Bend, 1993). Both αMB and BBT were found to be potent inhibitors of pulmonary 2B4 retaining their selectivity for the inactivation of 2B4 vs 1A1; 1 μmol/kg αMB caused 80% vs 5% inactivation, respectively, in β-NF-induced rabbits. The inactivation of pulmonary P450 4B1, which comprises 25-35% of the pulmonary P450 in β-NF-induced rabbits (Domin *et al.*, 1984), was not examined. In PB-induced animals, where benzphetamine N-demethylation (BND) is primarily catalyzed by P450 2B4 in both organs, BBT (100 μmol/kg) selectively inactivated pulmonary vs hepatic P450 2B4 (90% vs 50%, respectively). With αMB, P450 2B4 was inactivated by >85% in both lung and liver at 100 μmol/kg, the only dose tested in PB-induced rabbits; a dose 100-fold greater than that required to inactivate pulmonary 2B4 by 80% in PB-induced rabbits. These data suggest that 3BT, and possibly αMB (at doses lower than 100 μmol/kg), are isozyme-selective (2B4), lung-selective inactivators of the P450 system *in vivo*.

It is now apparent that BBT inactivates P450 by a number of mechanisms. a) BBT causes arylation of the heme by the reactive metabolite benzyne. An abnormal porphyrin pigment with physical properties identical to the phenylene-porphyrin adduct formed by ABT has been isolated from the livers of rats treated with BBT (Mathews and Bend, 1986). It is likely that the inactivation by this route involves N-debenzylation of BBT to form ABT, and subsequent oxidation of ABT to form the reactive benzyne. b) BBT can covalently modify P450 protein. In guinea pig hepatic and pulmonary microsomes incubated with either of two forms of radiolabelled BBT, N-benzyl-1-amino-[^{14}C]2,3-benzotriazole or N-[^{14}C]7-benzyl-1-aminobenzotriazole, in the presence of NADPH, radioactivity was covalently bound to microsomal protein that migrated in the molecular weight region corresponding to P450 on sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (K.J. Woodcroft and J.R. Bend, unpublished data). These data indicate that BBT is metabolized to at least two reactive species capable of covalent modification of the protein and that P450 apoprotein alkylation may be a significant route of inactivation of P450 by BBT. c) Recent studies conducted in our laboratory have shown that both BBT and *α*MB form a reversible metabolic intermediate complex (MI-complex) in hepatic microsomes *in vitro* and that this MI-complex is more abundant in microsomes from PB-treated guinea pigs, an inducer of hepatic 2Bx, than microsomes from untreated guinea pigs (C.J. Sinal and J.R. Bend, unpublished data). It is likely that this P450 catalysis-dependent MI-complex contributes to the inhibition of guinea pig 2Bx.

CHAPTER 2

AIMS AND APPROACHES

2.1 AIMS

The overall long-term goal of this research is to learn more about the contribution of individual pulmonary P450 isozymes to the *in vivo* metabolism of exogenous and endogenous compounds, and hence the toxicological, physiological and pathobiological roles of these isozymes.

The research presented in this thesis focuses on two specific areas related to this long-term goal. The first is detailed characterization of the *in vivo* effects of ABT and its N-aralkylated derivatives, BBT and *α*MB, on the pulmonary and hepatic P450 systems of guinea pig. Both BBT and *α*MB are isozyme-selective inactivators of guinea pig pulmonary P450 *in vitro* (Woodcroft *et al.*, 1990). Also, both derivatives are potent inactivators of pulmonary P450 2B4 in rabbit *in vivo* although the lowest dose tested also inactivated a lot of the hepatic 2B4 activity (>50% and >85% inhibition with BBT and *α*MB, respectively; Mathews and Bend, 1993). At lower doses, it is possible that these compounds may specifically inactivate pulmonary, vs hepatic P450. If so, these compounds may be useful probes for future *in vivo* and *in vitro* studies.

The second is characterization of P450-dependent metabolism of AA in lung microsomes of guinea pig at the isozyme level. A few experiments were also conducted using liver and kidney microsomes because some of the

isozymes found in the lung are also expressed in one, or both, of these organs.

2.2 APPROACHES

The guinea pig was used for these studies because it contains relatively high concentrations of pulmonary P450, second only to the rabbit (Philpot *et al.*, 1977), and is a much smaller species for detailed *in vivo* studies. Also, the guinea pig pulmonary P450 system contains immuno-orthologues of the three isozymes that comprise the majority of P450 in rabbit lung, P450 1A1, 2Bx and 4Bx (Domin *et al.* 1984; Philpot *et al.* 1985; Vanderslice *et al.* 1987), which are inactivated by BBT and *o*MB *in vitro* in an isozyme-selective manner (Woodcroft *et al.*, 1990) analogous to the rabbit (Mathews and Bend, 1986).

The activity of individual P450 isozymes was evaluated using isozyme selective/specific monooxygenase activities. In lung, one assay was used for each of the three P450 isozymes known to be important constituents of guinea pig lung: ERF for P450 1A1; 7-pentoxyresorufin O-depentylation (PRF) for P450 2Bx; and 4-aminobiphenyl N-hydroxylation (ABP) for P450 4Bx (Burke *et al.*, 1985; Lubet *et al.*, 1985; Vanderslice *et al.*, 1987; Domin and Philpot, 1986; Woodcroft *et al.*, 1990).

The hepatic P450 system is much more complex containing numerous P450 isozymes. Isozyme-selective monooxygenase activities were used for several forms of hepatic P450 including ERF for P450 1A1 (Guengerich *et al.*, 1982b; Burke *et al.*, 1985); ABP for P450 1A2 (Butler *et al.*, 1989); PRF for the P450 2Bx (Burke *et al.*, 1985; Lubet *et al.*, 1985) and erythromycin N-

demethylation (END) for the P450 3A subfamily (Bonfils *et al.*, 1983; Wrighton *et al.*, 1985). P450 4Bx, the isozyme responsible for ABP activity in lung, is not expressed in guinea pig liver (Vanderslice *et al.*, 1987; Gasser and Philpot, 1989).[2]

In some of the studies the guinea pigs were pretreated with β-NF or PB to induce specific P450 isozymes allowing better assessment of the ability of the mechanism-based inhibitors to inactivate these isozymes and/or the ability of these isozymes to metabolize AA. Inducers of the PAH-type, including β-NF, induce P450 1A1 in lung and kidney, and 1A1/1A2 in liver (Liem *et al.*, 1980; Domin *et al.*, 1984; Goldstein and Linko, 1984). PB-treatment increases the concentration of 2B isozymes in liver but not in lung (Domin *et al.* 1984; Yamada *et al.*, 1992). P450 2Bx is not expressed in guinea pig kidney (Yamada *et al.*, 1992; R.M. Philpot, personal communication).

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[2]We have confirmed that these assays are highly isozyme selective/specific for guinea pig P450. Antibodies to rabbit P450 2B4 inhibited PRF activity in hepatic microsomes from PB-treated guinea pigs and pulmonary microsomes by >90%. Antibodies to rabbit 4B1 inhibited ABP activity in pulmonary microsomes by >70%. α-Naphthoflavone (3×10^{-7}M *in vitro*), an inhibitor of 1A1 in lung and kidney and 1A1/1A2 in liver, inhibited pulmonary, renal and hepatic ERF activity, and hepatic ABP activity, by 75%, >95%, 80% and 70% respectively, in microsomes prepared from β-NF-treated animals.

CHAPTER 3

INACTIVATION OF CYTOCHROME P450 *IN VIVO* BY 1-AMINOBENZOTRIAZOLE ADMINISTERED INTRAPERITONEALLY

3.1 OBJECTIVE

The primary objective of this study was to investigate the potency, isozyme-selectivity, and tissue-selectivity (lung vs liver) of ABT administered intraperitoneally (i.p.) as a mechanism-based inhibitor of P450 in non-induced, PB-induced and β-NF-induced guinea pigs. The purpose of these studies was to determine how much of the pulmonary (vs hepatic) monooxygenase activity could be inactivated *in vivo* and the relative ability of ABT to inactivate a single isozyme, P450 1A1 in lung, liver and kidney of β-NF induced animals.

One experiment was also performed examining the potency, isozyme-selectivity and tissue-selectivity (lung vs liver) of BBT administered i.p. as a mechanism-based inhibitor of P450 in non-induced guinea pigs.

3.2 MATERIALS AND METHODS

3.2.1 Reagents

ABT and BBT were synthesized and purified as previously described (Mathews *et al.*, 1985; Mathews and Bend, 1986). 7-Ethoxyresorufin, 7-pentoxyresorufin and resorufin were obtained from Molecular Probes Inc. (Eugene, OR); β-NF, 4-aminobiphenyl and 2,4,6-tripyridyl-s-triazine were from the Aldrich Chemical Co. (Milwaukee, WI); and NADPH and erythromycin

estolate from the Sigma Chemical Co. (St. Louis, MO). Benzphetamine HCl was obtained from The Upjohn Company (Kalamazoo, MI).

3.2.2 Animal Treatment

Male Hartley guinea pigs (275-350 g), were obtained from Charles River Ltd. (St. Constant, Que.). They were housed in wire-bottom cages and fed Purina guinea pig chow and water *ad libitum*. They were allowed to acclimatize in our facilities for at least 4 days before use.

Guinea pigs were treated i.p. with 80 mg/kg sodium PB (2% in saline) or 80 mg/kg β-NF (2% in corn oil) daily for 4 days. Some animals received no inducing agent prior to ABT or BBT treatment. Animals were given a single i.p. injection of ABT (7.5-750 μmol/kg [1-100 mg/kg]) or BBT (7.5-75 μmol/kg) 24 hr following the last injection of inducer. ABT was dissolved in saline at concentrations so that each animal received an equivalent volume of saline (4 ml/kg). BBT was dissolved in ethanol so that each animal received 1 ml/kg. Animals used for 100% control values received saline (4 ml/kg) in the ABT experiments and ethanol (1 ml/kg) in the BBT experiment.

Guinea pigs were sacrificed 24 hr after the injection of ABT, BBT or vehicle by CO_2 asphyxiation. Livers, lungs and kidneys were removed immediately and stored at -80°C until preparation of microsomes (usually within one week).

3.2.3 Preparation of Microsomes

Washed hepatic, pulmonary and renal microsomes were prepared from individual tissues by differential centrifugation. All steps were carried out on ice or at 4°C. The tissues were homogenized in homogenization buffer (1.15% KCl in 50 mM potassium phosphate buffer, pH 7.4; 3 ml per g tissue weight) using a motor driven Potter-Elvehjem tissue homogenizer (5, 8 and 12 passes for liver, kidney and lung, respectively). The homogenate was centrifuged at 12,500 g for 20 min (10,500 rpm in a Beckman JA20.1 rotor or 11,000 rpm in a Beckman JA20 rotor). The resulting supernatant was recentrifuged at 125,000 g for 50 min (33,000 rpm in a Beckman Ti50.2 rotor or 38,000 rpm in a Beckman Ti50 rotor). The microsomal pellet was resuspended in homogenization buffer using a hand-held homogenizer. The suspension was centrifuged at 125,000 g for 50 min (liver and kidney; rpm and rotors as before) or 400,000 g for 15 min (lung; 100,000 rpm in a Beckman TL100.3 rotor). The microsomal pellet was resuspended in homogenization buffer (5, 2 and 1 ml for liver, kidney and lung microsomes, respectively). Aliquots were removed and stored at -80°C until determination of protein content, P450 content and P450-dependent monooxygenase activities (usually less than 2 weeks).

3.2.4 Determination of Microsomal Protein Content

The protein content of the microsomal suspensions was determined by the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as the

standard protein. To 0.5 ml of diluted microsomes (1/125, 1/60 and 1/50 dilution with water for liver, kidney and lung microsomes, respectively) 2.5 ml of Reagent A (3% Na_2CO_3 in 0.1 N NaOH:2% $CuSO_4 \cdot 5H_2O$:4% NaKtartrate$\cdot 4H_2O$, 100:1:1, v/v/v) were added. Following a 10 min incubation at room temperature 0.25 ml of 1 N phenol Folin-Ciocalteau reagent was added and immediately mixed. The absorbance at 630 nm was measured after a 30 min incubation at room temperature. BSA standards, ranging from 50-350 μg/ml, were processed in an identical manner.

3.2.5 Determination of Spectral P450 Content

Total microsomal P450 content was quantitated by the dithionite difference spectrum of carbon monoxide saturated microsomes (Estabrook *et al.*, 1972). Microsomes were diluted to 1 (hepatic) or 1.5 (pulmonary and renal) mg/ml with 0.1 M potassium phosphate buffer, pH 7.4. About 1 ml of the diluted microsomal suspension was placed in a cuvette and bubbled with carbon monoxide for 3 min. A baseline scan from 400-500 nm was determined using a Beckman DU-65 spectrophotometer. A few crystals of sodium dithionite were added to the cuvette and the 400-500 nm spectrum was determined. The concentration of P450 was calculated from the difference spectrum using an extinction coefficient of 100 $mM^{-1}cm^{-1}$ between the difference spectrum peak (448-450 nm) and 490 nm (Estabrook *et al.*, 1972).

3.2.6 Determination of 7-Pentoxyresorufin and 7-Ethoxyresorufin O-Dealkylation Activities

PRF and ERF activities were determined by measuring the formation of the fluorescent product, resorufin, as described by Burke *et al.* (1985). The reaction mixtures contained microsomal protein (for PRF activity 0.1-0.15 or 0.2 mg/ml of hepatic or pulmonary microsomal protein, respectively, and for ERF activity 0.05, 0.4 or 0.1 mg/ml of hepatic, pulmonary or renal microsomal protein, respectively), 5 μM pentoxyresorufin or 2 μM ethoxyresorufin, and 0.1 M potassium phosphate buffer, pH 7.8. The pentoxyresorufin and ethoxyresorufin were prepared as 1 mM and 0.4 mM stock solutions, respectively, in dimethyl sulfoxide (DMSO). The final reaction volume was 1.5 or 2 ml. The reactions were carried out in fluorimeter cuvettes at 37°C. After an initial 5-10 min preincubation a baseline scan was recorded for a few min at excitation and emission wavelengths of 550 and 580 nm, respectively, using a fluorescence spectrometer (Perkin-Elmer model LS-5B). The reaction was initiated with 0.1 mM NADPH and the change in fluorescence was monitored. After a suitable period of reaction, generally 5-10 min, resorufin (15 pmol in 5 μl DMSO) was added to the reaction mixture in duplicate and the rate for formation of resorufin was calculated from this internal standard.

3.2.7 Determination of 4-Aminobiphenyl N-Hydroxylation Activity

ABP activity was determined by measuring the rate of formation of the N-hydroxylation product by a modification of the colorimetric method described

for the N-hydroxylation of 2-aminofluorene (Belanger *et al.*, 1981; Vanderslice *et al.*, 1987). The reaction mixtures contained microsomal protein (20-50 μg/ml), 0.5 mM 4-aminobiphenyl and 0.1 M potassium phosphate buffer (pH 7.4) in a final volume of 1 ml. The 4-aminobiphenyl was prepared as a 0.1 mM stock solution in DMSO. Incubation blanks used to determine background absorbance contained DMSO instead of 4-aminobiphenyl because it was determined in preliminary experiments that both protein and NADPH, but not 4-aminobiphenyl, produced a color with the color reagent. After a 6 min preincubation at 37°C the reaction was initiated with 2 mM NADPH. After a further 6 min incubation 0.4 ml of the reaction mixture was added to 2 ml of color reagent (5 M Na acetate buffer, pH 5.3, 0.24 mM 2,4,6-tripyridyl-s-triazine, 80 μM $FeCl_3$ at 55°C). The color was developed for 40 min at 55°C. The samples were cooled at room temperature for 5-10 min and the absorbance at 595 nm determined.

Hydroxylamine HCl (0.1-0.2 mM in water) was used as a standard. To 0.4 ml of the standard solutions 2 ml of the color reagent was added. The color was developed and the absorbance determined as above.

3.2.8 Determination of Benzphetamine and Erythromycin N-Demethylation Activities

BND and END activities were determined by measuring the rate of formation of formaldehyde by the colorimetric method of Nash *et al.*, 1953. The reaction mixtures contained 1.5 mg/ml microsomal protein, 2 mM d-

benzphetamine HCl or 0.2 mM erythromycin estolate, and 0.1 M potassium phosphate buffer, pH 7.4, in a final volume of 2 ml. After a 5 min preincubation at 37°C the reaction was initiated with 1 mM NADPH. After a further 10-15 min at 37°C the reaction was terminated with 1 ml of 8.9% $ZnSO_4$ and 1.5 ml of saturate $Ba(OH)_2$ and 0.5 ml of saturated Na borate were added. The contents were mixed and then centrifuged at 2400 rpm for 10 min. An aliquot of the supernatant (1.5 ml) was removed and added to 1.5 ml of the color reagent (4 M ammonium acetate, 0.1 M acetic acid, 40 mM acetylacetone). The color was developed at 60° for 10 min. The samples were then cooled to room temperature, centrifuged at 2400 rpm for 10 min, and the absorbance at 412 nm was determined.

Standard samples, containing known amounts of formaldehyde (26-156 nmol), microsomal protein, and NADPH, but no benzphetamine or erythromycin, were processed in an identical manner.

3.2.9 Statistical Analysis

One-way analysis of variance (ANOVA) with Newman-Keuls test was used to analyze for significant difference in enzyme activities or P450 content between vehicle-treated and inhibitor-treated groups (Zar, 1984). In all cases, analyses were performed on the raw data and not the "percentage of control data" presented in the figures.

3.3 RESULTS

Treatment with β-NF or PB resulted in the expected marked and selective increases in hepatic microsomal monooxygenase activities relative to those of non-induced guinea pigs (legends for Figs. 3.1 and 3.2). PB-treatment increased PRF activity 4.8-fold with much smaller increases in BND and ABP activities (2.3- and 1.7-fold, respectively) and no increase in END or ERF activities (1.0- and 0.9-fold, respectively). On the other hand, β-NF-treatment markedly increased ABP and ERF activities (5.0- and 3.8-fold, respectively) with little change in PRF, BND and END activities (1.2-, 1.0- and 0.7-fold, respectively). Although PB treatment did not markedly alter any of the pulmonary monooxygenase activities (legends for Figs. 3.3 and 3.4), β-NF-treatment increased ERF activity (2.7-fold) in lung with a slight decrease in ABP activity (0.7-fold) and no change in PRF activity (54.3 ± 4.6 and 50.0 ± 8.3 pmol/min/mg protein in β-NF-induced and non-induced lung, respectively).

ABT (75-750 μmol/kg, i.p.) inhibited the hepatic monooxygenase system in non-induced, PB-induced and β-NF-induced animals in a dose-dependent manner (Figs. 3.1 and 3.2). At the highest dose of ABT studied the loss in spectral P450 content was 60-65%, with little loss seen at 75 μmol/kg (<25%). At each dose of ABT, the loss in P450 content was intermediate to the relative losses in the various monooxygenase activities.

In non-induced and PB-induced guinea pigs the loss of PRF activity (>70% at 750 μmol/kg dose) was greater than that of ERF, ABP, BND and END activities at all three doses of ABT studied (Figs. 3.1 and 3.2). In β-NF-

Figure 3.1

Effects of ABT, administered i.p., on hepatic ABP, ERF and PRF activities in non-induced (A), PB-induced (B) and β-NF-induced (C) guinea pigs. Control (100%) values were: for ABP 118 ± 11 (A, non-induced), 200 ± 12 (B, PB) and 589 ± 23 (C, β-NF) nmol/min/mg protein; for ERF 264 ± 18 (A, non-induced), 242 ± 17 (B, PB) and 999 ± 32 (C, β-NF) pmol/min/mg protein; for PRF 23.4 ± 2.3 (A, non-induced), 112 ± 24 (B, PB), 29.0 ± 1.2 (C, β-NF) pmol/min/mg protein. Data shown are means ± SE, N = 3-5. *Significantly different ($P < 0.05$) than corresponding vehicle-treated group.

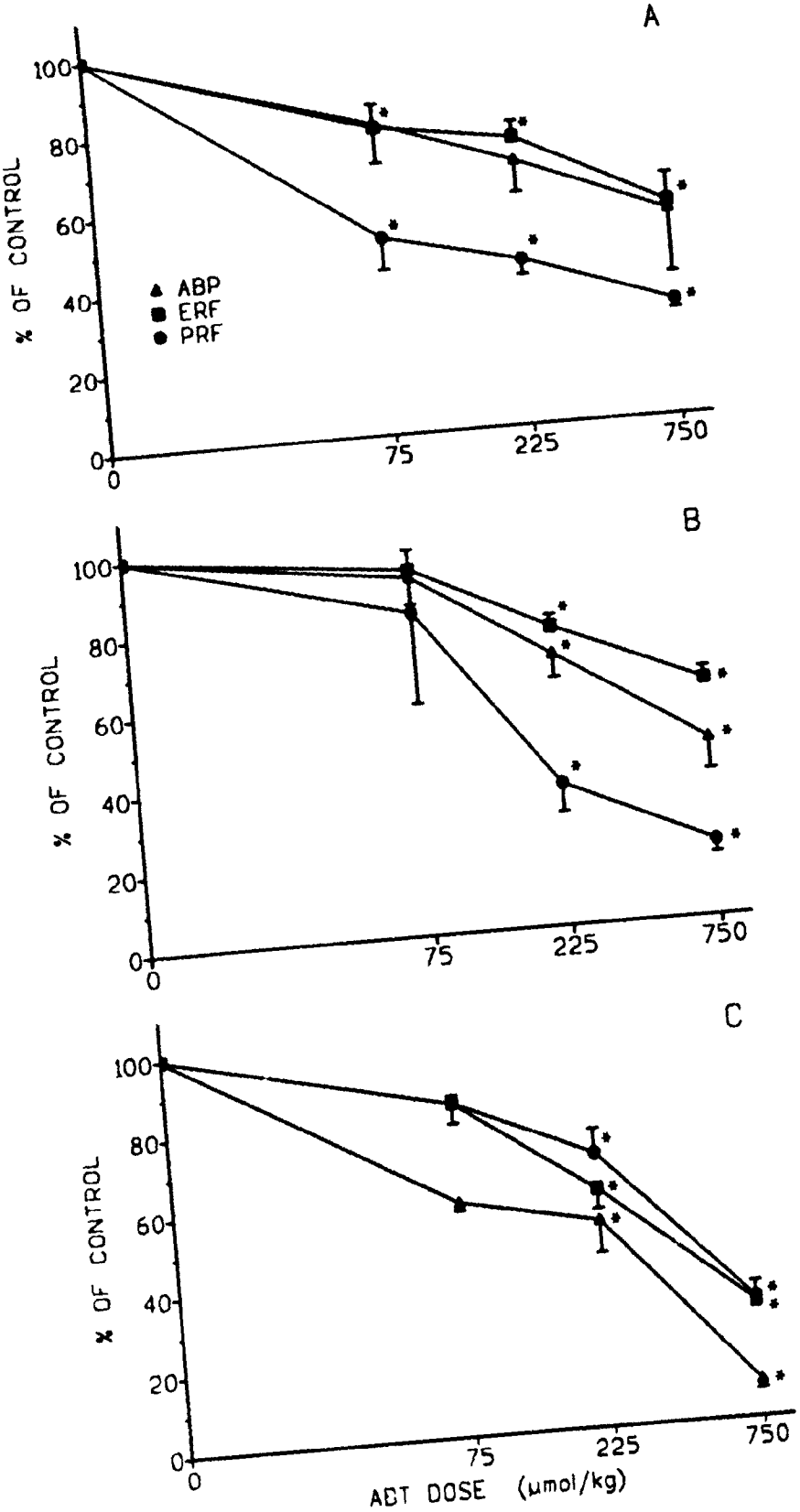

A
B
C
% OF CONTROL
100
80
60
40
20
0
ABP
ERF
PRF
75
225
750
ADT DOSE (μmol/kg)

Figure 3.2

Effects of ABT, administered i.p., on hepatic BND and END activities, and P450 content in non-induced (A), PB-induced (B) and β-NF-induced (C) guinea pigs. Control (100%) values were: for BND 5.93 ± 0.22 (A, non-induced), 13.8 ± 1.4 (B, PB) and 5.92 ± 0.20 (C, β-NF) nmol/min/mg protein; for END 2.52 ± 0.29 (A, non-induced), 2.51 ± 0.14 (B, PB) and 1.74 ± 0.15 (C, β-NF) nmol/min/mg protein; for P450 0.69 ± 0.03 (A, non-induced), 1.34 ± 0.12 (B, PB) and 1.19 ± 0.05 (C, β-NF) nmol/mg protein. Data shown are means ± SE, N = 3-5. *Significantly different ($P < 0.05$) than corresponding vehicle-treated group.

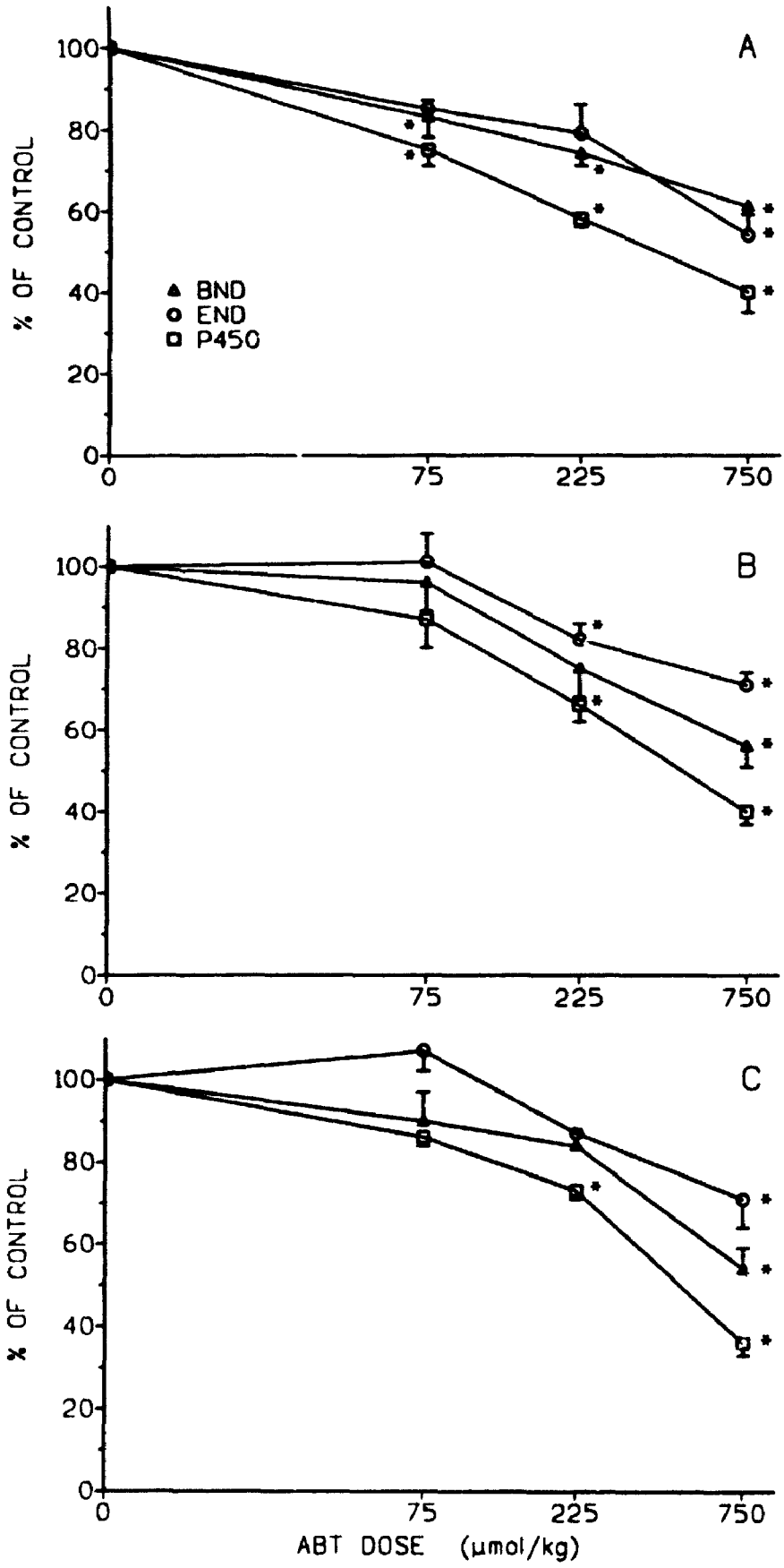

A
B
C
% OF CONTROL
100
80
60
40
20
0
0
75
225
750
BND
END
P450
ABT DOSE (µmol/kg)

Figure 3.3

Effects of ABT, administered i.p., on pulmonary monooxygenase activities and P450 content in PB-induced guinea pigs. Control (100%) values were: 267 ± 8 nmol/min/mg protein (ABP); 15.4 ± 2.4 pmol/min/mg protein (ERF); 50.2 ± 8.0 pmol/min/mg protein (PRF); and 0.09 ± 0.01 nmol/mg protein (P450). Data shown are means ± SE, N = 4. *Significantly different ($P < 0.05$) than corresponding vehicle-treated group.

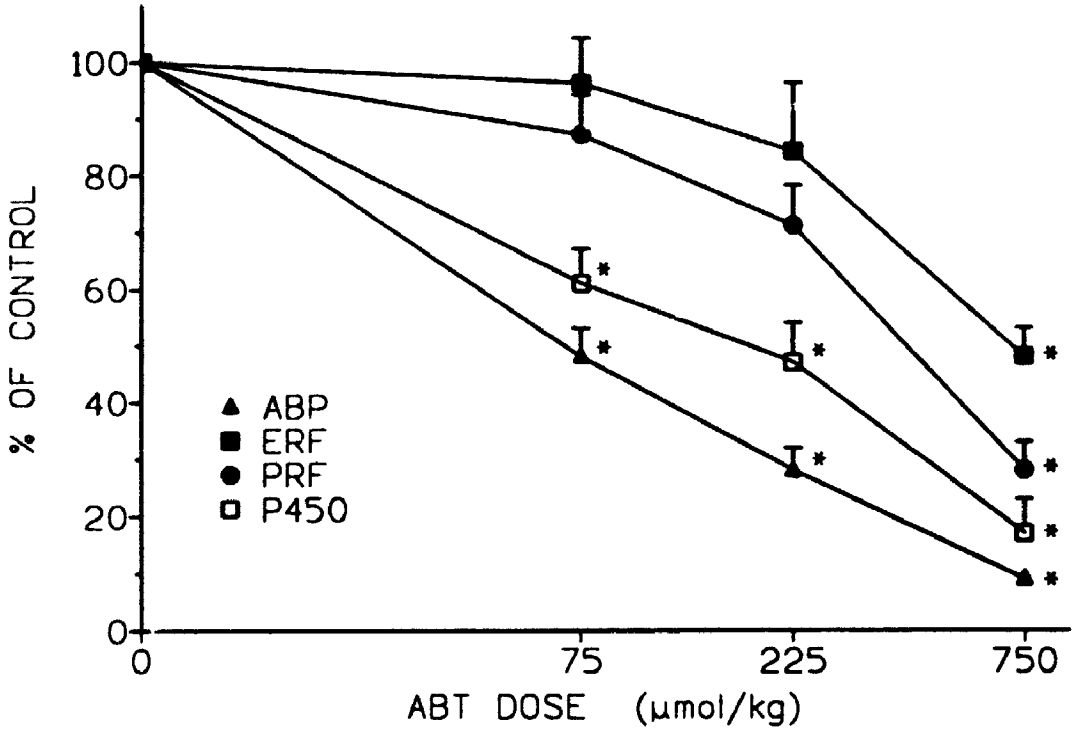
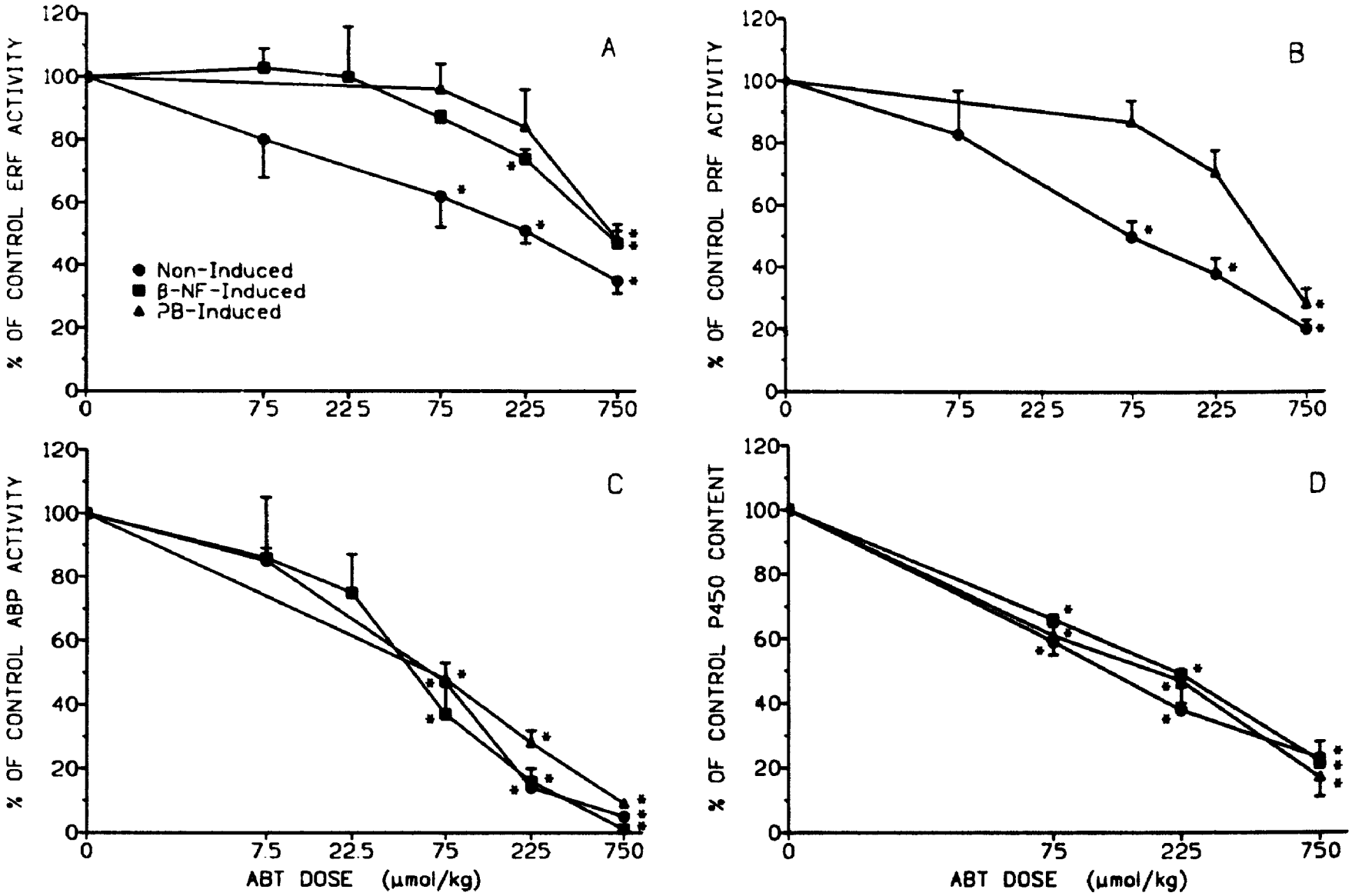

100
80
60
40
20
0
% OF CONTROL
ABP
ERF
PRF
P450
0
75
225
750
ABT DOSE (µmol/kg)

Figure 3.4

Effects of ABT, administered i.p., on pulmonary ERF (A), PRF (B) and ABP (C) activities, and P450 content (D) in non-induced, β-NF-induced and PB-induced guinea pigs. Control (100%) values were: for ERF (A) 16.4 ± 2.3 (non-induced), 44.4 ± 2.9 (β-NF) and 15.4 ± 2.4 (PB) pmol/min/mg protein; for PRF (B) 50.0 ± 8.3 (non-induced) and 50.2 ± 8.0 (PB) pmol/min/mg protein; for ABP (C) 268 ± 18 (non-induced), 198 ± 26 (β-NF) and 267 ± 8 (PB) pmol/min/mg protein; and for P450 (D) 0.111 ± 0.004 (non-induced), 0.135 ± 0.003 (β-NF) and 0.094 ± 0.006 (PB) nmol/mg protein. Data shown are means ± SE, N = 3-5. *Significantly different ($P < 0.05$) than corresponding vehicle-treated group.


A
B
C
D
% OF CONTROL ERF ACTIVITY
% OF CONTROL PRF ACTIVITY
% OF CONTROL ABP ACTIVITY
% OF CONTROL P450 CONTENT
Non-Induced
β-NF-Induced
PB-Induced
ABT DOSE (µmol/kg)
ABT DOSE (µmol/kg)

induced animals ABP activity was inhibited more than the other activities, with >90% inhibition at 750 μmol/kg. This change in the rank order of inhibition of the enzyme activities is primarily due to an increase in the loss of ABP activity in β-NF-induced animals compared to non-induced and PB-induced animals (91% vs 48% and 55%, respectively, at 75 μmol/kg). In non-induced and β-NF-induced animals the amount of ABP activity remaining at the highest dose of ABT (750 μmol/kg) was similar (62 ± 19 and 54 ± 11 nmol/min/mg protein, respectively).

Induction also altered the dose-response characteristics for the inactivation of other hepatic monooxygenase activities. For example, PRF was inhibited more in non-induced than PB-induced or β-NF-induced animals at 75 μmol/kg ABT (50% vs 18% and 16%, respectively) with near equal inhibition at 750 μmol/kg (71% vs 81% and 70%, respectively; Fig. 3.1). At 750 μmol/kg ABT, ERF activity was inactivated more in β-NF-induced than non-induced or PB-induced animals (71% vs 40% and 46%, respectively). P450 3A-dependent END activity was the monooxygenase activity most resistant to inactivation in induced guinea pigs and was inhibited to a lesser extent in PB- and β-NF-induced animals than non-induced animals (29% vs 46%, respectively, at 750 μmol/kg).

At each dose of ABT the loss of BND activity did not vary by more than 15% between non-induced, PB-induced and β-NF-induced animals (~40% at 750 μmol/kg; Fig. 3.2). In all cases BND activity was inhibited to a lesser degree than PRF activity (~25-35% less at 750 μmol/kg).

ABT also inhibited the pulmonary monooxygenase system in a dose-dependent manner (Figs. 3.3 and 3.4). Some selectivity in the inactivation of the activities was evident. In PB-induced animals ABT selectively inhibited ABP activity compared to ERF or PRF activities (72% vs 16% and 29%, respectively, at 225 μmol/kg; Fig. 3.3). Similar trends were seen in lungs from non-induced and β-NF-induced animals (Fig. 3.4).

Pulmonary ERF was inhibited less in PB-induced or β-NF-induced animals than in non-induced animals at both the 75 and 225 μmol/kg doses of ABT (Fig. 3.4). At the highest dose (750 μmol/kg) near equal inhibition of ERF activity (~55%) was observed in all three groups. PRF activity was also inhibited less in lung of PB-induced animals than in lung of non-induced guinea pigs at the 75 and 225 μmol/kg doses of ABT, with near equal inhibition at 750 μmol/kg (~75%). On the other hand, there were no marked differences between non-induced and PB- or β-NF-induced animals in the inhibition of pulmonary ABP activity (~55% at 75 μmol/kg and >90% at 750 μmol/kg) or loss of spectrally assayed P450 (~40% at 75 μmol/kg and ~80% at 750 μmol/kg). At each dose of ABT, the loss in P450 content was intermediate to the inhibition of the various monooxygenase activities.

To determine if ABT, administered i.p., inactivated P450 in a tissue selective manner we compared the inhibition of PRF activity in the lungs vs livers of PB-induced animals, and the inhibition of ERF activity in lungs, livers and kidneys of β-NF-induced animals. In PB-induced animals, PRF activity was inhibited to a greater extent in liver compared to lung with 225 μmol/kg ABT

(64% vs 29%; Figs. 3.1 and 3.3). However, at the 10 and 750 μmol/kg doses, there were no apparent differences in inhibition between the two organs (~15% at 75 μmol/kg and ~75% at 750 μmol/kg). At 225 and 750 μmol/kg ABT, ERF activity was inhibited more in liver than lung or kidney (71% vs 53% and 45%, respectively, at 750 μmol/kg; Figs. 3.1, 3.4 and 3.5) but there was little inhibition in any of these organs at the 75 μmol/kg dose (<20%). In the kidney, β-NF treatment increased ERF activity >100-fold compared to that of non-induced guinea pigs (216 ± 13 vs 2.0 ± 0.2 pmol/min/mg protein, respectively).

The effects of BBT, administered i.p., on the pulmonary and hepatic monooxygenase system were also dose dependent (Fig. 3.6). The lowest dose of BBT examined (7.5 μmol/kg) appeared to increase pulmonary ERF and PRF activities, as well as pulmonary P450 content (130-150%) but had little effect on ERF and PRF activities or P450 content in the liver. The intermediate dose (22.5 μmol/kg) had little effect on the pulmonary monooxygenase system (<15% loss in PRF or ERF activity) whereas the highest dose (22.5 μmol/kg), inhibited pulmonary PRF activity by ~50% with little effect on pulmonary ERF activity (<15% loss). This dose also inhibited the hepatic monooxygenase system (~60% loss of ERF activity).

3.4 DISCUSSION

There are two important differences between this study and earlier work examining the *in vivo* affects of ABT on the P450 monooxygenase system.

Figure 3.5

Effects of ABT, administered i.p., on renal ERF activity and P450 content in β-NF-induced guinea pigs. Control (100%) values were: 216 ± 13 pmol/min/mg protein (ERF) and 0.24 ± 0.02 nmol/mg protein (P450). Data shown are means ± SE, N=3-5. *Significantly different (P<0.05) than corresponding vehicle-treated group.

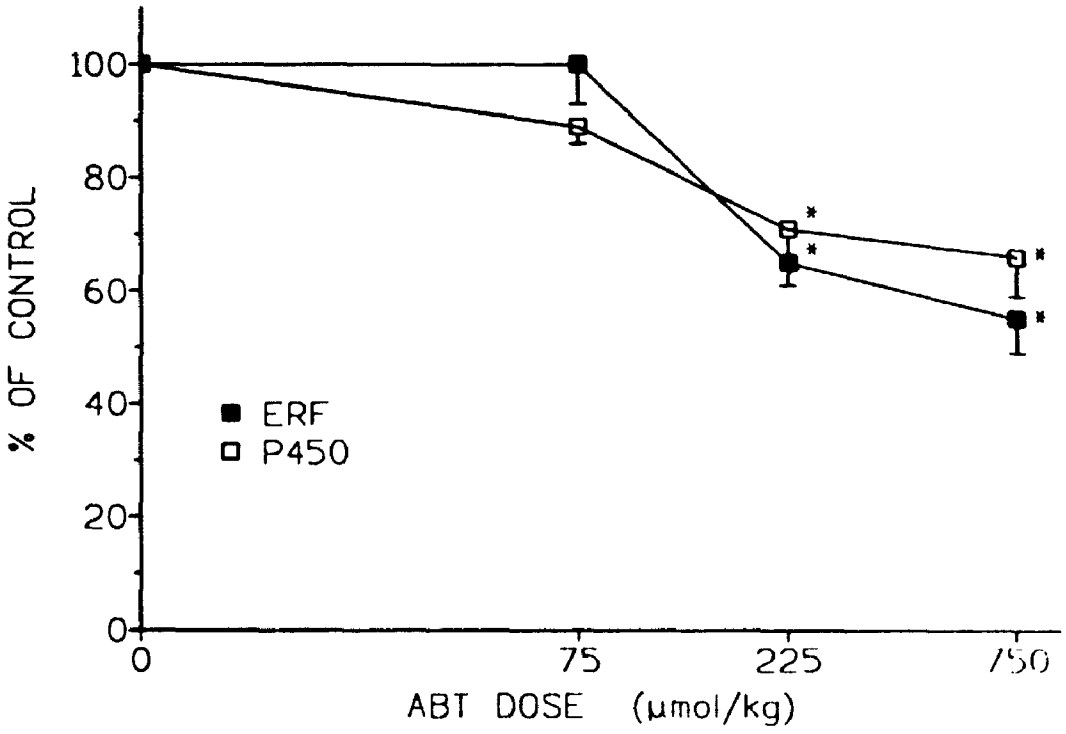
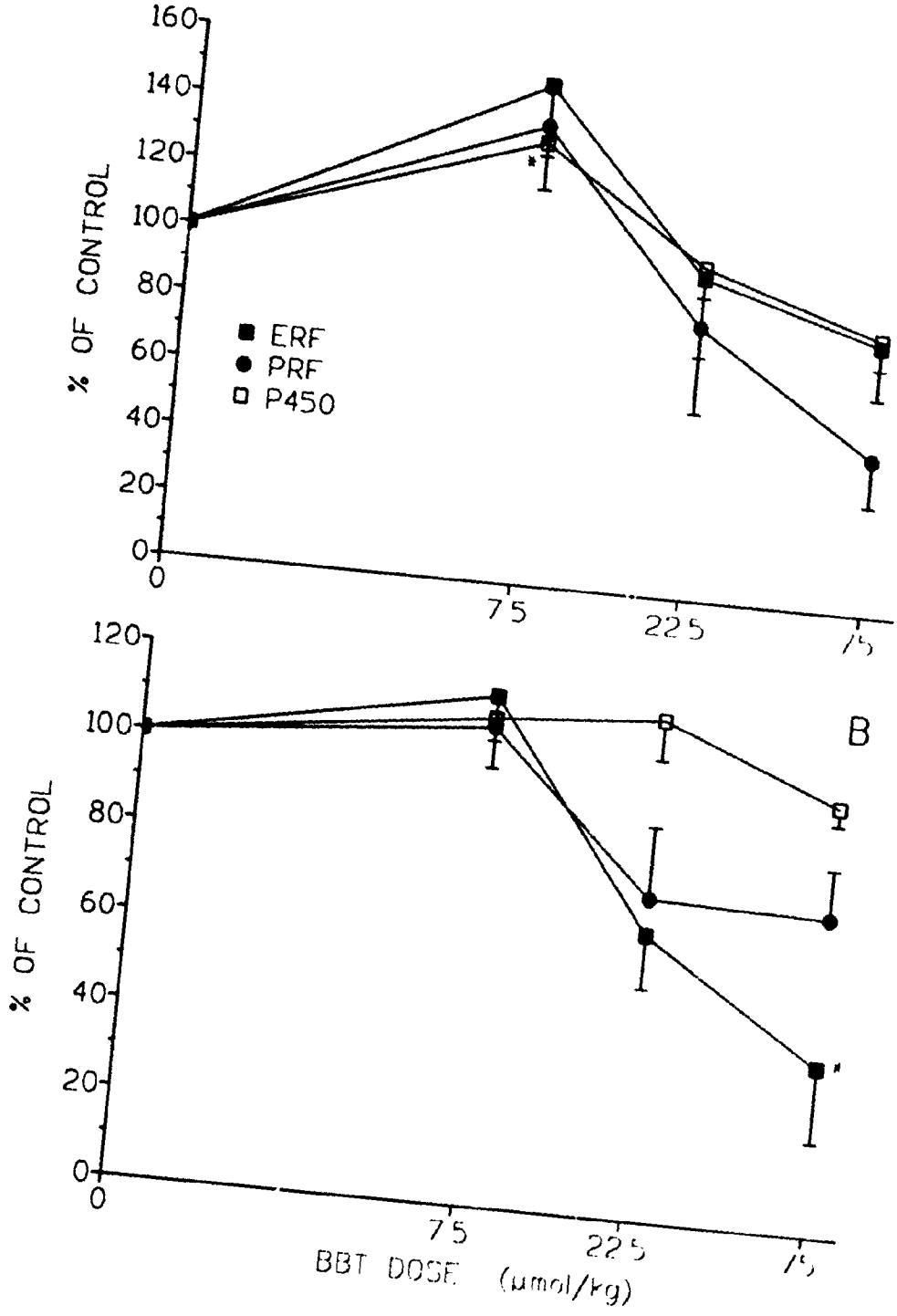

100
80
60
40
20
0
% OF CONTROL
ERF
P450
*
*
*
*
0
75
225
750
ABT DOSE (µmol/kg)

Figure 3.6

Effects of BBT, administered i.p., on pulmonary (A) and hepatic (B) monooxygenase activities and P450 content in non-induced guinea pigs. Control (100%) values were: for ERF 13.5 ± 3.7 (A, lung) and 119 ± 8 (B, liver) pmol/min/mg protein; for PRF 41.8 ± 8.2 (A, lung) and 9.9 ± 1.0 (B, liver) pmol/min/mg protein; for P450 0.09 ± 0.01 (A, lung) and 0.63 ± 0.03 (B, liver) nmol/mg protein. Data shown are means ± SE, N = 4. *Significantly different ($P < 0.05$) than corresponding vehicle-treated group.


A
B
% OF CONTROL
ERF
PRF
P450
BBT DOSE (μmol/kg)

First, these experiments examined the ability of ABT to inactivate pulmonary P450 in addition to hepatic P450. Second, the relative inhibition of a variety of isozyme-selective monooxygenase activities was studied in non-induced, PB-induced and β-NF-induced animals.

The maximal dose of ABT used in this study (750 μmol/kg or 100 mg/kg i.p.) was selected from preliminary experiments which demonstrated that this dose almost totally inactivated pulmonary ABP activity in guinea pig 24 hr after treatment. Based on previous work in the rat (Ortiz de Montellano and Costa, 1986; Mugford *et al.*, 1992) and mouse (Capello *et al.*, 1990), this dose was expected to cause maximal loss of hepatic P450 content. The magnitude of P450 modification/inactivation obtained in this study is comparable to the 75% loss in P450 content seen in rat liver 24 hr after 100 mg/kg i.p. ABT (Mugford *et al.*, 1992).

Studies examining the time-dependent loss of P450 in rats following i.p administration of ABT demonstrated that maximal loss of P450 content in liver and kidney occurs within 2 hr and is maintained for more than 24 hr (Mugford *et al.*, 1992). In this study the animals were treated with ABT for 24 hr to maximize mechanism-based inactivation while minimizing the possibility of residual ABT inhibiting the monooxygenase activities competitively/non-competitively.

In our studies all of the hepatic (P450 1A-, 2Bx- and 3A-dependent), pulmonary (P450 1A1-, 2Bx- and 4Bx-dependent) and renal (P450 1A1-dependent) monooxygenase activities examined were inhibited to some extent

by ABT. This is consistent with previous *in vitro* and *in vivo* studies which revealed that many, if not all P450 isozymes, are inactivated by ABT (Ortiz de Montellano *et al.*, 1981b; Mathews *et al.*, 1985; Ortiz de Montellano and Costa, 1986; Reich and Ortiz de Montellano, 1986; Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990; Tierney *et al.*, 1992). As determined by examining the inactivation of P450 2Bx (PRF) and 1A1 (ERF), in PB-induced and β-NF-induced animals, respectively, the extent of inhibition of the pulmonary P450 system was either lower than or equal to that of the hepatic system, but was never greater. Renal 1A1 was also inhibited to a lesser extent than hepatic 1A1.

In the lung, ABT selectively inhibited ABP activity compared to ERF and PRF activities. We previously reported a similar rank order for NADPH-dependent inactivation of P450 4Bx vs P450 2Bx and 1A1 by ABT in guinea pig pulmonary microsomes *in vitro* (Woodcroft *et al.*, 1990). The sensitivity of pulmonary P450 4Bx to mechanism-based inhibition by ABT appears to be related to the efficient oxidation of primary aromatic amines by this isozyme (Vanderslice *et al.*, 1987).

Of interest, pulmonary ERF and PRF activities were inhibited less in PB- or β-NF-induced guinea pigs than in non-induced animals. A similar effect was not observed in earlier *in vitro* experiments with ABT (Woodcroft *et al.*, 1990). The difference between the *in vivo* and the *in vitro* data may be related to increased hepatic metabolism of ABT by those P450 isozymes induced by PB or β-NF resulting in decreased delivery of ABT to lung. *In vitro* experiments

performed with [^{14}C]ABT and hepatic microsomes from guinea pigs demonstrated that ABT is converted to a [^{14}C]metabolite that co-chromatographs with benzotriazole on high pressure liquid chromatography (HPLC), and that metabolism is more efficient if the animals are pretreated with PB or β-NF (K.J. Woodcroft and J.R. Bend, unpublished observations), consistent with this possibility.

In the liver of non-induced and PB-induced animals PRF activity was inhibited more than ERF, ABP, END or BND activity. In β-NF-induced animals ABP activity was preferentially inhibited. The greater percentage of loss of ABP activity in β-NF-induced guinea pigs compared to non-induced animals, with comparable amounts of ABP activity (pmol/min/mg protein) remaining at the highest dose, strongly suggests higher susceptibility of P450 1A2, the major isozyme catalyzing ABP activity in β-NF-induced animals, compared to the isozymes that catalyze ABP constitutively, to inactivation by ABT. The sensitivity of P450 1A2 to inactivation by ABT, as for P450 4Bx in lung, reflects its efficiency in the oxidation of aromatic amines (Hammons *et al.*, 1985; Butler *et al.*, 1989).

In general, P450 3A-dependent END activity was fairly resistant to inhibition by *in vivo* ABT treatment and was inactivated less in animals pretreated with PB or β-NF than non-induced animals. Decreased inactivation of END in induced vs non-induced animals may be due to increased formation of the non suicidal metabolite benzotriazole in PB- and β-NF-induced guinea pigs (K.J. Woodcroft and J.R. Bend, unpublished data) and/or the increased levels

of 1A1/1A2 or 2Bx competing with isozymes of the P450 3A subfamily for the suicide substrate in the β-NF- and PB-induced animals.

In all cases hepatic PRF activity was inhibited to a greater extent than BND activity. Also, the percentage of loss in PRF activity varied between induction states whereas that of BND activity showed little variation. These findings, along with the ability of PB to selectively increase PRF (vs BND) activity, emphasize that 7-pentoxyresorufin is a much more isozyme-selective substrate for guinea pig P450 2Bx than is benzphetamine. This is consistent with other species; 7-pentoxyresorufin is also much more isozyme-selective for rat 2B1 than benzphetamine (Guengerich, 1987).

In liver, lung and kidney the ABT-mediated loss of P450 content corresponded quite well with the inhibition of monooxygenase activities. This indicates that in all three organs the major mechanism of inactivation of the P450 isozymes by ABT involves loss of heme iron from the P450 protein consistent with the known mechanism whereby ABT inactivates P450 via arylation of the prosthetic heme moiety by the reactive metabolite, benzyne (Ortiz de Montellano and Mathews, 1981; Ortiz de Montellano *et al.*, 1984).

BBT, designed as a potential isozyme-selective, lung-selective inhibitor of P450 *in vivo* (Mathews and Bend, 1986), inactivated the guinea pig pulmonary P450 system in an isozyme selective manner 24 hr following i.p. administration. PRF activity (P450 2Bx) was inhibited more than ERF activity (P450 1A1), consistent with previous *in vitro* studies (Woodcroft *et al.*, 1990). However, *in vitro* BBT was at least 10-fold more potent at inactivating

pulmonary 2Bx than ABT whereas *in vivo* both compounds inhibited PRF activity about equipotently (75 μmol/kg was required for ~50% inhibition). Perhaps following i.p. administration, a smaller percentage of the injected BBT, compared to the injected ABT, is reaching the lung due to differences in absorption, distribution, and/or hepatic first-pass metabolism. The more lipophilic BBT is a more potent inhibitor of pulmonary PRF activity than ABT when administered i.v. via the lateral ear vein to rabbit (Mathews and Bend, 1993). Also in rabbit BBT, administered i.v., selectively inactivated pulmonary vs hepatic P450 whereas in guinea pig no tissue selectively was evident following i.p. administration. When administered i.p. the BBT would pass through the liver, undergoing hepatic metabolism (and inactivating hepatic P450) before reaching the lung whereas when administered i.v. the BBT would pass through the lung, undergoing pulmonary metabolism (and inactivating pulmonary P450) before reaching the liver. BBT is likely a substrate for the facilitated uptake system for lipophilic basic amines located in lung. To take full advantage of this uptake system, BBT should be administered i.v. to maximize its uptake into the lung, thereby increasing the concentration of BBT in the lung and decreasing the amount delivered to the liver.

The increases in hepatic PRF and ERF activities obtained in guinea pig with PB-and β-NF-treatment, respectively, are much smaller than those reported for rats (Burke *et al.*, 1985; Lubet *et al.*, 1985) and C57/BL10 mice (Burke and Mayer, 1983). Consistent with the differences found in the induction of PRF activity between guinea pig and rat, immunoblotting studies have demonstrated

that PB induces P450 2Bx only a few-fold in guinea pig liver because of the high level of constitutive expression (Yamada *et al.*, 1992; Ohmura *et al.*, 1993) whereas 2B1 is poorly expressed constitutively in rat liver (Christou *et al.*, 1987; Ohmura *et al.*, 1993). Differences in the induction of ERF activity by 1A inducers between rat and other species, including the guinea pig, have also been reported by other research groups. 3-MC increased hepatic ERF activity ~60-fold in the rat but only by ~8-fold in the hamster (Burke and Mayer, 1974) and 3,4,5,3',5'-pentachlorobiphenyl (inducer of P450 1A1/1A2) increased hepatic ERF activity in the guinea pig <5-fold while causing ~60-fold increase in the rat (Huang and Gibson, 1991).

In summary, ABT is an effective inactivator of the pulmonary, hepatic and renal P450 systems in guinea pigs following i.p. administration, and P450 1A2 (liver) and P450 4Bx (lung) are preferentially inhibited by this suicide substrate. BBT is an 2Bx-selective inactivator in the lung of guinea pig following i.p. administration but is no more potent than ABT nor is it selective for pulmonary vs hepatic P450 under these conditions.

CHAPTER 4

INACTIVATION OF CYTOCHROME P450 *IN VIVO* BY 1-AMINOBENZOTRIAZOLE AND ITS N-ARALKYLATED DERIVATIVES ADMINISTERED INTRAVENOUSLY

4.1 OBJECTIVES

The primary objectives of this study were to characterize, in detail, the ability of BBT and *α*MB, relative to ABT, to selectively inhibit pulmonary P450 2Bx (vs 1A1 and 4Bx) and to inactivate pulmonary vs hepatic P450. To achieve this objective the dose response of P450 inactivation following i.v. injection of BBT, *α*MB or ABT into non-induced, *β*-NF-induced or PB-induced guinea pigs was evaluated.

4.2 MATERIALS AND METHODS

4.2.1 Reagents

ABT, BBT and *α*MB were synthesized and purified as previously described (Mathews *et al.*, 1985; Mathews and Bend, 1986). Emulphor EL-620 was obtained from the GAF Corporation (New York, NY); rabbit anti-goat IgG and goat peroxidase anti-peroxidase from the Sigma Chemical Co. (St. Louis, MO); Schleicher and Schuell nitrocellulose (0.2 *μ*m) from Mandel Scientific Co. Ltd. (Guelph, Ont.); acrylamide, bisacrylamide, SDS, tetramethylethylenediamine (TEMED) and ammonium persulfate from BIO-RAD Laboratories Inc. (Mississauga, Ont.); and ECL detection reagents from

Amersham Canada Ltd. (Oakville, Ont.). The sources of other chemicals are given in Section 3.2.1. Goat antibodies to rabbit P450 2B4 were generously provided by Dr. R.M. Philpot of the National Institute of Environmental Health Sciences, NIH (Research Triangle Park, NC).

4.2.2 Animal Treatment

Male Hartley guinea pigs (300-375 g) were obtained from Charles River Ltd. (St. Constant, Que.). They were housed as indicated in Section 3.2.1.

Guinea pigs were treated i.p. with 80 mg/kg PB (2% in saline) or 80 mg/kg β-NF (2% in corn oil) daily for 4 days. Some animals received no inducing agent. Approximately 24 hr after the last injection of inducer, urethane (1.5 g/kg) was administered i.p. Once 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% BSA/DMSO/Emulphor, 6/0.15/0.3, v/v/v) by sonication at concentrations so that each animal received an equivalent volume of vehicle (2.15 ml/kg). BBT and αMB were dissolved in the DMSO prior to their addition to the Emulphor/BSA mixture. The animals were sacrificed 4 hr after the inhibitor was administered. The lungs and livers were removed and stored at -80°C until preparation of microsomes (usually within one week).

4.2.3 Preparation of Microsomes

Washed hepatic and pulmonary microsomes were prepared from

individual tissues by differential centrifugation as described previously (Section 3.2.3) and the protein concentrations were determined by the method of Lowry *et al.*, 1951 (Section 3.2.4).

4.2.3 Determination of Cytochrome P450 Content and Related Monooxygenase Activities

Total microsomal P450 content was determined from the dithionite difference spectrum of carbon monoxide-saturated microsomes (Section 3.2.5). ERF, PRF, ABP and END activities were determined as previously described (Sections 3.2.6 to 3.2.8).

4.2.4 Western Blot Analysis

Microsomal protein was electrophoresed on a 7.5% polyacrylamide gel (1.5 mm thick) containing 0.1% SDS by the method of Laemmli (1970) using a "Protean II" apparatus (BIO-RAD Laboratories Inc., Mississauga, Ont.). The separating gel was prepared by degassing 24.25 ml of water, 12.5 ml of 1.5 M Tris-HCl buffer (pH 8.8), 0.5 ml of 10% (w/v) SDS and 12.5 ml of acrylamide/bisacrylamide (30% T, 2.67% C) for 15 min. This solution was poured immediately after adding 250 μl of 10% ammonium persulfate (freshly prepared) and 25 μl of TEMED, and overlayed with water. The separating gel was allowed to polymerize for at least 90 min before pouring the stacking gel. The stacking gel was prepared by degassing 6.1 ml of water, 2.5 ml of 0.5 M Tris-HCl buffer (pH 6.8), 100 μl 10% SDS and 1.3 ml acrylamide/bisacrylamide

(30% T, 2.67% C). This solution was poured immediately after adding 10% ammonium sulfate solution (50 μl) and TEMED solution (10 μl) and allowed to polymerize for at least 30 min.

Microsomal protein was diluted at least 1:4 with sample buffer containing 62.5 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol and 0.0012% (w/v) bromophenol blue. The samples were heated in boiling water for 3 min, cooled, and then loaded onto the gel. The stacking and separating gels were run at 25 and 35 mA, respectively, with cooling to 10°C. The running buffer (pH 8.3) contained 25 mM Tris, 192 mM glycine and 0.1% (w/v) SDS. Total run time was about 5 hr.

Following electrophoresis the separating gel was soaked in transfer buffer (25 mM Tris, 192 mM glycine and 20% (v/v) methanol) for 1 hr. The proteins were then electrophoretically transferred from the polyacrylamide gel to a 0.2 μm nitrocellulose membrane by the method of Towbin *et al.* (1979) in a "Trans Blot Cell" (BIO-RAD Laboratories Inc., Mississauga, Ont.) at 30 V, 100 mA overnight followed by 60 V, 200 mA for 1 hr.

The blot was soaked in 3% gelatin in Tris-buffered saline (TBS; 20 mM Tris-HCl, pH 7.4, 0.9% NaCl) containing 0.05% Tween-20 (TBS-Tween) for 1 hr, washed for 5 min in TBS-Tween, and incubated with antibodies to rabbit P450 2B4 diluted 1:5000 in antibody buffer (TBS-Tween containing 1% gelatin). After 1.5 to 2 hr the blot was washed 3 times in TBS-Tween (~5 min/wash) and then incubated for 15 min with rabbit anti-goat IgG diluted 1:1000 in antibody buffer. The blot was washed 3 times in TBS-Tween (~5

min/wash) and incubated for 15 min with goat peroxidase anti-peroxidase diluted 1:3000 in antibody buffer. After washing 3 times with TBS-Tween (~5 min/wash) immunoreactive protein bands were detected using the Amersham ECL detection reagents. Equal amounts of reagent A and reagent B (2-3 ml of each) where mixed together and poured on the blot. After 1 min the blot was covered with plastic wrap and exposed to Kodax X-OMAT AR film for 10-60 seconds.

Band density was determined on the film utilizing an LKB ultrascan XL enhanced laser densitometer with gel scan XL software (Pharmacia Biotech Inc., Baie d'Urfé, Que.). For each microsomal sample multiple amounts (2-4) of microsomal protein in the linear range (2.5-20 μg/lane) were assayed on the same blot and the slope (change in density/μg protein) calculated. For each inhibitor 2 blots were carried out and the slope for each sample was expressed as a ratio of the slope of a particular control sample (C2) on the same blot. This particular control sample (C2) was present on both blots. The slope of C2 on each blot was defined as 1 arbitrary unit (A.U.).

4.2.5 Statistical Analysis

One-way ANOVA with Newman-Keuls test was used to analyze for significant difference in enzyme activities or P450 content between vehicle-treated and inhibitor-treated groups (Zar, 1984). In all cases, analyses were performed on the raw data and not the "percentage of control data" presented in the figures. The unpaired Student's t-test was used to analyze for

significant difference in P450 2Bx apoprotein levels.

4.3 RESULTS

The effects of various doses of ABT, BBT and *o*MB on the pulmonary monooxygenase system of non-induced guinea pigs are shown in Fig. 4.1. Both *o*MB and BBT inhibited PRF and ERF activities whereas neither significantly inhibited ABP activity even at the highest doses examined (7.5 and 75 μmol/kg, respectively). At each dose of *o*MB (0.075-7.5 μmol/kg) and BBT (0.75-75 μmol/kg) PRF activity was inhibited 20-40% more than ERF activity. Also, ERF inactivation plateaued at about 60% with both analogues whereas PRF inactivation reached >80% with BBT and >95% with *o*MB. ABT was a much less potent inactivator of the pulmonary P450 system having little effect at 0.75 μmol/kg. However, at 75 μmol/kg all three enzyme activities were inhibited by >80%. At 7.5 μmol/kg, ABT showed limited isozyme selectivity; ABP activity was inhibited about 30% more than ERF or PRF activity.

*o*MB, BBT and ABT also inactivated the hepatic monooxygenase system in non-induced guinea pigs in a dose-dependent manner (Fig. 4.2). With *o*MB and BBT, 10- to 100-fold higher doses were required for inhibition of any of the hepatic monooxygenase activities examined (ie. ERF, PRF, ABP and END) than for pulmonary PRF activity. Pulmonary PRF was inactivated by >90% at 0.075 μmol/kg *o*MB but 7.5 μmol/kg was required to cause >25% inhibition of even one of the hepatic activities. With BBT, 0.75 μmol/kg

Figure 4.1

Effects of αMB (A), BBT (B) or ABT (C), administered i.v., on pulmonary monooxygenase activities and P450 content in non-induced guinea pigs. Control (100%) values were: for ABP 170 ± 23 (A, C) and 267 ± 15 (B) nmol/min/mg protein; for ERF 10.0 ± 1.3 (A, C) and 13.6 ± 0.9 (B) pmol/min/mg protein; for PRF 29.8 ± 4.9 (A, C) and 40.7 ± 0.9 (B) pmol/min/mg protein; for P450 0.11 ± 0.01 (A, B, C) nmol/mg protein. Data shown are means ± SE, N=4-5. *Significantly different ($P<0.05$) than corresponding vehicle-treated group.

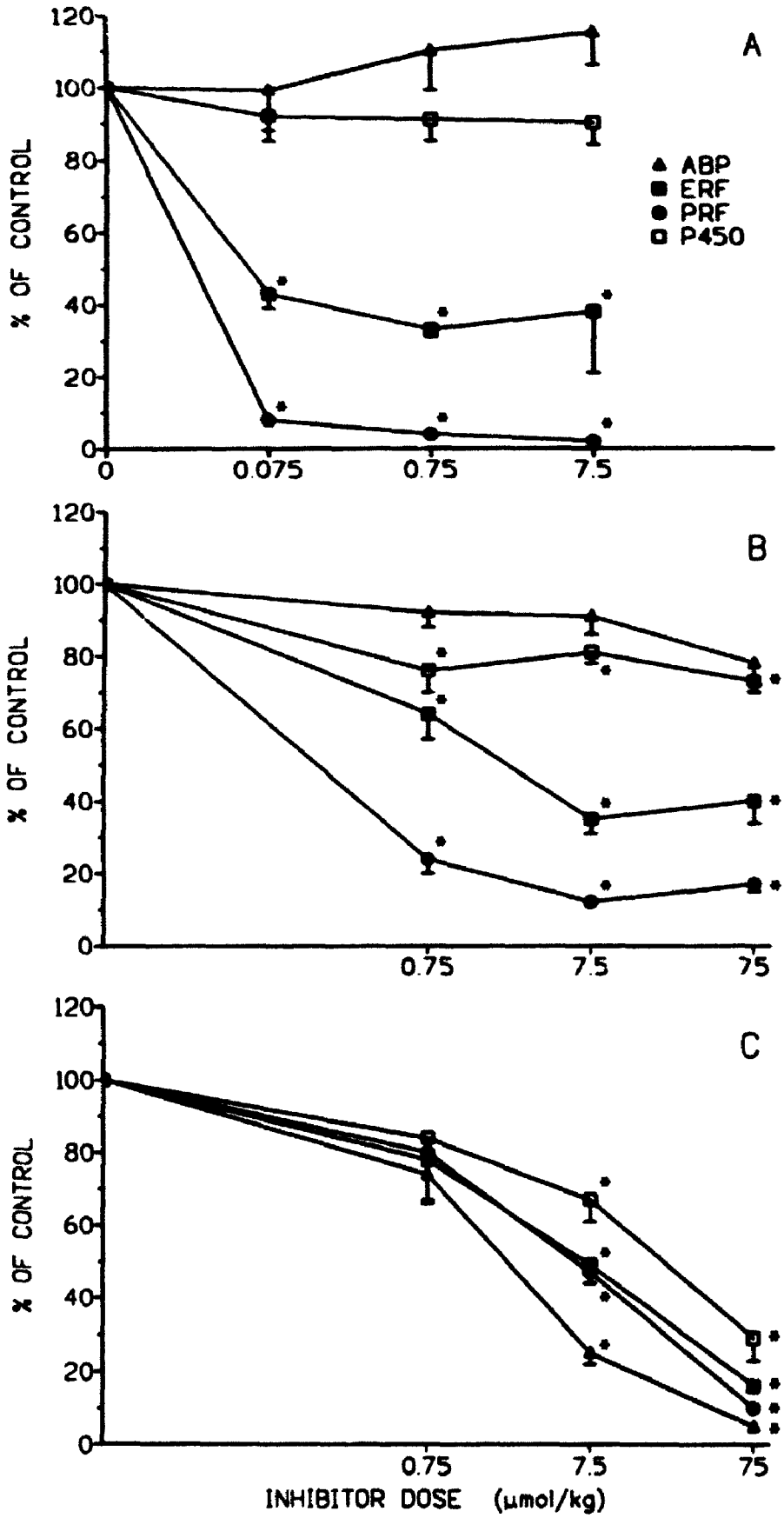
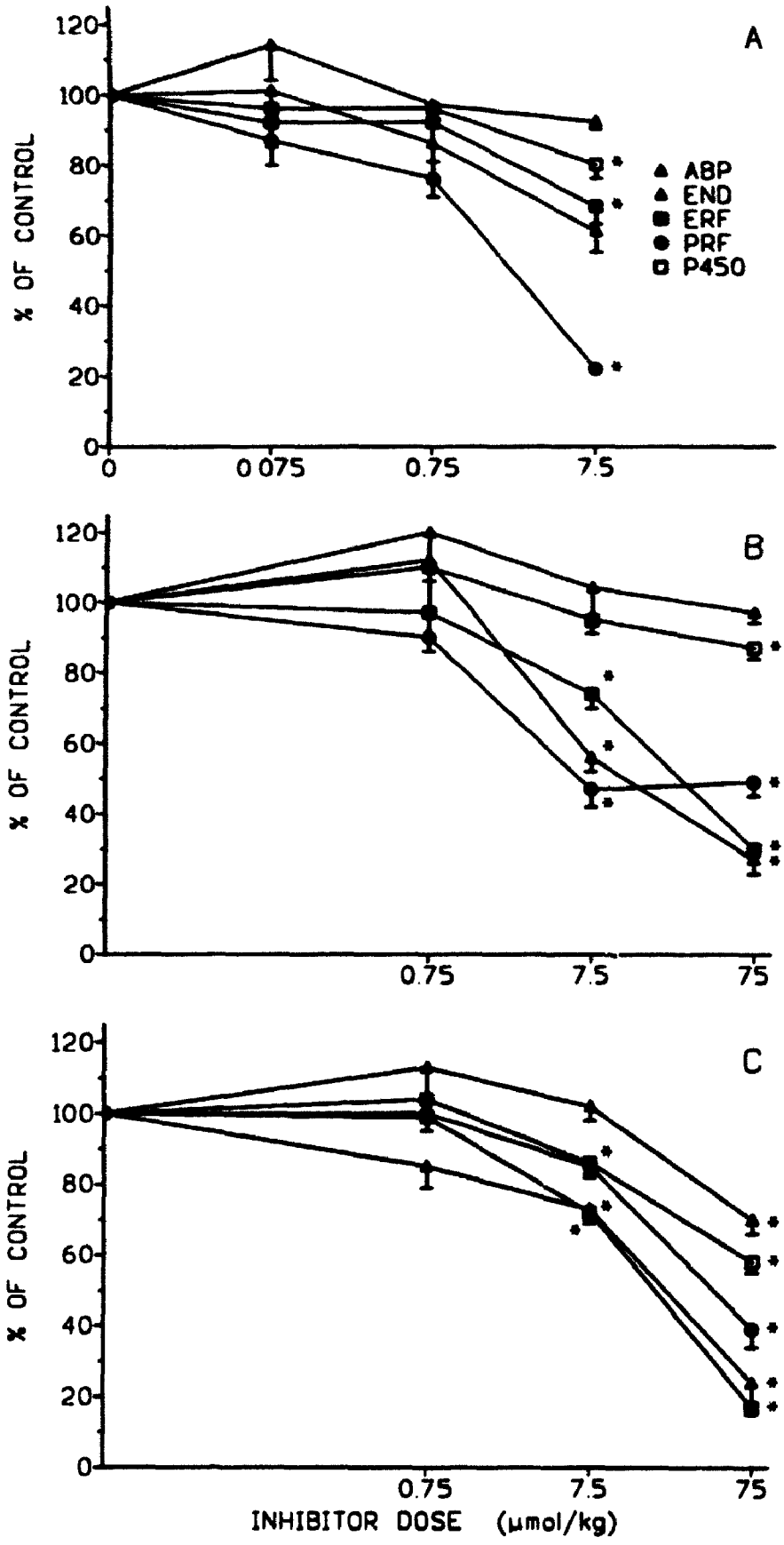

A
B
C
ABP
ERF
PRF
P450
% OF CONTROL
% OF CONTROL
% OF CONTROL
120
100
80
60
40
20
0
0
0.075
0.75
7.5
0.75
7.5
75
INHIBITOR DOSE (µmol/kg)

Figure 4.2

Effects of αMB (A), BBT (B) or ABT (C), administered i.v., on hepatic monooxygenase activities and P450 content in non-induced guinea pigs. Control (100%) values were: for ABP 111 ± 7 (A, C) and 73 ± 8 (B) nmol/min/mg protein; for END 2.48 ± 0.15 (A, C) and 3.00 ± 0.18 (B) nmol/min/mg protein; for ERF 276 ± 17 (A, C) and 231 ± 16 (B) pmol/min/mg protein; for PRF 16.7 ± 2.3 (A, C) and 22.8 ± 3.4 (B) pmol/min/mg protein; for P450 1.00 ± 0.02 (A, C) and 0.77 ± 0.02 (B) nmol/mg protein. Data shown are means ± SE, N=4-5. *Significantly different ($P<0.05$) than corresponding vehicle-treated group.


A
B
C
% OF CONTROL
120
100
80
60
40
20
0
0
0.075
0.75
7.5
75
ABP
END
ERF
PRF
P450
INHIBITOR DOSE (µmol/kg)

inactivated pulmonary PRF by >75% but 7.5 μmol/kg was required for inhibition of the hepatic activities. Some tissue-selectivity was also evident with ABT; the middle dose (7.5 μmol/kg) inhibited pulmonary ABP activity by >70% with <30% inhibition of any of the hepatic activities.

The ability of αMB, BBT and ABT to inactivate the pulmonary and hepatic P450 monooxygenase systems was also examined in β-NF-induced guinea pigs (Figs. 4.3 and 4.4, respectively). In lungs of β-NF-induced animals all doses of αMB tested specifically inhibited PRF (vs ERF or ABP) activity. At 0.075 μmol/kg greater than 90% of the PRF activity was inhibited whereas there was no loss in either ERF or ABP activities even at a 100-fold higher dose. This increase in P450 isozyme-selectivity in β-NF induced guinea pigs in comparison to non-induced animals is due to a decrease in the relative inhibition of ERF activity (0% vs 57% loss at the highest dose, 7.5 μmol/kg). BBT is also a more isozyme-selective inactivator of pulmonary P450 in β-NF-induced animals than non-induced animals at the 7.5 and 75 μmol/kg doses due to a decrease in the inhibition of ERF activity in β-NF-induced relative to non-induced guinea pigs. In non-induced animals 0.75 μmol/kg BBT inhibited ERF activity by >35% whereas 100-fold higher doses were required for an equivalent amount of inactivation in β-NF-induced animals. In β-NF-induced guinea pigs, ABT inactivated the pulmonary monooxygenase system with little isozyme-selectivity. In fact, the selectivity for ABP inactivation at 7.5 μmol/kg in non-induced guinea pigs did not occur in β-NF-induced animals due to a decrease in the inhibition of ABP activity in the latter (74% in non-induced vs

Figure 4.3

Effects of *α*MB (A), BBT (B) or ABT (C), administered i.v., on pulmonary monooxygenase activities and P450 content in *β*-NF-induced guinea pigs. Control (100%) values were: for ABP 204 ± 26 (A, C) and 151 ± 10 (B) nmol/min/mg protein; for ERF 25.7 ± 2.9 (A, C) and 35.9 ± 10.6 (B) pmol/min/mg protein; for PRF 31.8 ± 3.9 (A, C) and 60.2 ± 10.6 (B) pmol/min/mg protein; for P450 0.14 ± 0.01 (A, C) nmol/mg protein. Data shown are means ± SE, N=4-5. *Significantly different ($P<0.05$) than corresponding vehicle-treated group.

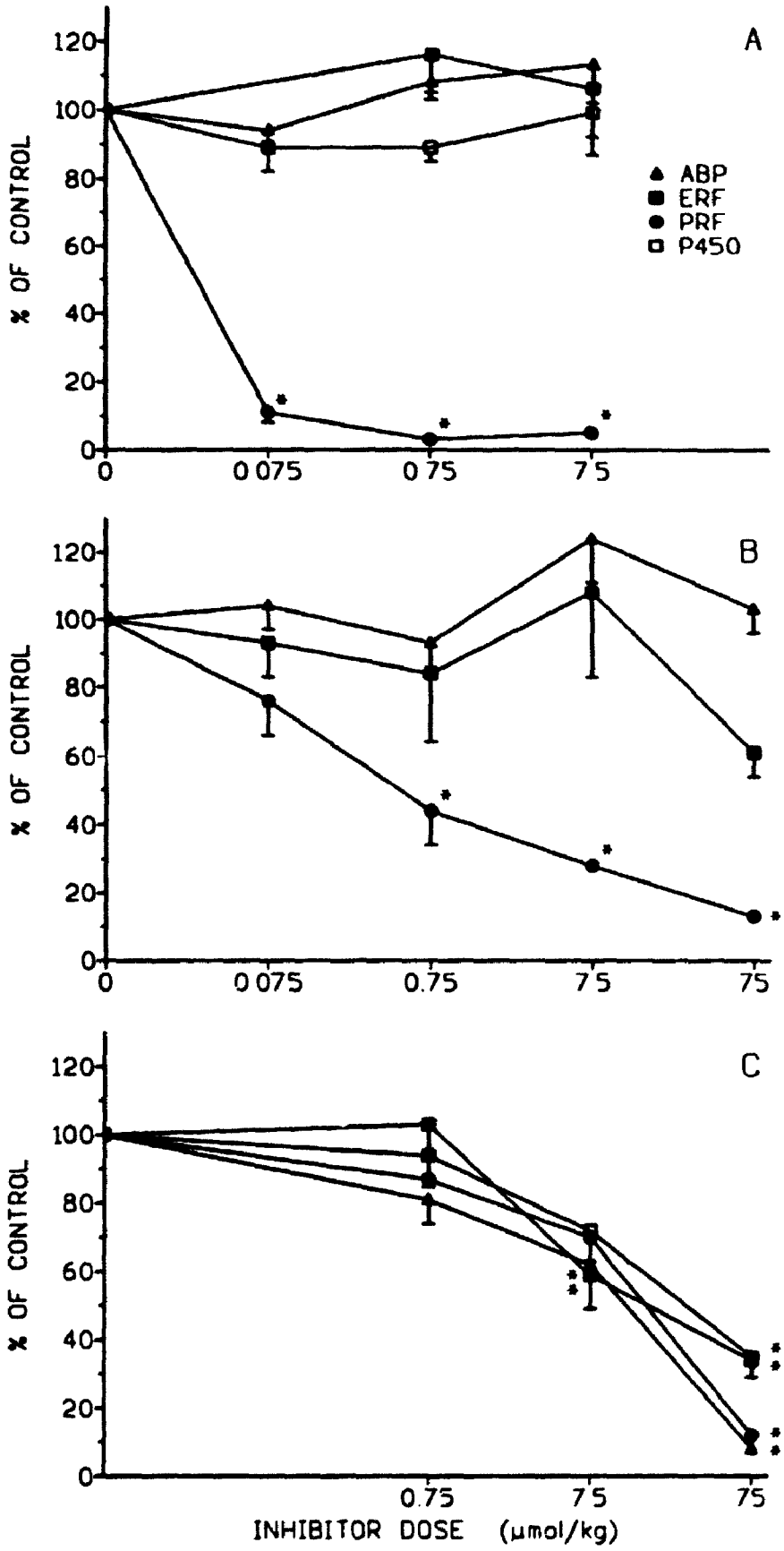
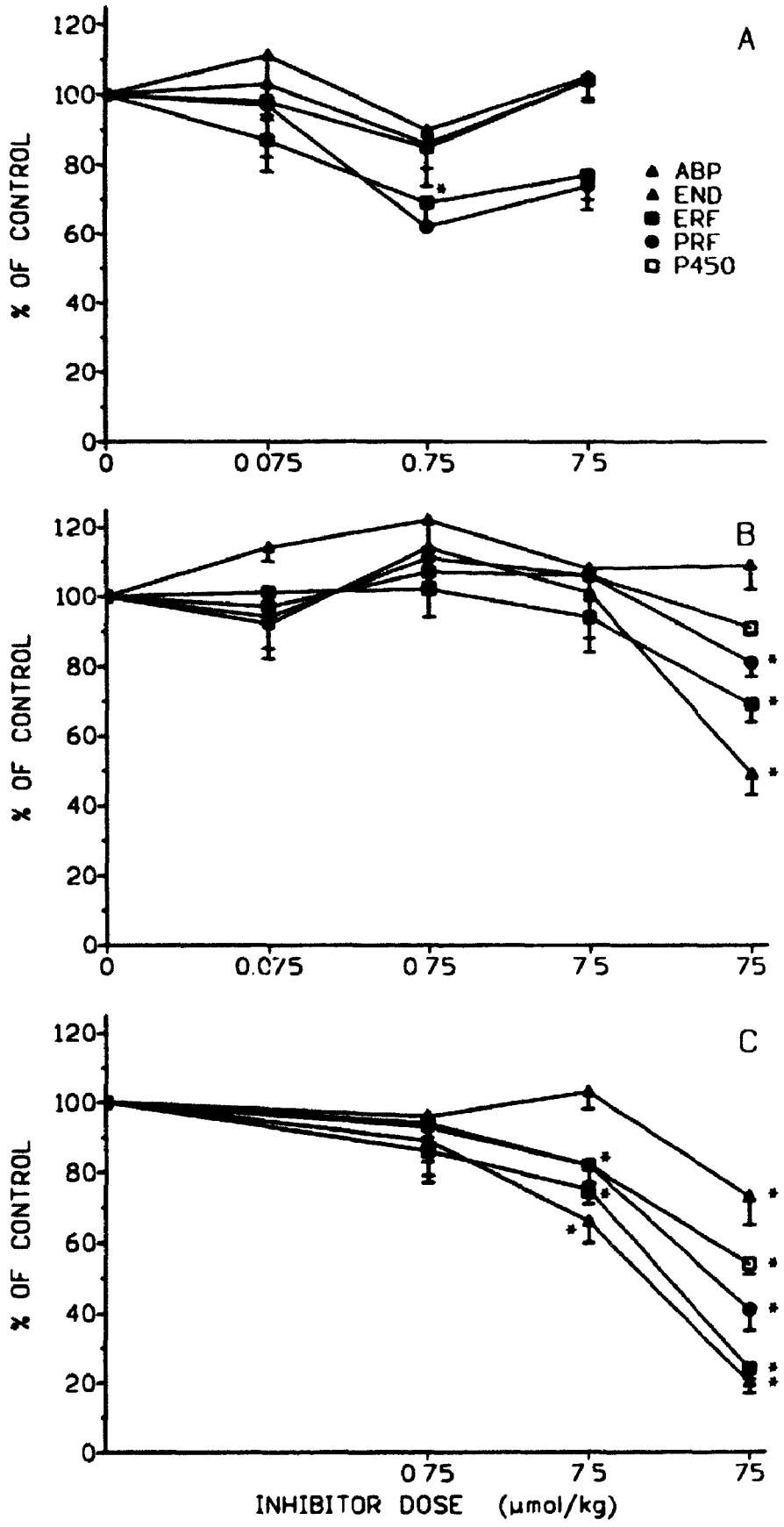

A
B
C
% OF CONTROL
120
100
80
60
40
20
0
ABP
ERF
PRF
P450
0
0 075
0 75
7 5
75
0.75
INHIBITOR DOSE (µmol/kg)

Figure 4.4

Effects of αMB (A), BBT (B) or ABT (C), administered i.v., on hepatic monooxygenase activities and P450 content in β-NF-induced guinea pigs. Control (100%) values were: for ABP 355 ± 22 (A, C) and 271 ± 31 (B) nmol/min/mg protein; for END 2.22 ± 0.11 (A, C) and 1.66 ± 0.18 (B) nmol/min/mg protein; for ERF 992 ± 40 (A, C) and 1108 ± 62 (B) pmol/min/mg protein; for PRF 16.7 ± 2.3 (A, C) and 22.5 ± 2.7 (B) pmol/min/mg protein; for P450 1.24 ± 0.05 (A, C) and 0.91 ± 0.06 nmol/mg protein. Data shown are means ± SE, N=4-5. *Significantly different ($P<0.05$) than corresponding vehicle-treated group. At 7.5 μmol/kg ABT (C) ABP and ERF activities and P450 content are significantly different than control.


A
B
C
% OF CONTROL
120
100
80
60
40
20
0
0
0 075
0.75
7 5
75
0.075
ABP
END
ERF
PRF
P450
INHIBITOR DOSE (µmol/kg)

38% in β-NF-induced guinea pigs).

In β-NF-induced animals, BBT and αMB retained their selectivity for the inactivation of pulmonary vs hepatic P450. For example, 0.075 μmol/kg αMB, which inhibited pulmonary PRF activity by >90%, did not inactivate any of the hepatic monooxygenase activities examined by more than 15% (Fig. 4.4). Also, 7.5 μmol/kg BBT, which inactivated >70% of the pulmonary PRF activity, did not inhibit any of the hepatic monooxygenase activities (< 10% loss). On the other hand, 75 μmol/kg ABT, the dose required for >50% inhibition of any of the pulmonary monooxygenase activities caused marked, near equivalent, inhibition of hepatic ABP, ERF and PRF activities (>50%).

Interestingly, little difference was apparent in the inhibition of hepatic monooxygenase activities between β-NF-induced and non-induced guinea pigs treated with ABT whereas marked differences were noted with BBT and αMB. For example, in non-induced animals 7.5 μmol/kg BBT caused about 45%, 25% and 50% inhibition of ABP, ERF and PRF, respectively, whereas a 10-fold higher dose (75 μmol/kg) was required for >10% inhibition of any of these activities in β-NF-induced animals. Also, 7.5 μmol/kg αMB inhibited hepatic PRF activity by >75% in non-induced guinea pigs whereas in β-NF-induced animals this dose caused <30% inhibition of PRF activity.

In PB-induced animals BBT and αMB retained their isozyme selectivity (inhibition of PRF activity vs ERF or ABP activity) in lung and also their tissue (lung vs liver) selectivity (Figs. 4.5 and 4.6). For example, 0.075 μmol/kg αMB inhibited pulmonary PRF, ERF and ABP activities by about 85%, 50% and 0%,

Figure 4.5

Effects of αMB (A), BBT (B) or ABT (C), administered i.v., on pulmonary monooxygenase activities and P450 content in PB-induced guinea pigs. Control (100%) values were: for ABP 231 ± 19 (A), 226 ± 10 (B) and 205 ± 23 (C) nmol/min/mg protein; for ERF 15.6 ± 1.9 (A), 13.2 ± 0.8 (B) and 13.6 ± 2.1 (C) pmol/min/mg protein; for PRF 52.6 ± 7.2 (A), 59.9 ± 4.5 (B) and 39.0 ± 4.5 (C) pmol/min/mg protein; for P450 0.13 ± 0.01 (A) and 0.10 ± 0.01 (B,C) nmol/mg protein. Data shown are means ± SE, N = 4-5. *Significantly different ($P < 0.05$) than corresponding vehicle-treated group. At 7.5 μmol/kg ABT (C) ABP and ERF activities are significantly different than control.

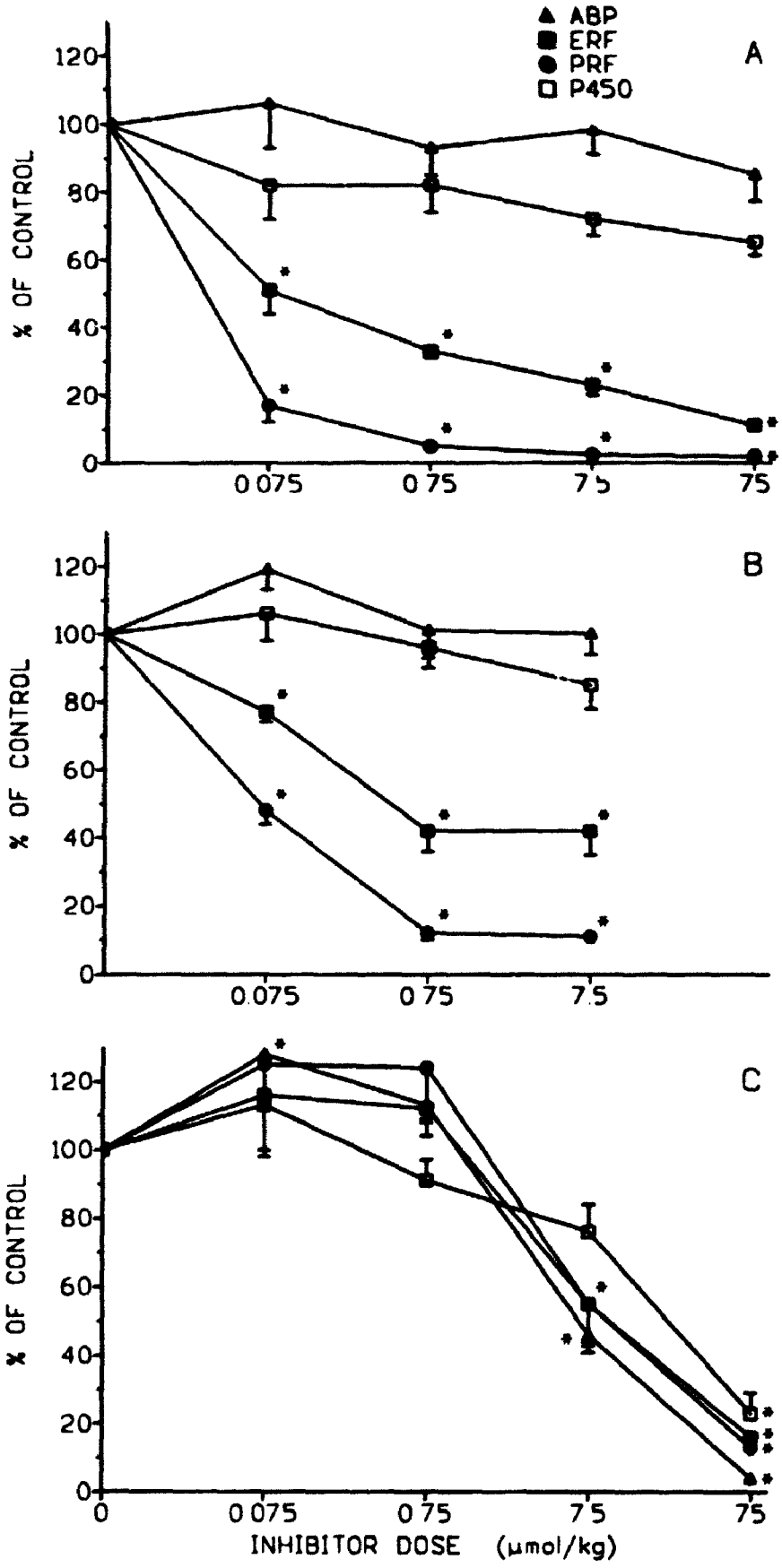
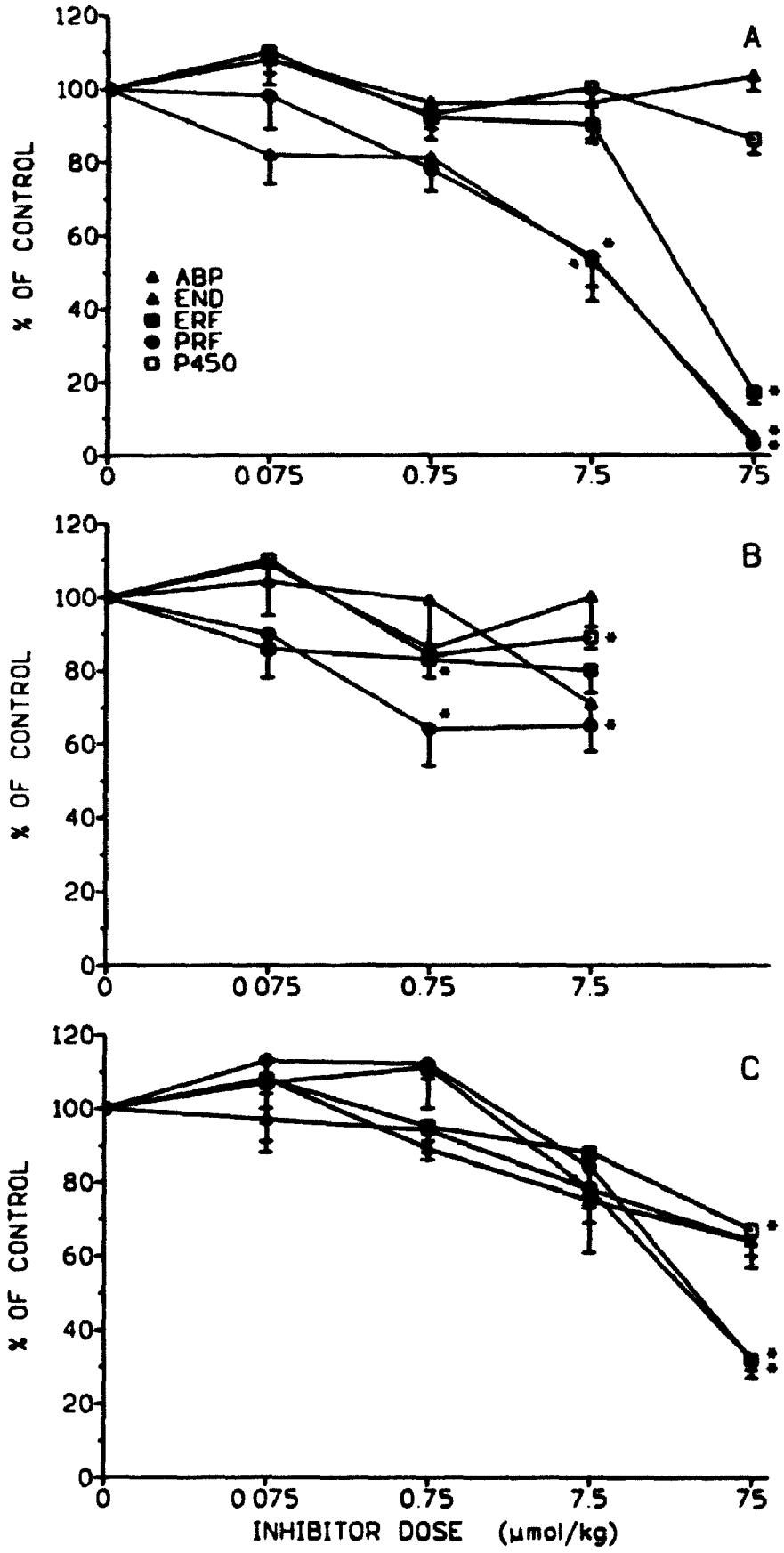

ABP
ERF
PRF
P450
A
B
C
% OF CONTROL
120
100
80
60
40
20
0
0 075
0 75
7 5
75
0.075
INHIBITOR DOSE (µmol/kg)

Figure 4.6

Effects of αMB (A), BBT (B) or ABT (C), administered i.v., on hepatic monooxygenase activities and P450 content in PB-induced guinea pigs. Control (100%) values were: for ABP 373 ± 32 (A), 248 ± 20 (B) and 240 ± 21 (C) nmol/min/mg protein; for END 5.71 ± 0.65 (A), 4.85 ± 0.19 (B) and 5.02 ± 0.79 (C) nmol/min/mg protein; for ERF 252 ± 20 (A), 210 ± 5 (B) and 191 ± 8 (C) pmol/min/mg protein; for PRF 193 ± 24 (A), 125 ± 14 (B) and 108 ± 23 (C) pmol/min/mg protein; for P450 1.35 ± 0.06 (A), 1.23 ± 0.04 (B) and 1.32 ± 0.10 (C) nmol/mg protein. Data shown are means ± SE, N=4-5. *Significantly different ($P<0.05$) than corresponding vehicle-treated group. At 0.75 μmol/kg BBT (B) PRF activity and P450 content are significantly different than control. At 7.5 μmol/kg ABT (C) ERF activity is significantly different than control.


A
B
C
% OF CONTROL
ABP
END
ERF
PRF
P450
0
0.075
0.75
7.5
75
INHIBITOR DOSE (µmol/kg)

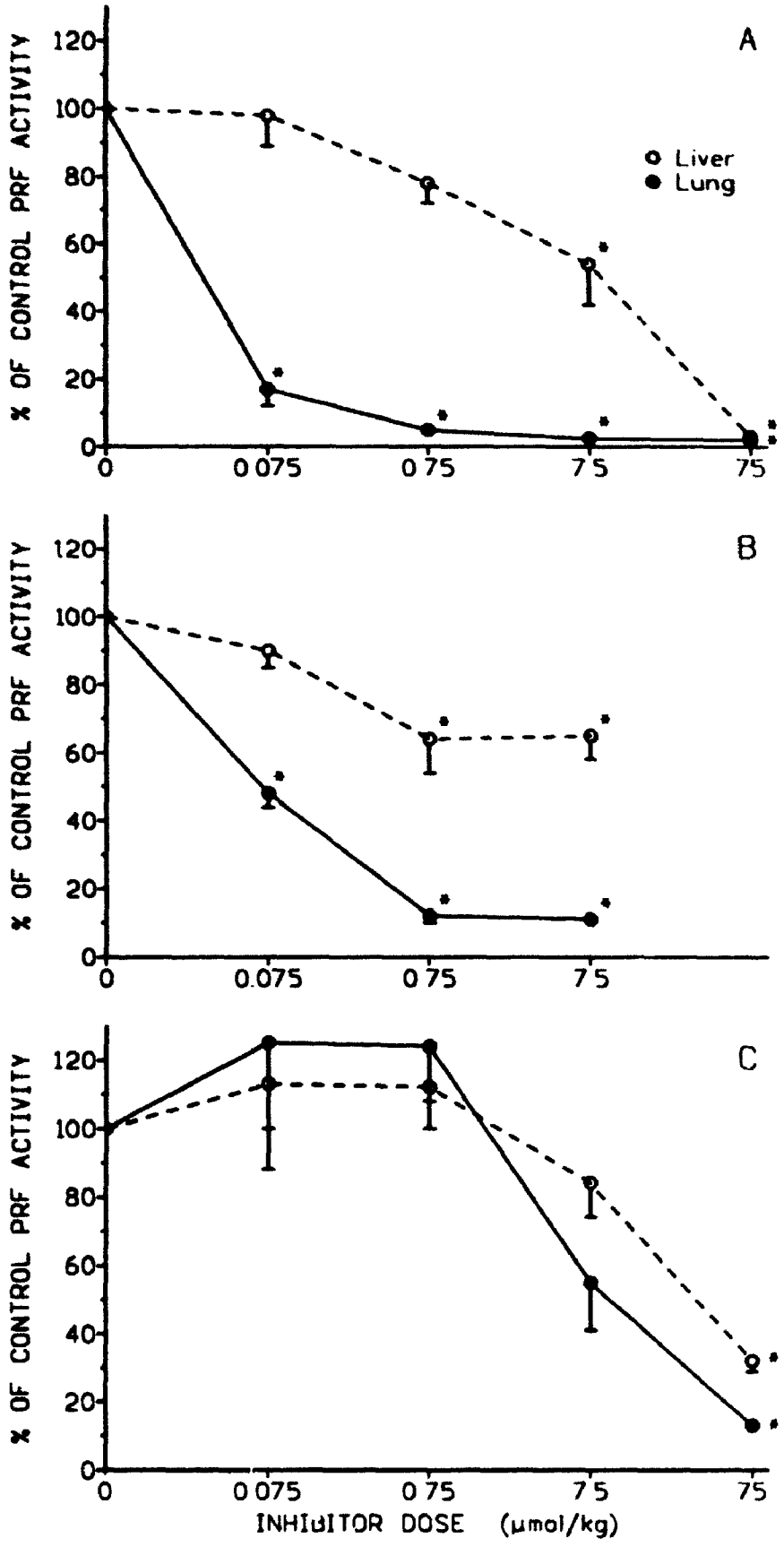
respectively, whereas a 100-fold higher dose was required for >20% inhibition of any of the hepatic monooxygenase activities. The maximal amount of isozyme selectivity obtained with BBT and *α*MB in PB-induced animals was similar to that obtained in non-induced animals (ie. PRF activity inhibited 20-40% more than ERF activity with no inhibition of ABP activity). In comparison, as seen in non-induced and *β*-NF-induced animals, ABT displayed low isozyme-selectivity (45-55% inhibition of each of the pulmonary activities at 7.5 *μ*mol/kg and >80% inhibition at 75 *μ*mol/kg) and limited tissue-selectivity.

To examine the tissue selectivity of the inhibitors more closely the inhibition of PRF activity in the liver and lung of PB-treated guinea pigs was compared (Fig. 4.7). At the lowest dose studied (0.075 *μ*mol/kg), *α*MB was highly selective for pulmonary vs hepatic P450 2Bx, inhibiting >80% of pulmonary PRF activity with no loss in hepatic PRF activity. At higher doses the magnitude of this selectivity decreased as more inhibition was noted in liver (>95% inactivation of PRF in both tissues at 75 *μ*mol/kg). BBT also inhibited pulmonary much more than hepatic PRF although the maximal difference, ~55% at 7.5 and 75 *μ*mol/kg, was less than that obtained with 0.075 *μ*mol/kg *α*MB. With ABT, hepatic and pulmonary PRF were inactivated almost equally at each dose.

The differences in tissue selectivity of these 3 compounds in PB-induced guinea pigs *in vivo* is attributed mainly to the marked differences in their abilities to inactivate pulmonary PRF. Both BBT and *α*MB are much more potent inhibitors of pulmonary PRF than is the parent compound, ABT (Fig.

Figure 4.7

Effects of αMB (A), BBT (B) or ABT (C), administered i.v., on hepatic and pulmonary PRF activity in PB-induced guinea pigs. Control (100%) values were: for liver 108 ± 23 (A), 125 ± 14 (B) and 193 ± 24 (C) pmol/min/mg protein; for lung 39.0 ± 4.5 (A), 59.9 ± 4.5 (B) and 52.6 ± 7.2 (C) pmol/min/mg protein. Data shown are means ± SE, N=4-5. *Significantly different ($P<0.05$) than corresponding vehicle-treated group.


A
B
C
Liver
Lung
% OF CONTROL PRF ACTIVITY
120
100
80
60
40
20
0
0
0.075
0.75
7.5
75
INHIBITOR DOSE (µmol/kg)

4.7). αMB inhibited greater than 80% of PRF activity at the lowest dose tested (0.075 μmol/kg) whereas 10-fold (0.75 μmol/kg) and 1000-fold (75 μmol/kg) doses of BBT and ABT, respectively, were required to produce equivalent amounts of inactivation. The marked differences in the abilities of these compounds to inhibit pulmonary PRF activity is not retained in the liver; αMB was only about 10-fold more potent than ABT (7.5 and 75 μmol/kg, respectively, for ~50% inactivation), and BBT did not cause more than 40% inactivation even at the highest dose examined (75 μmol/kg).

In the lung of non-induced and induced animals treated with ABT, the loss of spectrally assayed P450 content paralleled the inhibition of the monooxygenase activities (Figs. 4.1, 4.3 and 4.5). For example, in PB-induced animals 75 μmol/kg ABT inactivated ERF, PRF and ABP by >80% and caused a ~75% loss in P450 (Fig. 4.5). On the other hand, in BBT and αMB treated animals there was little loss in spectral P450 in lung at doses that inhibited most of the PRF and ERF activity (Figs. 4.1, 4.3 and 4.5). For example, in PB-induced animals 7.5 μmol/kg BBT inactivated ERF and PRF activities by ~60% and ~90%, respectively, with no apparent loss in P450 content (<15% loss) and 75 μmol/kg αMB inactivated both ERF and PRF activities by ~90% with <25% loss in P450 content (Fig. 4.5). Similar results were also obtained in the liver (Figs. 4.2, 4.4 and 4.6). For example, in PB-treated animals 75 μmol/kg αMB markedly inhibited ERF, PRF and ABP activities (>80%) in the liver with <15% loss in P450 content (Fig. 4.6).

Western blot analysis was conducted to determine if ABT, BBT or αMB

caused a loss of the P450 2Bx protein moiety in lung of PB-induced guinea pigs 4 hr after i.v. administration. For each inhibitor the lowest dose that inactivated pulmonary PRF activity in PB-induced animals by >80% was examined (0.075, 0.75 and 75 μmol/kg for αMB, BBT and ABT, respectively). BBT caused an apparent 42% loss in the amount of P450 2Bx protein without emergence of any other immunoreactive band on the Western blots (Fig. 4.8). In contrast, neither αMB (Fig. 4.9) nor ABT (Fig. 4.10) caused a significant loss in the amount of P450 2Bx protein (20% and 24% loss, respectively).

4.4 DISCUSSION

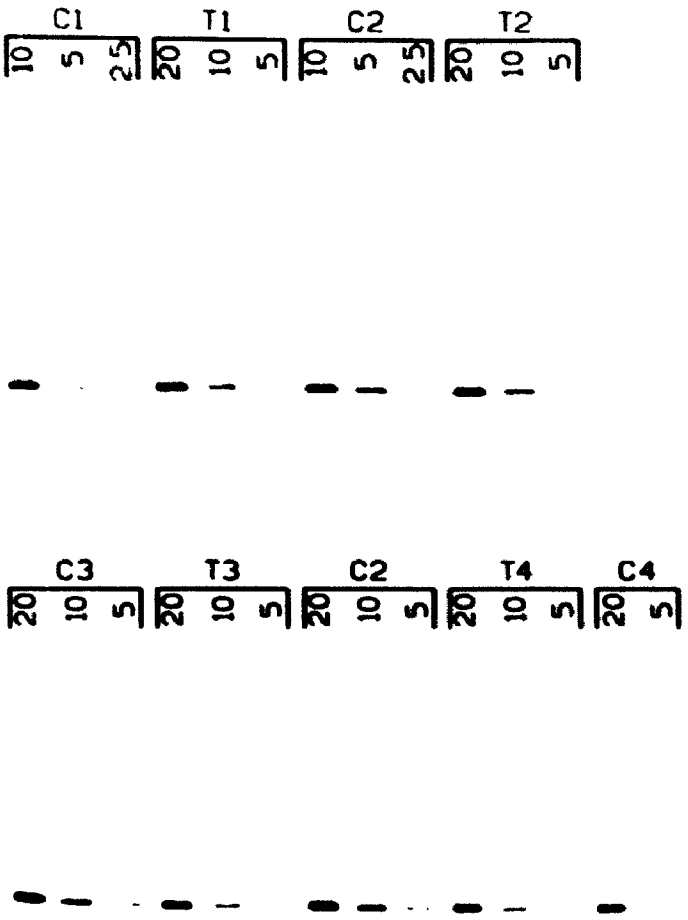
There are important differences between this study and earlier *in vivo* work in the rabbit (Mathews and Bend, 1993). First, the inactivation of the pulmonary P450 4B isozyme was examined in addition to pulmonary P450 2B and 1A isozymes. Second, PRF, a much more isozyme-selective monooxygenase activity for hepatic P450 2B4 than BND was used to monitor P450 2Bx-dependent activity. Third, multiple doses of each inhibitor were studied for their ability to inactivate P450 in an isozyme selective and tissue selective manner in non-induced, β-NF-induced and PB-induced guinea pigs. Finally, Western blotting with antibodies to P450 2B4 was used to analyze pulmonary microsomes for any changes in 2Bx protein content 4 hr after administration of the mechanism-based inhibitors.

BBT and αMB are suspected substrates for the facilitated uptake system for basic lipophilic amines in the lung (Orton *et al.*, 1973). Therefore, the

Figure 4.8

Effect of BBT (0.75 μmol/kg, i.v.) on the amount of pulmonary P450 2Bx apoprotein in PB-induced guinea pigs. A. Western blots of guinea pig pulmonary microsomes immunostained with antibodies to rabbit P450 2B4. C1-C4 indicate microsomes from 4 individual control (vehicle-treated) animals and T1-T4 indicate microsomes from 4 individual BBT-treated animals. The amount of microsomal protein loaded is indicated above each lane (2.5, 5, 10 or 20 μg). B. Quantitation of P450 2Bx apoprotein in pulmonary microsomes from control (C1-C4) and BBT-treated (T1-T4) animals based on densitometry of the above Western blots. Data shown are means ± SE. *Significantly different ($P<0.05$) than vehicle-treated control determined by unpaired Student's t-test.

A.


C1
10 5 25
T1
20 10 5
C2
10 5 25
T2
20 10 5
C3
20 10 5
T3
20 10 5
C2
20 10 5
T4
20 10 5
C4
20 5

B.

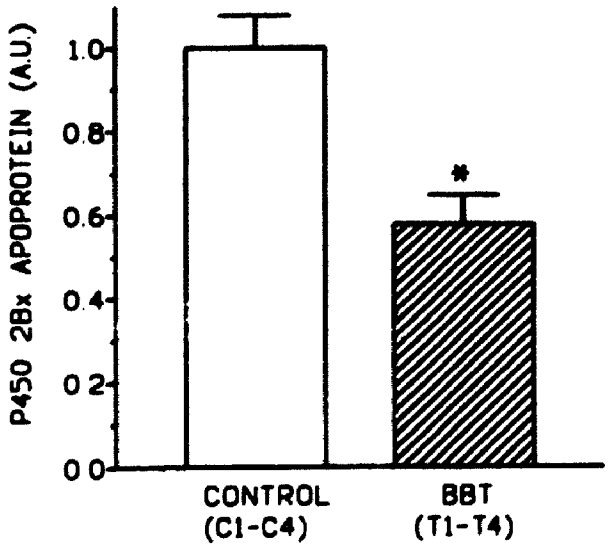
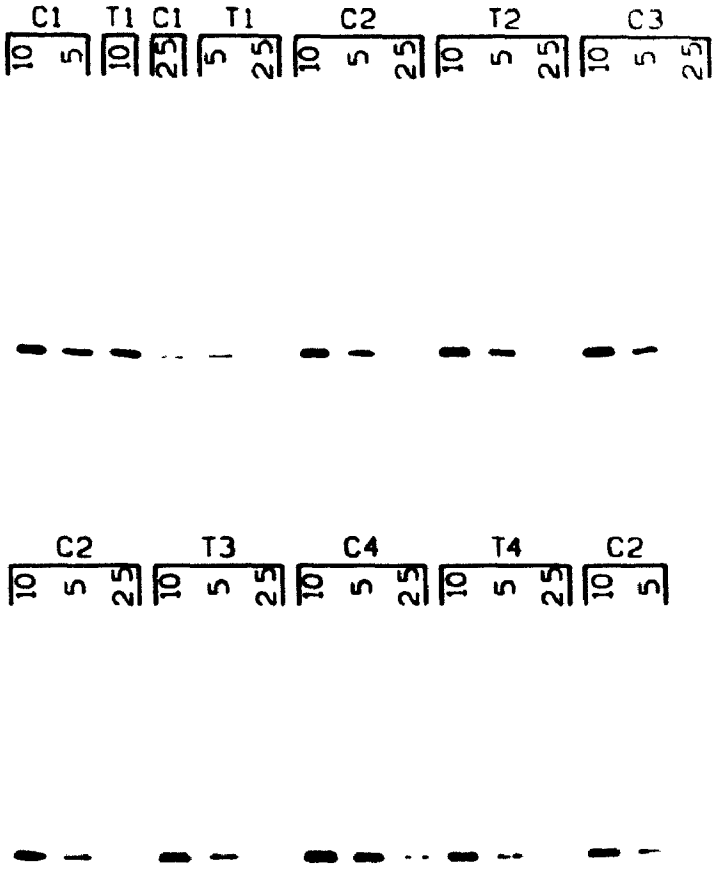

P450 2Bx APOPROTEIN (A.U.)
1.0
0.8
0.6
0.4
0.2
0.0
*
CONTROL
(C1-C4)
BBT
(T1-T4)

Figure 4.9

Effect of *o*MB (0.075 μmol/kg, i.v.) on the amount of pulmonary P450 2Bx apoprotein in PB-induced guinea pigs. A. Western blots of guinea pig pulmonary microsomes immunostained with antibodies to rabbit P450 2B4. C1-C4 indicate microsomes from 4 individual control (vehicle-treated) animals and T1-T4 indicate microsomes from 4 individual *o*MB-treated animals. The amount of microsomal protein loaded is indicated above each lane (2.5, 5, 10 or 20 μg). B. Quantitation of P450 2Bx apoprotein in pulmonary microsomes from control (C1-C4) and *o*MB-treated (T1-T4) animals based on densitometry of the above Western blots. Data shown are means ± SE.

A.


C1 T1 C1 T1 C2 T2 C3
10 5 10 25 5 25 10 5 25 10 5 25 10 5 25
C2 T3 C4 T4 C2
10 5 25 10 5 25 10 5 25 10 5 25 10 5

B.

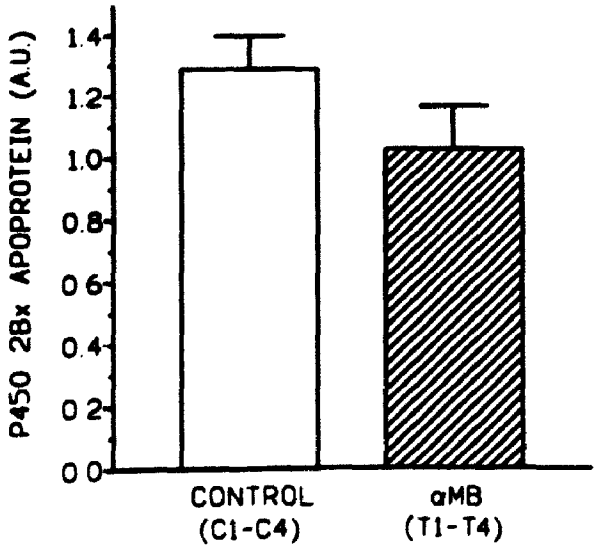
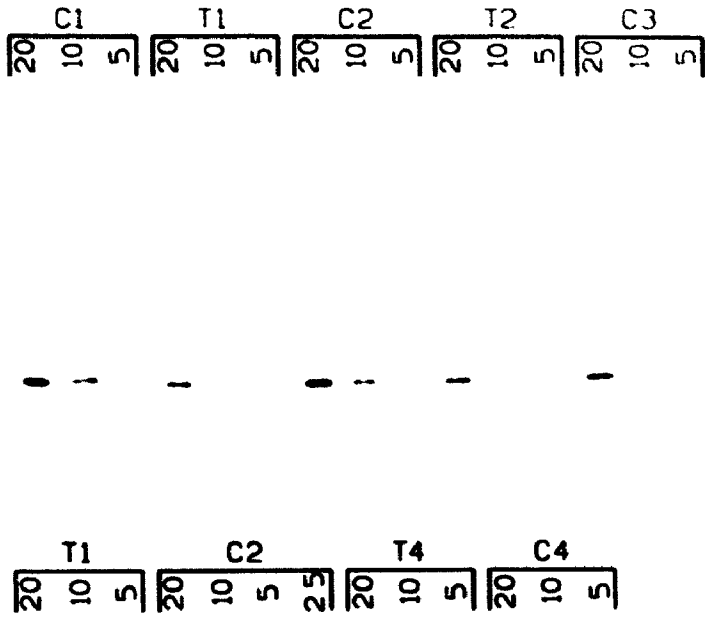

P450 28x APOPROTEIN (A.U.)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
CONTROL
(C1-C4)
αMB
(T1-T4)

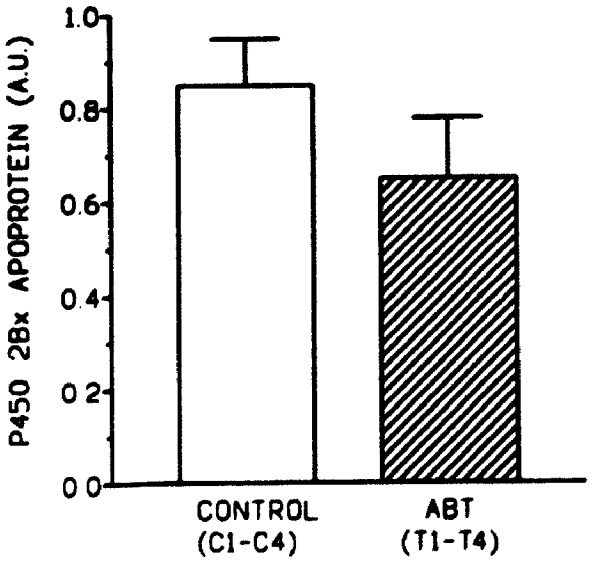
Figure 4.10

Effect of ABT (75 μmol/kg, i.v.) on the amount of pulmonary P450 2Bx apoprotein in PB-induced guinea pigs. A. Western blots of guinea pig pulmonary microsomes immunostained with antibodies to rabbit P450 2B4. C1-C4 indicate microsomes from 4 individual control (vehicle-treated) animals and T1-T4 indicate microsomes from 4 individual ABT-treated animals. The amount of microsomal protein loaded is indicated above each lane (2.5, 5, 10 or 20 μg). B. Quantitation of P450 2Bx apoprotein in pulmonary microsomes from control (C1-C4) and ABT-treated (T1-T4) animals based on densitometry of the above Western blots. Data shown are means ± SE.

A.


C1
T1
C2
T2
C3
20
10
5
T1
C2
T4
C4
20
10
5
25

B.


P450 2Bx APOPROTEIN (A.U.)
1.0
0.8
0.6
0.4
0.2
0.0
CONTROL
(C1-C4)
ABT
(T1-T4)

mechanism-based inhibitors were administered i.v. (jugular vein) to allow access to this accumulation system before reaching the liver. In preliminary experiments it was found that the anesthetic, urethane, and the drug vehicle had no effect on the P450 monooxygenase activities monitored in this study. In other preliminary experiments it was found that the maximal loss in monooxygenase activities occurred within 1 hr after their administration and persisted for at least 4 hr. Here the effects of these mechanism-based inhibitors on the monooxygenase activities were determined 4 hr after injection. In the previous rabbit study monooxygenase activities were monitored 1 hr after inhibitor administration (Mathews and Bend, 1993).

Both BBT and αMB inhibited the pulmonary monooxygenase system in an isozyme-selective manner with αMB displaying greater selectivity. In all cases P450 2Bx was inhibited more than P450 1A1, and P450 4Bx was virtually resistant to inactivation. In non-induced and PB-induced animals, at each dose of αMB (0.075-7.5 μmol/kg) PRF activity was inhibited 20-40% more than ERF activity with greater than 80% inhibition of PRF activity. In β-NF-induced guinea pigs αMB inhibited the pulmonary monooxygenase system in an isozyme-specific manner; the lowest dose studied inhibited 80% of the P450 2Bx catalyzed PRF activity whereas 100-fold higher doses did not inactivate either P450 4Bx catalyzed ABP activity or P450 1A1 catalyzed ERF activity (under conditions where the PRF activity was totally inhibited). In contrast, ABT inhibited the pulmonary monooxygenase system in non-induced and induced guinea pigs with little isozyme-selectivity.

The higher isozyme-selectivity of *α*MB and BBT in lung of *β*-NF-induced guinea pigs, compared to non-induced and PB-induced animals, is due to a decrease in the degree of inhibition of pulmonary ERF activity in *β*-NF-induced animals. Several factors may contribute to this decrease in ERF inactivation. 1) Pulmonary endothelial cells contain P450 1A1, which is induced by *β*-NF-treatment, but no or very little NADPH-P450 reductase (Overby *et al.*, 1992). Therefore, in *β*-NF-induced guinea pigs it is possible that a greater percentage of the 1A1 may not be coupled to reductase *in vivo* due to cellular localization and integrity. Thus, the endothelial P450 might be catalytically inactive and immune to suicidal inactivation *in vivo*, but may be coupled to reductase in microsomes prepared from total lung *in vitro* where ERF activity is determined. 2) BBT and *α*MB are suspected substrates for the facilitated uptake system for basic lipophilic amines located in the lung (Orton *et al.*, 1973). Like other uptake systems in lung, this system is probably differentially distributed between cell types. It is possible that it is absent in some cell types where P450 1A1 levels are induced. This would also explain why ERF activity is inhibited less in *β*-NF-induced animals than non-induced or PB-induced animals by BBT and *α*MB but not ABT because the latter is not a substrate for this accumulation system (Mathews *et al.*, 1985). Differences in the cellular localization of P450 1A1 and P450 2Bx, and limiting P450 reductase and/or absence of the uptake system in some pulmonary cell types containing 1A1, may also explain why P450 2Bx catalyzed PRF activity is virtually abolished in the lung of non-induced animals by *α*MB and BBT whereas the inhibition of ERF

activity plateaued at about 60%. 3) However, cellular localization of induced P450 1A1 probably does not account for the entire decrease in ERF inactivation in lung of β-NF-induced compared to non-induced animals because ERF activity is also inhibited less in pulmonary microsomes from β-NF induced vs non-induced guinea pigs incubated with BBT or αMB *in vitro* (Woodcroft *et al.*, 1990). It is possible that P450 1A1 catalyzes the conversion of BBT and αMB to metabolites incapable or less capable of inactivating P450. (Preliminary studies with radiolabelled BBT suggest that this is the case; K.J. Woodcroft and J.R. Bend, unpublished data). Thus, more of the inhibitor is metabolized to suicidally inactive (or less active) products in lungs of β-NF-induced vs non-induced or PB-induced animals. The efficient conversion of BBT and αMB to suicidally inactive (or less active) metabolites by 1A1 could also explain the decrease in inactivation of hepatic P450 in β-NF-induced vs non-induced guinea pigs observed both *in vivo* (this study) and *in vitro* (Woodcroft and Bend, 1990) as β-NF also induces P450 1A1 (as well as P450 1A2) in liver.

Both αMB and BBT also inactivated P450 in a tissue-selective manner; in each case at least one of the doses administered caused marked inactivation of pulmonary P450 2Bx without inhibiting the hepatic monooxygenase activities examined. For example, in non-indu ed and induced animals the lowest dose of αMB (0.075 μmol/kg) inactivated pulmonary PRF activity by >80% with no inhibition of hepatic PRF, ERF, ABP or END activity and in fact, even a 100-fold higher dose (7.5 μmol/kg) did not inhibit any of these hepatic activities to this extent (>80%). With BBT, 50-70% of the pulmonary P450

2Bx could be inactivated with no apparent inhibition of the hepatic P450 system. At the higher doses of inhibitor studied (7.5-75 μmol/kg) generally one or more of the hepatic monooxygenases was inhibited by >50%. For example, in PB-induced animals 75 μmol/kg αMB inactivated hepatic ABP, ERF and PRF activities by >80%. This is of significance because it demonstrates that the selectivity of BBT and αMB for pulmonary 2Bx is not due to the inability of these compounds to inhibit 2Bx and other isozymes in the liver.

To examine the tissue selectivity of the inhibitors more closely we compared the inhibition of PRF activity in the liver and lung of PB-induced guinea pigs, a situation where this activity is almost totally catalyzed by the same P450 isozyme in the two tissues, P450 2Bx. At lower doses (0.075-7.5 μmol/kg) both BBT and αMB are highly selective for the inactivation of pulmonary vs hepatic P450 2Bx *in vivo*. The highest selectivity was obtained with 0.075 μmol/kg αMB, which inhibited pulmonary PRF activity by more than 80% with no loss in hepatic PRF activity. In marked contrast hepatic and pulmonary PRF are inactivated almost equally at each dose by ABT. These differences in tissue selectivity of P450 inhibition between the lipophilic, N-aralkylated derivatives of ABT and the parent compound are mainly due to marked differences in their ability to inhibit pulmonary P450 2Bx; αMB and BBT are about 1000-fold and 100-fold more potent, respectively, than ABT at inhibiting pulmonary PRF activity. On the other hand, all three compounds are about equipotent at inhibiting hepatic PRF activity. This is consistent with accumulation of αMB and BBT, but not ABT, in lung.

BBT and *α*MB inactivated the pulmonary and hepatic monooxygenase systems with very little loss of spectral P450 content whereas with ABT, the loss of spectral P450 corresponds quite well with the loss of the monooxygenase activities. This was also seen in pulmonary (Mathews and Bend, 1986; Woodcroft *et al.*, 1990) and hepatic microsomes (Woodcroft and Bend, 1990) of the rabbit and guinea pig *in vitro*. This suggests that BBT and *α*MB function as mechanism-based inhibitors by an alternative or additional mechanism(s) to benzyne-mediated alteration of protoporphyrin IX known for ABT (Ortiz de Montellano and Mathews, 1981; Ortiz de Montellano *et al.*, 1984). Recent *in vitro* studies conducted in our laboratory have indicated that these derivatives may inactivate P450 by covalent modification of the protein and/or MI-complex formation (Section 1.3.3). It is unlikely, however, that MI-complex formation accounts for the majority of the P450 inactivation in these studies. Firstly, MI-complex formation, like protoporphyrin alkylation, would result in a loss in spectral P450. Secondly, no MI-complex was detectable in liver microsomes from PB-induced guinea pigs treated with 75 μmol/kg *α*MB (C.J. Sinal and J.R. Bend, unpublished data), even though PRF activity was inhibited by >90%.

Some mechanism-based inhibitors not only inactivate P450, but also result in a loss of the P450 protein moiety from endoplasmic reticulum. The loss of apoprotein can be both inhibitor and isozyme specific. For example, 3,5-dicarbethoxy-2,6-dimethyl-4-ethyl-1,4-dihydropyridine inactivates P450 2B, 2C11, 3A and 2C6 isozymes by about the same amount but only causes a loss

in the 2C11 and 3A apoproteins whereas AIA inactivates P450 3A without apoprotein loss but causes a loss in P450 2C6 apoprotein (Correia *et al.*, 1992b). In this study we analyzed for the loss of pulmonary P450 2Bx by ABT, BBT and *α*MB, at doses that caused comparable inactivation of pulmonary PRF activity (83-88% loss) and found that BBT, but not *α*MB or ABT, caused a significant loss of the P450 2Bx protein moiety in lung by 4 hr after treatment. For comparison, a previous study showed that ABT inactivates hepatic P450 2E1 in acetone-pretreated mice within 1 hr following i.p. administration with no increased loss, compared to vehicle-treated controls, in 2E1 protein for at least 9 hr following administration (Tierney *et al.*, 1992). The structural modification(s) that targets BBT inactivated P450 2Bx in lung for rapid proteolysis is not known although it must involve a reactive metabolite that is formed from BBT and not ABT. If the same metabolite is formed from *α*MB it is presumably at a lower concentration than from BBT.

In summary, we have shown that BBT and *α*MB, at appropriate low doses, are isozyme-selective/specific (P450 2Bx) and lung-specific mechanism-based inhibitors of the P450 monooxygenase system in guinea pig *in vivo*.

CHAPTER 5

SUBACUTE EFFECTS OF N-BENZYL-1-AMINOBENZOTRIAZOLE ON CYTOCHROME P-450

5.1 OBJECTIVES

The objectives of this study were to determine the duration of the inactivation of pulmonary PRF activity following a single dose of BBT (0.75 μmol/kg, i.v.) and if isozyme-selective induction of the pulmonary (vs hepatic) P450 system occurs.

5.2 MATERIALS AND METHODS

5.2.1 Reagents

Methoxyflurane (Metofane) was obtained from M.T.C. Pharmaceuticals (Mississauga, Ont.). The sources of the other chemicals used are given in Sections 3.2.1 and 4.2.1.

5.2.2 Animal Treatment

Male Hartley guinea pigs (300-375 g) were obtained from Charles River Ltd. (St. Constant, Que.). They were housed as indicated in Section 3.2.1.

The animals were anesthetized using the inhalable anesthetic, methoxyflurane. The jugular vein was exposed using sterile surgical techniques and BBT (in 5% BSA/DMSO/Emulphor, 6/0.15/0.3; 0.75 μmol/kg) or the vehicle was injected into the vein (2.15 ml/kg). The incision was closed with

3-4 stitches and the animal was removed from the methoxyflurane-containing nose cone. All animals recovered from the anesthetic within 30 min. The animals (4 vehicle and 4 BBT treated) were sacrificed at 6, 12, 18, 24, 48 or 96 hr after i.v. injection. The lungs and livers were removed and stored at -80°C until preparation of microsomes (within 1 week).

5.2.3 Preparation of Microsomes

Washed hepatic and pulmonary microsomes were prepared from individual tissues by differential centrifugation as described previously (Section 3.2.3) and protein concentrations were determined by the method of Lowry *et al.*, 1951 (Section 3.2.4).

5.2.4 Determination of Isozyme Selective/Specific Cytochrome P450 Monooxygenase Activities

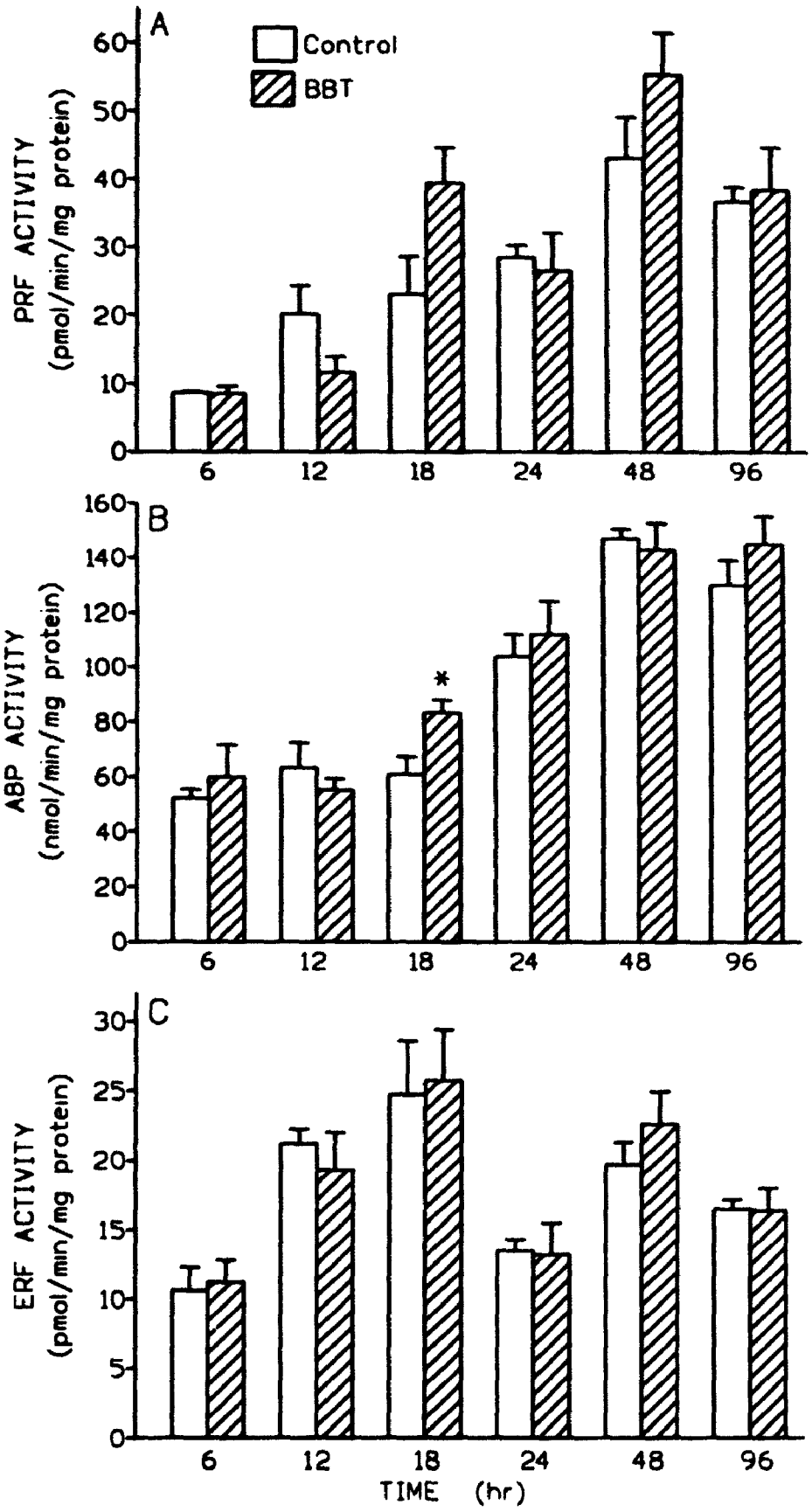
PRF and ERF activities were determined by measuring the formation of the fluorescent product, resorufin, as previously described (Section 3.2.6). ABP activity was assayed colorimetrically as described in Section 3.2.7.

5.3 RESULTS

The effects of BBT, 0.75 μmol/kg i.v., on the pulmonary isozyme selective/specific monooxygenase activities are shown in Fig. 5.1. There was no significant difference in pulmonary PRF activity in BBT-treated animals compared to the time-matched control (vehicle-treated) animals at any of the

Figure 5.1

Effects of BBT on pulmonary PRF (A), ABP (B) and ERF (C) activities 4-96 hr following i.v. administration. Both control (vehicle-treated) and BBT-treated animals received methoxyflurane as an anesthetic. Data shown are means ± SE, N=4. *Significantly different ($P<0.05$) than control determined by unpaired Student's t-test.


A
Control
BBT
PRF ACTIVITY
(pmol/min/mg protein)
60
50
40
30
20
10
0
6
12
18
24
48
96
B
ABP ACTIVITY
(nmol/min/mg protein)
160
140
120
100
80
60
40
20
0
*
C
ERF ACTIVITY
(pmol/min/mg protein)
30
25
20
15
10
5
0
TIME (hr)

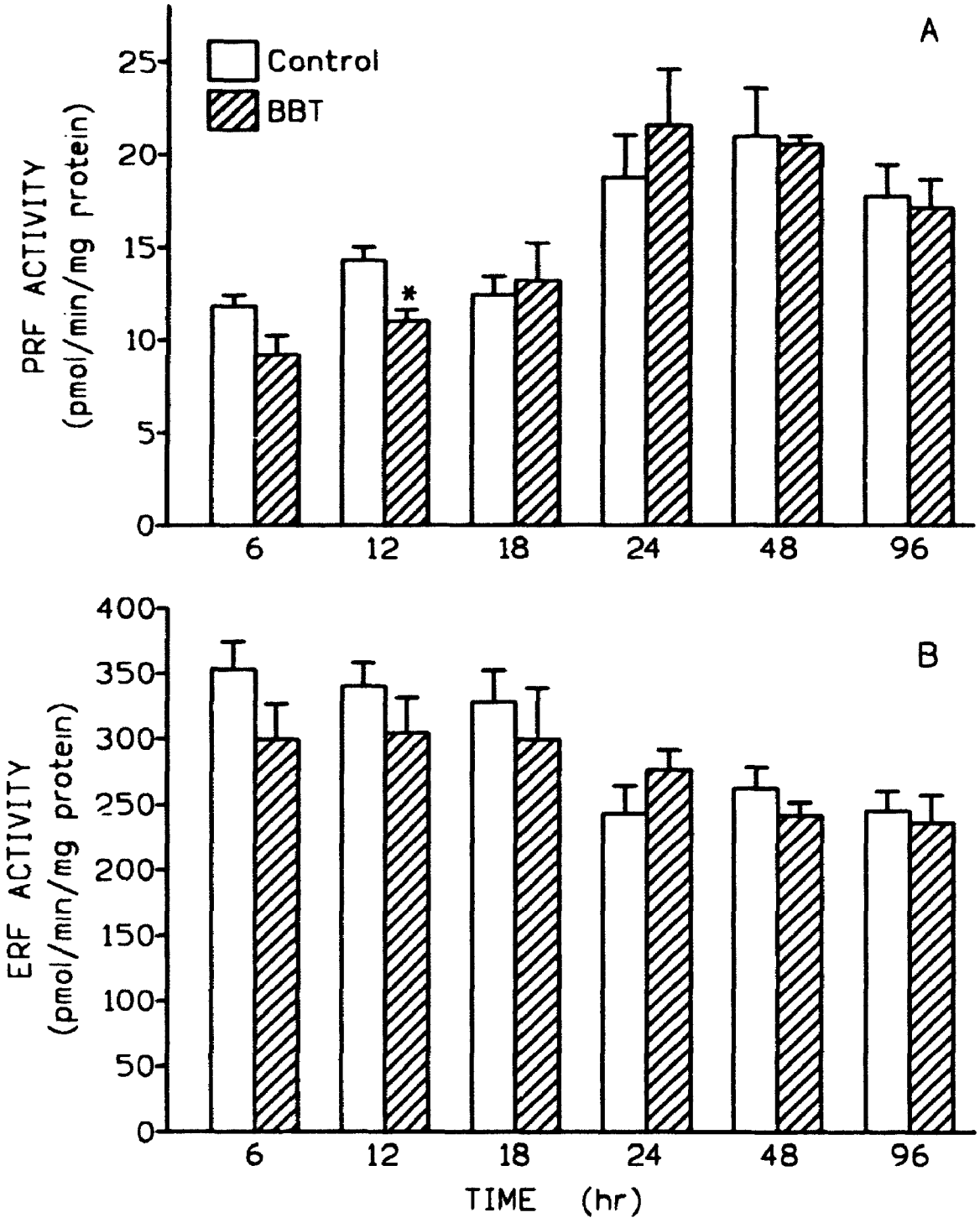
times examined (6, 12, 18, 24, 48 or 96 hr). Interestingly, pulmonary PRF activity was also inhibited in the vehicle-treated animals in a time-dependent manner. For example, the 6 hr control value was significantly different (lower) than the 18, 24, 48 or 96 hr values, and the 48 hr control value was significantly different (higher) than the 6, 12, 18 or 24 hr values ($P<0.05$, one way ANOVA followed by Newman Keul's test). In fact, the highest specific activity (48 hr) was more than 4-fold greater than the lowest activity (6 hr).

Variation in pulmonary ABP and ERF activities amongst the vehicle-treated groups was also evident ($P<0.05$, ANOVA). With ABP, specific activity appeared to increase with time and plateau at 48 hr. Similar to PRF activity, the highest activity (48 hr) was ~3-fold greater than the lowest activity (6 hr). ERF activity varied ~2-fold but did not appear to follow any trend. The only significant difference in any of these pulmonary monooxygenase activities between BBT-treated and the appropriately timed control animals was at 18 hr where a small increase (~35%) in ABP activity was apparent.

The effects of BBT on PRF and ERF activities in liver of these same animals are shown in Fig. 5.2. Both PRF and ERF activities varied amongst the vehicle-treated groups ($P<0.05$, ANOVA). PRF activity appeared to increase with time, similar to the pulmonary data, plateauing at 24 hr (~2-fold increase overall) whereas ERF activity was higher (~1.4-fold) at the earlier times (6-18 hr) than the later times (24-96 hr). The only significant difference in hepatic PRF or ERF activity between BBT-treated and the appropriately timed control

Figure 5.2

Effects of BBT on hepatic PRF (A) and ERF (B) activities 4-96 hr following i.v. administration. Both control (vehicle-treated) and BBT-treated animals received methoxyflurane as an anesthetic. Data shown are means ± SE, N = 4. *Significantly different ($P < 0.05$) than vehicle-treated control determined by unpaired Student's t-test.


A
Control
BBT
PRF ACTIVITY
(pmol/min/mg protein)
25
20
15
10
5
0
*
6
12
18
24
48
96
B
ERF ACTIVITY
(pmol/min/mg protein)
400
350
300
250
200
150
100
50
0
6
12
18
24
48
96
TIME (hr)

animals was at 12 hr where a small decrease (~25%) in PRF activity was apparent.

5.4 DISCUSSION

In vivo some mechanism-based inhibitors not only inactivate P450, but also cause P450 induction. This includes both reversible inhibitors, such as dihydrosafrole (Murray *et al.*, 1983), erythromycin (Danan *et al.*, 1981), piperonyl butoxide (Kamienski and Murphy, 1971), SKF-525A (Buening et al., 1976) and troleandomycin (Passayre *et al.*, 1981), as well as irreversible inhibitors such as diallyl sulfide (Brady *et al.*, 1990b; Pan *et al.*, 1993), 8-methoxyporsalen (Fouin-Fortunet *et al.*, 1985; Apseloff *et al.*, 1991) and AIA (DeMatteis, 1973; Srivastava *et al.*, 1989). The effects on the hepatic P450 monooxygenase system after a single dose of dihydrosafrole (Murray *et al.*, 1983), piperonyl butoxide (Kamienski and Murphy, 1971) or AIA (DeMatteis, 1970) have been shown to be biphasic with acute inhibition followed by induction. With AIA, the increase in P450 2B1/2 in rat is tissue selective; an increase in 2B1/2 mRNA is seen in both liver and kidney but not in the lung (Srivastava *et al.*, 1989).

A previous study showed that ABT, administered as single or multiple i.p. injections, inactivates the rat hepatic P450 system but does not induce this system (Ortiz de Montellano and Costa, 1986). The study presented here examines the time course of pulmonary P450 inactivation by a single dose of BBT in guinea pigs and the possibility of isozyme-selective induction of P450

in lung vs liver. The dose of BBT chosen for this study, 0.75 μmol/kg, was that which caused the greatest isozyme (2Bx) and lung (vs liver) selectivity 4 hr after i.v. administration inactivating pulmonary P450 2Bx and 1A1 by 75% and 35% respectively, with little effect on pulmonary P450 4Bx or hepatic P450 activities (Chapter 4; Fig. 4.1 and 4.2). If BBT is used as an *in vivo* probe to determine the role of pulmonary 2Bx in the metabolism of a particular compound this is the dose that would likely be administered. A previous study in which ^{14}C-labelled BBT (0.75 μmol/kg) was administered i.v. to guinea pigs showed that 70%-75% of the radioactivity was excreted in the urine within 12 hr and after 48 hr, little radioactivity was found in either liver (<1% of administered ^{14}C) or lung (<0.1%; K.J. Woodcroft and J.R. Bend, unpublished data). Therefore, in this study times ranging from 6 hr to 96 hr were examined.

In this study no inactivation of pulmonary P450 2Bx, 4Bx or 1A1 was evident in BBT-treated guinea pigs compared to appropriately timed vehicle-treated animals 6-96 hr after administration. Interesting, the time between the injection and sacrifice affected these activities. Marked differences were seen in each of the isozyme selective/specific monooxygenase activities (>4-fold with PRF activity and >3-fold with ABP activity) amongst the vehicle-treated groups. With both pulmonary PRF and ABP activity, a time-dependent increase in activity was apparent. In both cases, this was likely not due to induction at the latter time points but due to inhibition of these activities at the earlier time points. For example, at 6 hr the mean PRF activity was 8.6 pmol/min/mg

protein with individual values ranging from 8.1 to 8.8 pmol/min/mg protein whereas the means for vehicle-treated animals (no inducer) in the 4 hr i.v. study (Chapter 4) and 24 hr i.p. study (Chapter 3) were 40.7, 29.8 and 50.0 pmol/min/mg protein with individual values ranging from 16.3 to 65.5 pmol/min/mg protein. With ABP activity, at 6 hr the mean activity was 52 nmol/min/mg protein with individual values ranging from 43 to 58 nmol/min/mg protein whereas the means from the vehicle-treated animals in the previous chapters (Chapters 3 and 4) were 267, 170 and 268 nmol/min/mg protein with individual values ranging from 104 to 308 nmol/min/mg protein. In both cases the mean value obtained in this study at 6 hr was ~50% of the lowest activity obtained from an individual vehicle-treated animal in the previous studies. Variation in P450-dependent monooxygenase activities amongst the vehicle-treated groups were also seen in the liver although the magnitude of the variation was much less in this organ (<2-fold).

The variation in the pulmonary monooxygenase activities seen among the vehicle-treated groups in this study is almost certainly due to the anesthetic, methoxyflurane because the surgical procedure and vehicle used in this study had no effect of these activities 4 hr following i.v. administration in guinea pigs anesthetized with urethane. Guinea pigs require an anesthetic for i.v. injection because all of their superficial veins collapse very easily and therefore an incision is required. However, urethane is a non-recoverable anesthetic and for long term studies it is necessary to employ a recoverable anesthetic. Methoxyflurane was chosen for these studies because it is an

effective anesthetic, even though it is known to be O-demethylated by P450. In fact, both P450 2B4 and P450 4B1 are known to contribute to the metabolism of methoxyflurane in rabbit pulmonary microsomes (Waskell *et al.*, 1986). However, the anesthetic did not interfere with the ability to demonstrate that BBT, at least at the low dose tested here, did not cause induction of P450 2Bx in guinea pig lung up to 96 hr after administration.

Unfortunately, due to the variation in pulmonary PRF activities amongst control groups we are unable to conclude if the inactivation of pulmonary 2Bx by BBT, which requires metabolism, persists for less than 6 hr following i.v. administration or if the BBT is not metabolized by pulmonary 2Bx in these anesthetized animals due in previous inhibition of this isozyme by another mechanism. It appears unlikely that BBT directly causes marked isozyme-selective induction in the lung although the possibility of rebound induction in response to inactivation cannot be ruled out. Perhaps in this study methoxyflurane is present in the pulmonary microsomes at the earlier times resulting in competitive/noncompetitive inhibition, or methoxyflurane has inactivated these isozymes *in vivo* by an unknown mechanism.

We are unaware of a recoverable anesthetic that is not metabolized by P450 or does not alter P450 monooxygenase activities. Therefore we feel that for long-term studies such as the one presented here it is necessary to surgically implant a catheter into the vein of the anesthetized guinea pig and then to allow the animal to recover for several days before injecting the vehicle/inhibitor.

CHAPTER 6

METABOLISM OF ARACHIDONIC ACID BY PULMONARY CYTOCHROME P450

6.1 OBJECTIVE

The primary objective of this study was to characterize the P450 metabolites of AA by class in guinea pig lung microsomes and to elucidate the role of each of the three P450 isozymes known to be important constituents of guinea pig lung (1A1, 2Bx and 4Bx) in the formation of AA metabolites.

6.2 MATERIALS AND METHODS

6.2.1 Reagents

[1-^{14}C]AA (50-60 mCi/mmol, >99% radiochemical purity) was purchased from Amersham Canada Ltd. (Oakville, Ont.); AA, diethylene-triaminepentaacetic acid (DETAPAC), metyrapone, nordihydroguaiaretic acid (NDGA), 5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium from the Sigma Chemical Co. (St. Louis, MO); α-naphthoflavone (α-NF) from the Aldrich Chemical Co. (Milwaukee, WI); goat anti-rabbit IgG (H + L) alkaline phosphatase conjugate from BIO-RAD Laboratories Ltd. (Mississauga, Ont.); and SKF-525A from Biomol Research Lab. Inc. (Plymouth Meeting, PA). The sources of other chemicals are given in Sections 3.2.1 and 4.2.1. Goat antibodies to rabbit P450 2B4 and P450 4B1, and rabbit antibodies to mouse rabbit P450 2A4/2A5 were generously provided by Drs. R.M. Philpot and M.

Negishi, respectively, of the National Institute of Environmental Health Sciences, NIH (Research Triangle Park, NC). Recombinant yeast expressing mouse P450 2A4 were also provided by Dr. M. Negishi.

6.2.2 Animal Treatment

Male Hartley guinea pigs (300-375 g) were obtained from Charles River Ltd. (St. Constant, Que.) and housed as indicated in Section 3.2.2.

Some guinea pigs were treated i.p. with 80 mg/kg β-NF (2% in corn oil) or 80 mg/kg PB (2% in saline) daily for 4 days. In experiments studying the ability of the mechanism-based inhibitors to inactivate the pulmonary and/or hepatic system *in vivo*, the animals were treated exactly as outlined in Section 4.2.2 and sacrificed 4 hr after the inhibitor was administered. The lungs and livers were removed immediately and stored at -80°C.

6.2.3 Preparation of Microsomes

Washed pulmonary and hepatic microsomes were prepared by differential centrifugation (Section 3.2.3) and protein concentrations were determined by the method of Lowry *et al.*, 1951 (Section 3.2.4).

6.2.4 Determination of Isozyme Selective/Specific Monooxygenase Activities

ERF, PRF, ABP and END activities were determined as previously described (Sections 3.2.6 to 3.2.8).

6.2.5 Determination of Cytochrome P450-Dependent Arachidonic Acid Metabolism

For determination of P450-dependent AA metabolism microsomal protein (1 mg/ml pulmonary or 0.25 mg/ml hepatic) was incubated with 100 μM of [1-^{14}C-AA] at 37°C for 5 min in 0.1 M potassium phosphate buffer, pH 7.4 in the presence of 1 mM NADPH. The reaction, with a final volume of 1 ml, was set up and carried out as follows. Unlabelled and radiolabelled AA, prepared as stock solutions of 1 mg/ml ethanol and 20 μCi/ml toluene, respectively, were added in the reaction vial to give 0.5 μCi (pulmonary microsomes) or 0.1 μCi (hepatic microsomes) of radioactivity and 0.1 μmol of AA. The ^{14}C-AA was analyzed before use by the HPLC system described below to ensure >99% purity. The solvents were removed from the reaction vial under a stream of N_2. Sodium bicarbonate (5 μl of 20 mM) was added to the vial. Microsomal protein (1 mg pulmonary or 0.25 mg hepatic), 200 μl of 0.5 M potassium phosphate buffer (pH 7.4), and water (to give a final reaction volume of 1 ml) were added. The mixture was preincubated at 37°C for 5 min and then the reaction was initiated with 1 mM NADPH (final concentration). After a further 5 min at 37°C the reaction was terminated by the addition of 50 μl of 1 N HCl and 1 ml of ethyl acetate containing 0.01% butylated hydroxytoluene. The mixture was transferred to a test tube, mixed by vortexing, and then centrifuged at 2000 rpm for 5 min. The upper organic phase was removed and the aqueous phase was extracted 2 more times with ethyl acetate containing 1% butylated hydroxytoluene. The organic phases were combined, dried under

a stream of N_2 and then reconstituted in 100 µl of ethanol. Some incubations were carried out in the absence of NADPH to determine NADPH-independent metabolism.

The extracted metabolites were separated by HPLC on ε Waters 5 µm Resolve C18 column (8 X 100 mm; Waters, Mississauga, Ont.) by a modification of the method of Capdevila *et al.* (1981, 1990b) 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 quantitation of cyclooxygenase and lipooxygenase metabolites in addition to P450 metabolites. The injection volumes were 80 and 45 µl for hepatic and pulmonary samples, respectively. Radioactivity was monitored by an on-line flow through radiochemical detector (Beckman Model 171; Beckman (Canada) Inc., Mississauga, Ont.). Initially, the metabolite classes were identified based on their retention times in the HPLC system described by Capdevila and coworkers (Capdevila *et al.*, 1990b; Falck *et al.*, 1990). Resolution of aliquots of the same reaction mixtures on the modified system provided the retention times in this system. Moreover, the retention times of radiolabelled 5,6-, 8,9-, 11,12- and 14,15-EET, synthesized (Fitzpatrick *et al.*, 1986) and separated (Oliw and Moldeus, 1982; Turk *et al.*, 1990) in our laboratory by C. Webb, were determined in our modified system.

For determination of the regioisomeric composition of the EETs, fractions containing the EETs were collected from the above HPLC separation and

pooled. The acetonitrile was removed under N_2 and 2 ml of water was added. The aqueous phase was extracted 3 times with equivolumes 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°C. Immediately prior to the separation of the regioisomers aliquots were taken to dryness under N_2 and 3% isopropyl alcohol in hexane containing 0.1% acetic acid was added. Regioisomers of the EETs were separated isocratically by HPLC on a Waters 10 μm radial-pak μPorasil column (8 X 100 mm; Waters, Mississauga, Ont.) with a mobile phase of 0.35% isopropyl alcohol in hexane containing 0.1% acetic acid at a flow rate of 1 ml/min (Turk *et al.*, 1990). Radioactivity was monitored by an on-line flow through radiochemical detector (Beckman Model 171; Beckman (Canada) Inc., Mississauga, Ont.). The regioisomers were identified based on the retention times of synthetic radiolabelled EETs (35.8, 39.4, 53.6 and 77.2 min for 14,15-, 11,12-, 8,9- and 5,6-EET, respectively).

Where indicated, 10^{-5} M SKF-525A, 10^{-5} M metyrapone or 3×10^{-7} M α-NF was present in the incubations with NADPH. Metyrapone (10^{-4} M stock solution in ethanol) was added to the reaction vial containing AA. The ethanol was removed under a stream of N_2 before addition of the microsomes. α-NF (1.2×10^{-5} M stock solution in ethanol) was added to a test tube and the solvent removed under a stream of N_2. Microsomal protein was added to the test tube and incubated with the α-NF (on ice) for at least 30 min before the addition of the protein to the reaction vial containing AA. SKF-525A (10^{-4} M

stock solution in water) was added to the reaction vial before addition of the microsomes. In some cases the microsomal protein was preincubated with 10^{-5} M SKF-525A and 1 mM NADPH in 0.1 M potassium phosphate buffer (pH 7.4) for 5 min at 37°C, and 1 ml of this mixture was added to the vial containing AA (at 37°C) to start the reaction.

Where indicated, 10^{-5} M indomethacin, 10^{-5} M NDGA, 1 mM DETAPAC, or 1 mM DETAPAC and 0.1 mM H_2O_2, was present in the incubations without NADPH. The indomethacin, NDGA and DETAPAC were prepared as stock solutions in acetone so that 5 μl was added to the reaction vial. This volume of acetone had no effect on the NADPH-independent HPLC chromatogram.

6.2.6 *In Vitro* Incubations with 1-Aminobenzotriazole and its N-Aralkylated Derivatives

Microsomal protein (~11 mg) was incubated with 1 mM NADPH (no NADPH in controls) and various concentrations of ABT, BBT and αMB (no inhibitor in controls) at 37°C for 45 min as previously described (Woodcroft *et al.*, 1990). The inhibitors were dissolved in methanol and added to the reaction vial. The methanol was removed under a gentle stream of N_2 before addition of the other incubation components. The final reaction volume was 2 ml. After 45 min at 37°C the incubation was cooled on ice and the microsomal protein was sedimented by centrifugation at 400,000 g (100,000 rpm in a Beckman TL100.3 rotor) for 15 min at 4°C. To remove excess inhibitor, the pellet was resuspended in ice cold 0.1 M potassium phosphate

buffer, pH 7.4, using a hand-held Potter-Elvehjem homogenizer and then recentrifuged. The resulting microsomal pellet was resuspended in 1.5 ml of 0.1 M potassium phosphate buffer, pH 7.4, and the protein concentration of this suspension was determined by the method of Lowry *et al.*, 1951 (Section 3.2.4). Aliquots of the microsomal suspensions were stored at -80°C until ERF, PRF and ABP activities, and AA metabolism were determined.

6.2.7 Inhibition of Cytochrome P450 with Antibodies

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 (Serabjit-Singh *et al.*, 1979).

6.2.8 Western Blot Analysis

Microsomal protein was electrophoresed on a 7.5% polyacrylamide gel containing SDS and then transferred to a nitrocellulose membrane as previously described (Section 4.2.4).

The blot was soaked in 3% gelatin in TBS, washed with TBS-Tween, and incubated with antibodies to mouse P450 2A4/2A5 diluted 1:5000 in antibody buffer (TBS-Tween containing 1% gelatin). After 1.5 to 2 hr the blot was washed 3 times in TBS-Tween (~5 min/wash) and then incubated for 15 min with rabbit anti-goat IgG diluted 1:1000 in antibody buffer. The blot was washed 3 times in TBS-Tween (~5 min/wash) and incubated for 15 min with

goat anti-rabbit IgG alkaline phosphatase conjugate diluted 1:3000 in antibody buffer. After washing twice with TBS-Tween and once with TBS, the immunoreactive protein bands were detected by addition of a phosphatase substrate system (5-bromo-4-chloro-3-indoyl phosphate and nitroblue tetrazolium).

Microsomes from recombinant yeast expressing mouse P450 2A4 (Iwasaki *et al.*, 1991) grown in our laboratory were prepared by K.J. Woodcroft and used as a positive control for this Western blot analysis.

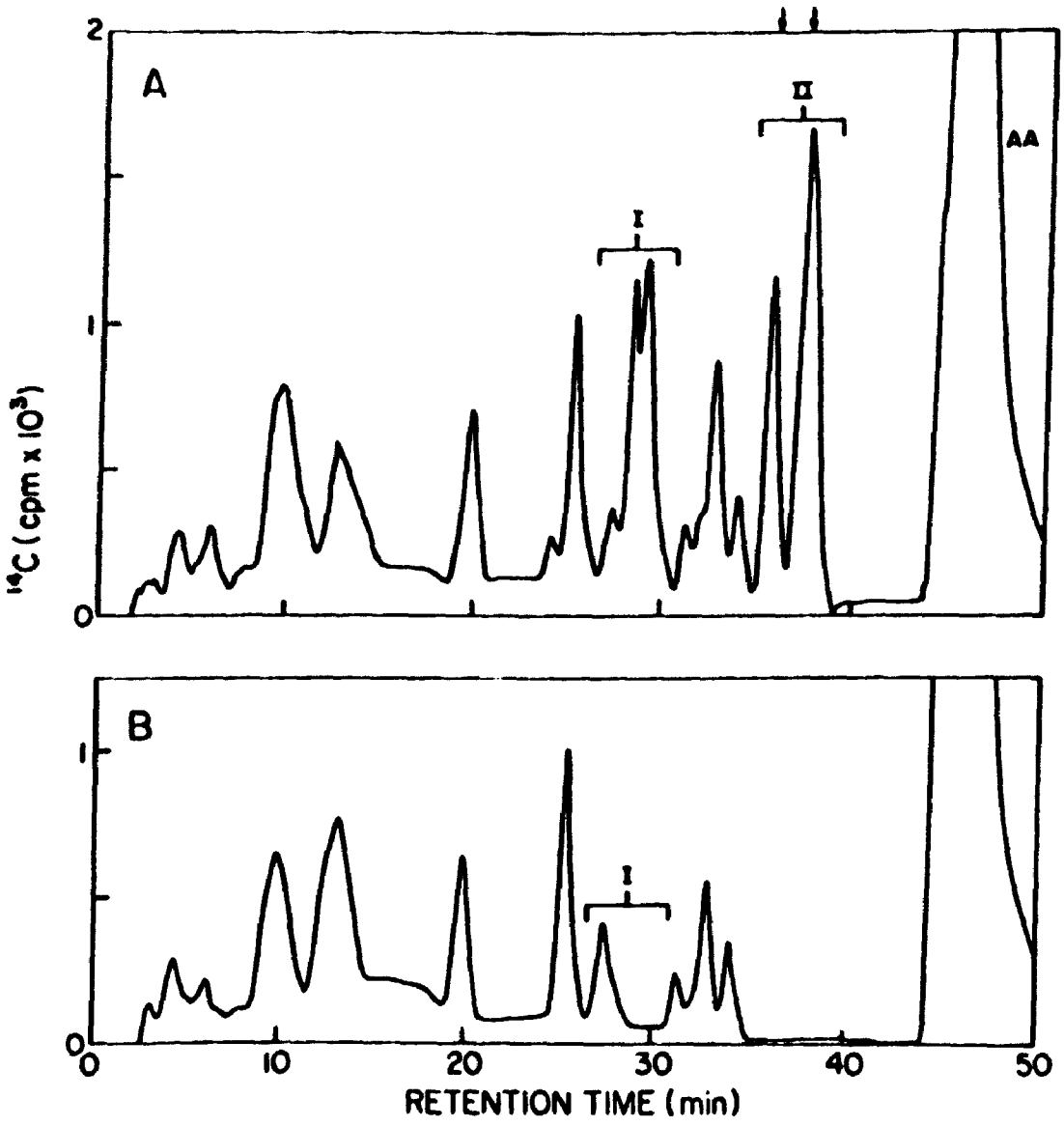
6.3 RESULTS

HPLC chromatograms (Fig. 6.1) 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 show the presence of two classes of NADPH-dependent metabolites, peak I and peak II. Peak II is composed EETs and the NADPH-dependent component of peak 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 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) and H_2O_2 (0.1 mM), in the AA incubation (data not shown).

In pulmonary microsomes from untreated animals the mean rates of

Figure 6.1

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. The arrows indicate the retention times of synthetic 14,15-EET (left), and 5,6-, 8,9- and 11,12-EET (right).


A
B
I
II
AA
2
1
0
^{14}C (cpm x 10^3)
0
10
20
30
40
50
RETENTION TIME (min)

formation of EETs, the major class of metabolites, and (16-20)-OH-AA were 0.18 and 0.04 nmol/min/mg protein, respectively (Table 6.1). β-NF-induction increased the rate of formation of (16-20)-OH-AA by ~3-fold but had little effect on EETs formation (0.23 nmol/min/mg protein). The regioisomeric composition of the EETs produced by guinea pig pulmonary microsomes was determined. The 11,12-, 14,15- and 8,9- regioisomers represented approximately 55%, 35% and 10%, respectively, of the total EETs formed by a set of microsomes pooled from untreated animals. The 11,12- and 14,15-regioisomers represented 70% and 30%, respectively, of the total EETs formed by a set of microsomes pooled from β-NF-induced animals.

The ability of classical P450 inhibitors to attenuate the formation of the NADPH-dependent AA metaoolites in pulmonary microsomes was examined (Table 6.2). 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-525A required preincubation with microsomal protein and NADPH, as SKF-525A without preincubation inhibited EETs formation by <30%. Neither metyrapone nor SKF-525A inhibited the formation of (16-20)-OH-AA. α-NF (3×10^{-7} M) did not 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 was shown to inhibit ERF activity by ~50% and ~75% in microsomes from untreated and β-NF-induced animals, respectively (data not shown).

The ability of ABT and its N-aralkylated derivatives, BBT and αMB, to

Table 6.1 **NADPH-dependent formation of AA metabolites in pulmonary microsomes from untreated and β-NF-induced guinea pigs.**

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

*Data obtained from 2 sets of microsomes each pooled from ≥8 animals.

Table 6.2 **Inhibition of AA metabolism by various P450 inhibitors in pulmonary microsomes from untreated and β-NF-induced guinea pigs *in vitro*.**

	Untreated[a]		β-NF-Induced[a]	
	Peak II (EETs)	Peak I[b]	Peak II (EETs)	Peak I[b]
No Preincubation				
No NADPH	-[c]	0.05	-	0.01
NADPH	0.19	0.09	0.21	0.14
Metyrapone (10^{-5} M)	0.03 (85)[d]	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 (0)	0.11	0.21 (0)	0.12
Preincubation[e]				
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]Data obtained from 1 set of microsomes pooled from ≥8 animals.

[b](16-20)-OH-AA and/or NADPH-independent metabolite.

[c]Below detection limit.

[d]% Inhibition.

[e]Microsomes were incubated for 5 min at 37°C with SKF-525A in the presence of 1 mM NADPH immediately before determination of AA metabolism.

inhibit the formation of (16-20)-OH-AA and EETs was also examined (Table 6.3). The inhibitor concentrations were chosen based on earlier experiments: ABT at a high concentration to ensure complete inactivation of P450 2Bx, 4Bx and 1A1, and BBT and *o*MB at concentrations where they are P450 2Bx selective inactivators in lung (Woodcroft *et al.*, 1990). 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% vs 65% and 55% inactivation at 10 μM), inhibited the formation of both classes of P450 metabolites of AA; the inhibition of EETs formation was equivalent to the inhibition of PRF activity (>95% at both 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. *o*MB, the most 2Bx-selective of these inhibitors inactivating PRF activity by >95% with little loss of ERF and ABP activities (<25%), abolished the formation of EETs (>95% loss) but had little effect on (16-20)-OH-AA formation (<10% loss).

ABT, BBT and *o*MB administered i.v. (jugular vein) to PB-induced guinea pigs were also effective *in vivo* inhibitors of EETs formation in lung (Table 6.4). ABT (75 μmol/kg) inhibited the formation of EETs as well as ERF, PRF and ABP by ≥85%. With BBT (0.075 and 7.5 μmol/kg) and *o*MB (75 nmol/kg), the inhibition of EETs formation approximated the loss in PRF activity which was

Table 6.3 **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 or αMB.**

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

*Data obtained from 1 set of microsomes pooled from ≥8 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 protein.

Table 6.4 **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 or αMB.**

Inhibitor Dose (μmol/kg)	EETs nmol/min/mg	PRF pmol/min/mg	ERF pmol/min/mg	ABP nmol/min/mg
ABT				
0 (N=3)[a]	0.29 ± 0.05[b]	41.6 ± 3.0	13.1 ± 3.0	218 ± 26
7.5 (N=3)	0.08 ± 0.02* (72)[c]	19.0 ± 6.6* (54)	6.4 ± 1.4* (52)	96 ± 9* (56)
75 (N=3)	0.01 ± 0.03* (>95)	5.4 ± 0.3* (88)	2.2 ± 0.2* (85)	8 ± 2* (>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* (59)	10.1 ± 0.1* (25)	278 ± 9* (<10)
7.5 (N=3)	0.03 ± 0.01* (88)	5.8 ± 1.5* (91)	5.2 ± 0.8* (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)

*P<0.05 (different from 0 μmol/kg control; one-way ANOVA followed by Newman-Keuls test)

[a]Number of individual animals.

[b]Mean ± SE.

[c]% Inhibition (compared to 0 μmol/kg control).

about 30% greater than the loss in ERF activity. Neither BBT nor *o*MB 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 nor its analogues suggesting that its formation is not P450 mediated in PB-induced guinea pig (0.12 ± 0.01 vs 0.15 ± 0.02 nmol/min/mg protein at 0 vs 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 6.5). Antibodies to P450 2B4 inhibited EETs formation and PRF activity in a concentration dependent manner with >95% inhibition at 2 mg IgG/mg microsomal protein. ERF activity was also inhibited by P450 2B4 antibodies but to a much lesser degree than PRF activity and EETs formation (28% at 2 mg IgG/mg 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 IgG/mg 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.

Antibodies to mouse P450 2A4/2A5 recognized a single protein band in guinea pig pulmonary microsomes by Western blot analysis (Fig. 6.2) and this band was more intense in pulmonary microsomes from β-NF-induced guinea pigs than from untreated guinea pigs (at equivalent protein concentrations). This band co-migrated with the protein band recognized by these antibodies in

Table 6.5 **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.**

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

[a]Data obtained from 1 set of microsomes pooled from ≥8 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 protein.

[b]<10% inhibition.

Figure 6.2

Western blot of guinea pig pulmonary microsomes immunostained with antibodies to mouse P450 2A4/2A5. Lane 1 - microsomes from recombinant yeast expressing mouse P450 2A4 (20 μg); Lane 2 - pulmonary microsomes from β-NF-induced guinea pigs (70 μg); Lane 3 - pulmonary microsomes from untreated guinea pigs (70 μg).

microsomes from recombinant yeast expressing mouse P450 2A4. This guinea pig isozyme is referred to as P450 2Ax throughout the remainder of this thesis.

Liver microsomes from guinea pig produce (16-20)-OH-AA (peak I), EETs (peak II), and two other classes of NADPH-dependent AA metabolites (peaks III and IV, Figure 6.3). Peak IV contains HETEs and peak III, more polar than the other classes of P450 metabolites of AA, is comprised of secondary AA metabolites (DiHETEs and/or 20-COOH-AA). In hepatic microsomes from untreated guinea pigs the rates 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 protein, respectively (Table 6.6). PB-induction markedly increased the rate of EETs 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 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 EETs formation (Table 6.7). The highest concentration of α-NF studied, 3×10^{-7} M, inhibited ERF and ABP activities by ~75% in hepatic microsomes from β-NF-induced guinea pigs.

Antibodies to rabbit P450 2B4 inhibited EETs formation and PRF activity in hepatic microsomes from PB-induced guinea pigs in a concentration dependent manner (Table 6.8). At each concentration EETs formation was inhibited much less than PRF activity; 5 mg IgG/mg microsomal protein, which inhibited PRF activity by >90% only inhibited EETs formation by 36%.

Figure 6.3

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. The arrows indicate the retention times of synthetic 14,15-EET (left), and 5,6-, 8,9- and 11,12-EET (right).

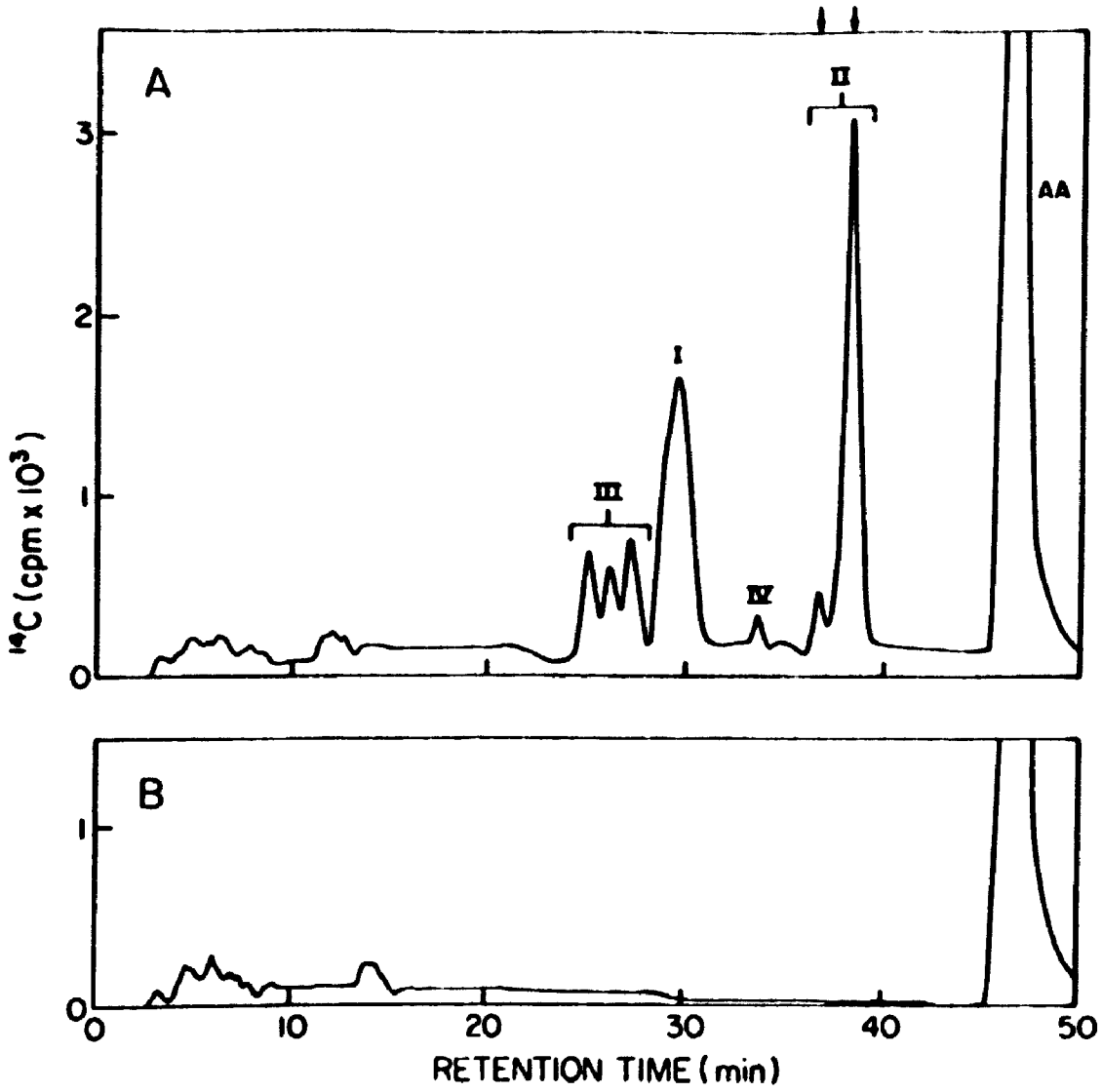

A
B
I
II
III
IV
AA
^{14}C (cpm x 10^3)
0
1
2
3
0
10
20
30
40
50
RETENTION TIME (min)

Table 6.6 **Comparison of P450-dependent AA metabolism and PRF activity in hepatic microsomes from untreated vs PB-induced guinea pigs.**

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

*P<0.05 (different than untreated; unpaired Student's t-test).

**P<0.01 (different than untreated; unpaired Student's t-test).

[a]Number of individual animals.

[b]Mean ± SE.

[c]Fold increase in activity compared to microsomes from untreated animals.

Table 6.7 **Inhibition of EETs 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%) activity was 8.99 nmol/min/mg protein. Data obtained from microsomes from 1 animal.

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

Table 6.8 **Inhibition of EETs formation and PRF activity in hepatic microsomes from PB-induced guinea pigs incubated *in vitro* with antibodies to rabbit P450 2B4.**

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

*% Inhibition compared to determination in the presence of the same amount of preimmune serum (ie. mg IgG/mg microsomal protein). Control (100%) values were 8.01 (EETs) and 0.124 (PRF) nmol/min/mg protein for 2 mg IgG/mg microsomal protein and 8.01 (EETs) and 0.101 (PRF) nmol/min/mg protein for 5 mg IgG/mg microsomal protein. Data obtained from microsomes from 1 animal.

At much higher doses (75 μmol/kg) than cause near complete inhibition of pulmonary P450 2Bx (75 nmol/kg), αMB is also an effective *in vivo* inactivator of the hepatic P450 2Bx when administered i.v. (jugular vein) to PB-induced guinea pigs (Chapter 4; Table 6.9) This high dose, which inactivated P450 2Bx catalyzed PRF by >95%, inhibited the formation of EETs by only 49% in liver; ERF and ABP activities were also inhibited by αMB (>80%) at 75 μmol/kg).

6.4 DISCUSSION

In this study pulmonary microsomes from guinea pig lung were shown to convert AA to two classes of P450 metabolites, EETs and (16-20)-OH-AA. These metabolites were formed only in the presence of NADPH, a required cofactor for the P450 monooxygenase system. 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 microsomes from untreated animals the rates of formation of EETs and (16-20)-OH-AA were 0.18 and 0.04 nmol/min/mg 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 regio-selective manner. In microsomes from untreated or β-NF-induced guinea pigs 11,12-EET was the most abundant regioisomer (≥55% of total EETs) with 11,12- and 14,15-EET accounting for ≥80% of

Table 6.9 **Inhibition of P450-dependent AA metabolism and isozyme-selective monooxygenase activities in hepatic microsomes from PB-induced guinea pigs treated *in vivo* with *α*MB.**

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

*P<0.05 (different from 0 μmol/kg control; ANOVA followed by Newman-Keuls test)

[a]Number of individual animals.

[b]Mean ± SE.

[c]% Inhibition (compared to 0 μmol/kg control).

the total EETs. 8,9-EET was detected in low amounts (10% of total) in microsomes from untreated animals. Although 5,6-EET was not detected it is possible that this regioisomer is also formed because it is a known substrate for cyclooxygenase (Oliw, 1984a, 1984b; Oliw and Benthin, 1985) which is abundant in lung.

Experiments examining the role of individual pulmonary P450 isozymes in AA metabolism were primarily carried out using microsomes from β-NF-induced guinea pigs to allow better assessment of the ability of P450 1A1 to metabolize AA. Also, the rate of (16-20)-OH-AA formation was greater (~3-fold) in pulmonary microsomes from β-NF-induced vs untreated guinea pigs.

EETs were formed solely by P450 2Bx in this organ. This is supported by several experimental findings. 1) Metyrapone and SKF-525A, both P450 2B selective inhibitors (Lubet *et al.*, 1985; Netter, 1980), 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(s) that forms a stable inhibitory P450-metabolite complex (Schenkman *et al.*, 1972). 2) αMB, a highly selective P450 2Bx mechanism-based inhibitor in guinea pig lung, inactivated 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 IgG/mg microsomal protein), likely due to this guinea pig

2B isozyme possessing a small amount of ERF activity. 4) Neither α-NF, a potent and highly selective inhibitor of 1A1 in lung (Domin and Philpot, 1986) nor antibodies to rabbit P450 4B1 inhibited the formation of EETs demonstrating that P450 1A1 and P450 4Bx, respectively, 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 EETs formation ~4-fold. Also metyrapone, SKF-525A and antibodies to P450 2B4 inhibited EETs formation in microsomes from PB-induced guinea pigs. However, P450 2Bx accounts for <50% of total EETs formation in liver of PB-induced guinea pigs. *In vitro*, antibodies to 2B4 which inhibited PRF activity by >90% inhibited EETs formation by only 36%. *In vivo*, αMB (75 μmol/kg i.v.) inhibited PRF activity by >95% with <50% loss of EETs 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 rabbit P450 2B4 and rat 2B1 in reconstituted systems (Oliw *et al.*, 1982; Capdevila *et al.*, 1990a; Falck *et al.*, 1990). Purified rat P450 2C11, which is expressed constitutively in liver, also converts AA to EETs in reconstituted systems (Capdevila *et al.*, 1990a; Falck *et al.*, 1990) and an orthologue may be contributing to EETs 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 P450 2B4 antibodies to inhibit this reaction.

P450 1A1 and 4Bx also do not contribute because neither α-NF nor antibodies to rabbit 4B1 inhibited the formation of (16-20)-OH-AA. These data suggest that a P450 isozyme other than guinea pig 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 rat P450 2B1 were identified as EETs (Capdevila *et al.*, 1990a; Falck *et al.*, 1990). In contrast, the finding that P450 1A1 does not contribute to the formation of (16-20)-OH-AA was not expected since β-NF, an inducer of 1A1 in guinea pig lung, increased the formation of (16-20)-OH-AA by ~ 3-fold. Also, purified P450 1A1 from rat and rabbit liver converts AA to (16-20)-OH-AA in reconstituted systems (Oliw *et al.*, 1982; Capdevila *et al.*, 1990a; Falck *et al.*, 1990; Tanaka *et al.*, 1990).

P450 2A3 mRNA is expressed in rat lung where it is induced by 3-MC (Kimura *et al.*, 1989a). Here, by Western blot analysis with antibodies to mouse P450 2A4/2A5, we have provided evidence that cytochrome P450 2Ax is present in guinea pig pulmonary microsomes and that this isozyme is induced by β-NF. P450 2Ax is likely the β-NF-inducible isozyme that metabolizes AA to (16-20)-OH-AA in guinea pig lung.

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 4A4, P450 2F1/2 and P450 2E1 although none of these isozymes have been reported to be β-NF-inducible (Section 1.1.2). Purified rat and rabbit hepatic P450 2E1 convert AA to (16-20)-OH-AA in reconstituted systems

(Tanaka *et al.*, 1990; Laethem *et al.*, 1993) but the ability of 4A4 and 2F1/2 to convert AA to (16-20)-OH-AA is currently unknown.

A variety of peaks was present in the HPLC chromatograms obtained from incubating pulmonary microsomes with AA in the absence of NADPH. The majority was abolished by boiling 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 as inhibitors of these enzymes (indomethacin and nordihydroguaiaretic acid, respectively) did not block their formation. P450 can metabolize AA independent of NADPH if AA hydroperoxides or presumably other lipid hydroperoxides are present (Weiss *et al.*, 1987; Capdevila *et al.*, 1990b) and perhaps this accounts for these products.

In summary, we have shown that guinea pig pulmonary microsomes convert AA to two classes of P450-dependent metabolites, EETs and (16-20)-OH-AA. P450 2Bx is solely responsible for the bioactivation of AA to EETs in this system. A P450 isozyme(s) other than 2Bx, 4Bx or 1A1, possibly an orthologue of mouse P450 2A4/2A5, is responsible for (16-20)-OH-AA formation.

CHAPTER 7

METABOLISM OF ARACHIDONIC ACID BY RENAL CYTOCHROME P450

7.1 OBJECTIVE

The primary objective of this study was to determine the ability of guinea pig renal P450 1A1 to metabolize AA.

7.2 MATERIALS AND METHODS

7.2.1 Reagents

Chemicals were obtained from the sources previously indicated (Sections 3.2.1, 4.2.1 and 6.2.1).

7.2.2 Animal Treatment

Male Hartley guinea pigs (300-375 g) were obtained from Charles River Ltd. (St. Constant, Que.) and housed as indicated in Section 3.2.2.

Most guinea pigs were treated i.p. with 80 mg/kg β-NF (2% in corn oil) daily for 4 days. In experiments studying the ability of the mechanism-based inhibitors to inactivate the renal system *in vivo*, the animals were treated exactly as outlined in Section 4.2.2 and sacrificed 4 hr after the inhibitor was administered. The kidneys and livers were removed and stored at -80°C.

7.2.3 Preparation of Microsomes

Washed renal and hepatic microsomes were prepared by differential

centrifugation as previously described (Section 3.2.3) and the protein concentrations were determined by the method of Lowry *et al.*, 1951 (Section 3.2.4).

7.2.3 Determination of 7-Ethoxyresorufin O-Deethylation and 4-Aminobiphenyl N-Hydroxylation Activities

ERF activity was determined fluorimetrically (Section 3.2.6) and ABP activity was determined colorimetrically (Section 3.2.7).

7.2.4 Determination of Cytochrome P450-Dependent Arachidonic Acid Metabolism

P450-dependent AA metabolism was carried out as described previously (Section 6.2.5). For renal microsomes the initial incubation contained 1 mg microsomal protein and 0.5 μCi of [1-^{14}C]AA. The HPLC injection volume was 40 μl for the renal samples.

In studies examining the ability of α-NF to inhibit ERF and ABP activities and AA metabolism, α-NF (in ethanol) was added to a test tube and the solvent removed under a stream of N_2. Microsomal protein was added to the test tube and incubated with the α-NF (on ice) for at least 30 min before the addition of the protein to the reaction vials.

7.2.5 *In Vitro* Incubations with 1-Aminobenzotriazole and its N-Aralkylated Derivatives

In vitro incubations with ABT, BBT and αMB were carried out as previously described (Section 6.2.6).

7.3 RESULTS

HPLC chromatograms (Fig. 7.1) of the ^{14}C-AA metabolites formed by renal microsomes of guinea pigs in the presence (A) and absence (B) of NADPH show the presence of two classes of P450 metabolites, (16-20)-OH-AA (peak I) and EETs (peak II). In renal microsomes from untreated animals the rates of formation of (16-20)-OH-AA and EETs were 0.47 and 0.34 nmol/min/mg protein, respectively (Table 7.1). β-NF-treatment did not affect the rates of formation of these metabolites significantly, although there was a tendency towards decreased EETs formation. β-NF induction routinely increases ERF activity, an activity associated with P450 1A1, ~100-fold in the guinea pig kidney (1-2 and 100-250 pmol/min/mg protein in microsomes from untreated and β-NF-treated guinea pigs, respectively). ERF activity was 168 ± 8 pmol/min/mg protein in the β-NF-induced microsomes used to study AA metabolism.

The ability of ABT, BBT and αMB to inhibit ERF activity and the formation of P450 metabolites of AA was determined in renal microsomes from β-NF-induced guinea pigs (Fig. 7.2). All three compounds inhibited ERF activity in a concentration dependent manner. At 10 μM, BBT and αMB were

Figure 7.1

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

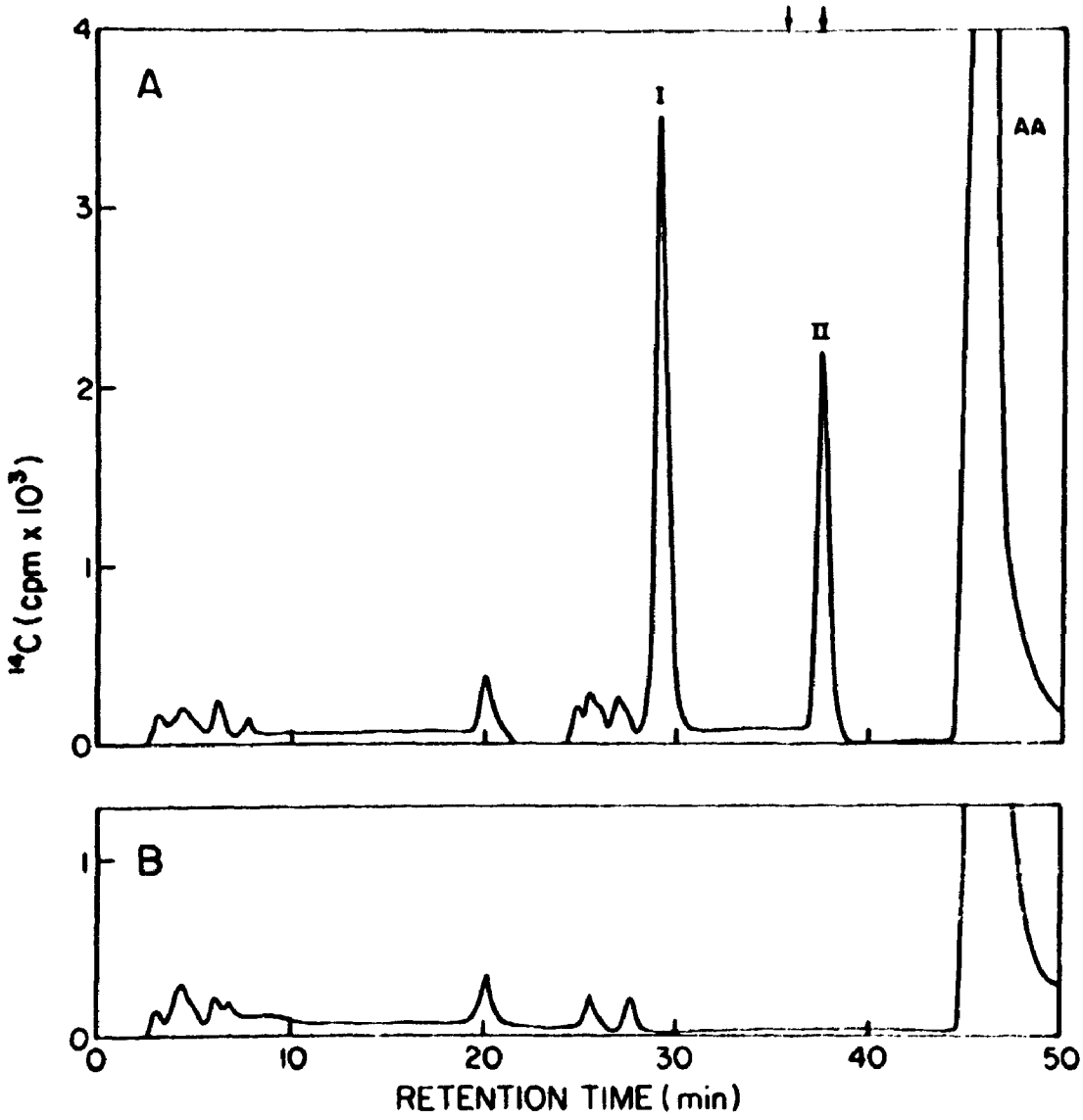

A
B
I
II
AA
^{14}C (cpm x 10^3)
RETENTION TIME (min)
0
1
2
3
4
10
20
30
40
50

Table 7.1 **Comparison of P450-dependent AA metabolism in renal microsomes from untreated and β-NF-induced guinea pigs.**

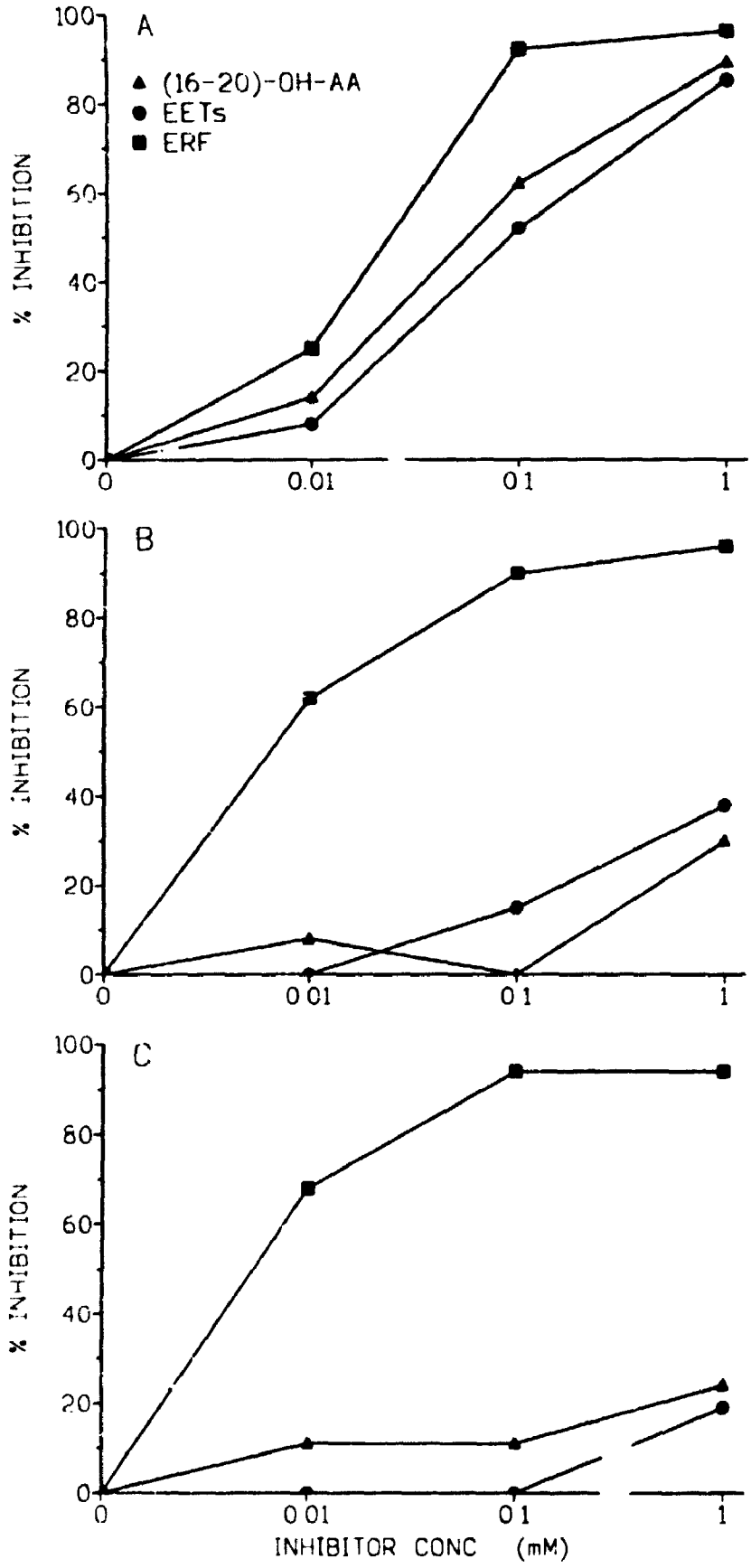
	(16-20)-OH-AA nmol/min/mg	EETs nmol/min/mg
Untreated (N = 5)[a]	0.47 ± 0.05[b]	0.34 ± 0.05
β-NF-Induced (N = 10)	0.45 ± 0.02	0.25 ± 0.03

[a]Number of individual animals.

[b]Mean ± SE.

Figure 7.2

Inhibition of P450-dependent AA metabolism and ERF activity in renal microsomes from β-NF-induced guinea pigs incubated *in vitro* with ABT (A), BBT (B) or αMB (C). Data were obtained from 1 set of microsomes pooled from ≥8 animals. Control (100%) values were 0.19 (ERF), 0.37 ((16-20)-OH-AA) and 0.26 (EETs) nmol/min/mg protein.


A
▲ (16-20)-OH-AA
● EETs
■ ERF
% INHIBITION
100
80
60
40
20
0
0
0.01
0 1
1
B
% INHIBITION
100
80
60
40
20
0
0
0 01
0 1
1
C
% INHIBITION
100
80
60
40
20
0
0
0 01
0 1
1
INHIBITOR CONC (mM)

more effective inhibitors of ERF activity than ABT (~65% vs 25% inhibition) whereas at 100 μM all three compounds inhibited ERF activity by >90%. ABT was also an effective inhibitor of the formation of both (16-20)-OH-AA and EETs. Approximately 50% of EETs and 60% of (16-20)-OH-AA formation were inhibited by 100 μM ABT, considerably less than the amount of ERF (>90%) inactivated. At 1 mM ABT, both ERF activity and the formation of P450 metabolites of AA were inhibited by >80%. BBT and αMB were more selective in the inhibition of ERF vs AA metabolism; at 100 μM, αMB inactivated almost all of the 1A1 activity with no effect on EETs formation and minimal effect on (16-20)-OH-AA (<10% inhibition) formation. Even at 1 mM, a 10-fold higher concentration than that required to inhibit ERF activity by >90%, the formation of both classes of P450 metabolites of AA was inhibited <40% and <25% by BBT and αMB, respectively.

ABT, BBT and αMB were also effective *in vivo* inhibitors of the renal P450 monooxygenase system when administered i.v. (jugular vein) to guinea pigs (Table 7.2). In β-NF-induced guinea pigs ABT (75 μmol/kg), BBT (75 μmol/kg) and αMB (7.5 μmol/kg) inhibited ERF activity by 62%, 44% and 58%, respectively. ABT also inhibited the formation of (16-20)-OH-AA (by 55%) and EETs (by 37%) whereas BBT and αMB had no inhibitory effect on the formation of either of these P450 metabolites of AA. An equivalent dose of ABT (75 μmol/kg) also inhibited the formation of (16-20)-OH-AA and EETs in non-induced guinea pigs, and to nearly the same extent as in β-NF-induced animals.

Table 7.2 **Inhibition of ERF activity and P450-dependent AA metabolism in renal microsomes from guinea pigs treated *in vivo* with ABT, BBT or *a*MB.**

Inhibitor Dose (μmol/kg)	ERF nmol/min/mg	(16-20)-OH-AA nmol/min/mg	EETs nmol/min/mg
BBT - β-NF-Induced			
0 (N=4)[a]	165 ± 9[b]	0.45 ± 0.03	0.16 ± 0.02
75 (N=4)	92 ± 9** (44)[c]	0.51 ± 0.04	0.16 ± 0.03
*a*MB - β-NF-Induced			
0 (N=5)	169 ± 16	0.44 ± 0.04	0.27 ± 0.03
7.5 (N=2)	81, 64 (58)	0.52, 0.48	0.24, 0.33
ABT - β-NF-Induced			
0 (N=5)	169 ± 16	0.44 ± 0.04	0.27 ± 0.03
75 (N=4)	64 ± 2** (62)	0.20 ± 0.02** (55)	0.17 ± 0.01* (37)
ABT - Non-Induced			
0 (N=5)	-[d]	0.47 ± 0.05	0.34 ± 0.05
75 (N=4)	-	0.21 ± 0.02** (55)	0.26 ± 0.02 (24)

*P<0.05 (different than 0 μmol/kg control; unpaired Student's t-test).

**P<0.01 (different than 0 μmol/kg control; unpaired Student's t-test).

[a]Number of individual animals.

[b]Mean ± SE.

[c]% Inhibition (compared to 0 μmol/kg control).

[d]Not determined.

Liver microsomes from guinea pig produce (16-20)-OH-AA, EETs, HETEs and secondary metabolites (Chapter 6, Fig. 6.3). In hepatic microsomes from untreated guinea pigs the rates of formation of EETs and (16-20)-OH-AA, the two classes of P450-dependent metabolites formed in kidney, were 1.97 and 1.74 nmol/min/mg protein, respectively. β-NF-induction increased the rate of (16-20)-OH-AA formation by hepatic microsomes 3.7-fold (Table 7.3); there was also an increase (1.7-fold) in the rate of EETs formation but this was not significant due to considerable variation in the β-NF-induced guinea pigs. By comparison, β-NF-induction increased hepatic ERF activity 4.7-fold, and ABP activity 4.0-fold.

The ability of α-NF, a selective inhibitor of the P450 1A subfamily (Johnson, 1979; Testa and Jenner, 1981), to inhibit ERF activity, ABP activity and the formation of the P450-dependent AA metabolites was determined in renal and hepatic microsomes from β-NF-induced guinea pigs (Table 7.4). In kidney microsomes α-NF inhibited ERF activity in a concentration dependent manner with 81% and 96% inhibition at 10^{-7} M and at 3×10^{-7} M α-NF, respectively. These concentrations of α-NF had little effect on the formation of (16-20)-OH-AA and EETs in renal microsomes ($< 15\%$ and $< 5\%$ inhibition, respectively). In liver microsomes ERF and ABP activities were inhibited about equally at each concentration of α-NF. The formation of EETs and (16-20)-OH-AA was inhibited by α-NF in liver microsomes although at both concentrations examined (10^{-7} and 3×10^{-7} M) the formation of the P450-dependent AA metabolites was inhibited less than ABP and ERF activities.

Table 7.3 **Comparison of P450-dependent AA metabolism and ERF activity in hepatic microsomes from β-NF-induced vs untreated guinea pigs.**

	Untreated (N = 5)[a]	β-NF-Induced (N = 4)
(16-20)-OH-AA nmol/min/mg	1.74 ± 0.18[b]	6.42 ± 0.31** (3.7)[c]
EETs nmol/min/mg	1.97 ± 0.25	3.38 ± 0.69 (1.7)
ERF nmol/min/mg	0.23 ± 0.02	1.08 ± 0.08** (4.7)
ABP nmol/min/mg	73 ± 8	289 ± 33** (4.0)

**P < 0.01 (different than untreated; unpaired Student's t-test).

[a]Number of individual animals.

[b]Mean ± SE.

[c]Fold increase in activity compared to microsomes from untreated animals.

Table 7.4 **Inhibition of ERF activity, ABP activity and AA metabolism by α-NF in renal and hepatic microsomes from β-NF-induced guinea pigs.**

α-NF Conc. (M)	% Inhibition						
	Kidney[a]			Liver[b]			
	ERF Activity	(16-20)-OH-AA Formation	EETs Formation	ERF Activity	ABP Activity	(16-20)-OH-AA Formation	EETs Formation
1×10^{-8}	25	-[c]	-	23	20	-	-
3×10^{-8}	46	-	-	21	26	-	-
1×10^{-7}	81	12	<5	48	49	37	26
3×10^{-7}	96	8	<5	80	71	53	36
1×10^{-6}	98	-	-	83	96	-	-

[a] Data obtained from 1 set of microsomes pooled from ≥8 animals. Control (100%) values were 0.15 (ERF), 0.48 ((16-20)-OH-AA) and 0.38 (EETs) nmol/min/mg protein.

[b] Data obtained from 1 set of microsomes pooled from 4 animals. Control (100%) values were 1.07 (ERF), 4.36 ((16-20)-OH-AA) and 4.90 (EETs) nmol/min/mg protein.

[c] Not determined.

7.4 DISCUSSION

Data presented in the previous chapter (Chapter 6) indicated that guinea pig pulmonary P450 1A1 does not metabolize AA. This was not expected since purified P450 1A1 from rat or rabbit liver converts AA to (16-20)-OH-AA and EETs in reconstituted monooxygenase systems (Oliw *et al.*, 1982, Capdevila *et al.*, 1990a; Falck *et al.*, 1990; Tanaka *et al.*, 1990). Therefore, the metabolism of AA by P450 1A1 was examined in another guinea pig tissue. The kidney was chosen because, like the lung, this organ expresses P450 1A1 but not the other member of the P450 1A subfamily, P450 1A2 (Liem *et al.*, 1980; Goldstein and Linko, 1984; Christou *et al.*, 1987; Pasco *et al.*, 1988).

In this study guinea pig renal microsomes were shown to convert AA to two classes of P450 metabolites, EETs and (16-20)-OH-AA. Formation of these metabolites was dependent on the presence of NADPH, a required cofactor for the P450 monooxygenase system, and was inhibited by ABT, a mechanism-based inhibitor of P450. These metabolite classes also constitute >90% of the P450-dependent metabolites formed by renal microsomes from rabbit (Schwartzman *et al.*, 1986; Lapuerta *et al.*, 1988) and rat (Takahashi *et al.*, 1990). It is likely that in guinea pig kidney only one, or at most two, individual metabolites of the (16-20)-OH-AA class are formed because the HPLC chromatograms contained a single narrow peak in this area instead of the multiple overlapping peaks that would be expected if all of the metabolites were formed (Falck *et al.*, 1990). Probably only 19-OH-AA and/or 20-OH-AA

are formed because all studies conducted to date with renal tissue that have identified the individual components of this metabolite class using gas chromatography-mass spectrometry have detected only these two metabolites (Morrison and Pascoe, 1981; Oliw *et al.*, 1981; Schwartzman *et al.*, 1986; Takahashi *et al.*, 1990; Omata *et al.*, 1992). In microsomes from untreated guinea pigs total P450 metabolites of AA were formed at a rate of ~0.81 nmol/min/mg protein [~60% (16-20)-OH-AA and ~40% EETs]. This rate is comparable to those reported for renal microsomes from rat (1.0 and 0.2 nmol/min/mg protein for cortical and medullary microsomes, respectively; Takahashi *et al.*, 1990) and rabbit (0.68, 0.49 and 0.10 for cortical, outer medullary and inner medullary microsomes, respectively; Schwartzman *et al.*, 1986).

In guinea pig kidney both BBT and *α*MB were found to be highly selective for the inactivation of P450 1A1, in comparison to the isozyme(s) that metabolize AA. *In vitro*, 100 *μ*M BBT and *α*MB inhibited 1A1-catalyzed ERF activity by >90% without inhibiting the formation of (16-20)-OH-AA or EETs. *In vivo*, BBT and *α*MB (75 and 7.5 *μ*mol/kg, respectively) inhibited ERF activity by ~50% without inhibiting the formation of either class of P450-dependent metabolites. BBT and *α*MB are also more isozyme-selective inhibitors of P450 than ABT in guinea pig lung and liver (Woodcroft and Bend, 1990; Woodcroft *et al.*, 1990; Chapter 4).

The ability of BBT and *α*MB to inactivate 1A1-catalyzed ERF activity without inhibiting the formation of either (16-20)-OH-AA or EETs demonstrates

that P450 1A1 does not contribute to the formation of either (16-20)-OH-AA or EETs in guinea pig kidney. This is supported by other experimental data. β-NF-induction, which increased 1A1-catalyzed ERF activity ~ 100-fold, did not increase the rate of formation of either class of P450-dependent AA metabolites in guinea pig kidney. α-NF, an inhibitor of 1A1 in the kidney, did not inhibit the rate of formation of AA metabolites by P450 monooxygenases in microsomes from β-NF-induced animals. This is consistent with the finding that P450 1A1 does not contribute to the formation of (16-20)-OH-AA or EETs in lung of β-NF-induced guinea pigs (Chapter 6). In contrast, total P450 metabolism of AA is about 2-fold higher in renal microsomes prepared from rabbits treated with 1A1 inducers (β-NF and 3-methylcholanthrene) compared to controls (Schwartzman *et al.*, 1986). Also, as previously mentioned, purified 1A1 from rat or rabbit liver converts AA to (16-20)-OH-AA and EETs in reconstituted monooxygenase systems (Oliw *et al.*, 1982, Capdevila *et al.*, 1990a; Falck *et al.*, 1990; Tanaka *et al.*, 1990). The apparent discrepancy in the ability of P450 1A1 from different species to metabolize AA may be due to differences in the P450 1A1 protein. Guinea pig 1A1 is about 3 kilodaltons shorter than either rabbit or rat 1A1 (Cheng *et al.*, 1986; Nelson *et al.*, 1993) and is likely missing an amino acid sequence(s) critical for AA binding and/or oxidation.

The P450 isozyme(s) which convert(s) AA to (16-20)-OH-AA and EETs in guinea pig kidney is unknown, but ABT is a much more potent inactivator of this isozyme(s) than either BBT or αMB. Isozymes of the 4A subfamily,

which is constitutively expressed in rat and rabbit kidney, have been shown to $\omega/(\omega-1)$-hydroxylate fatty acids including AA (Capdevila *et al.*, 1985; Bains *et al.*, 1985; Sharma *et al.*, 1989; Capdevila *et al.*, 1990b; Sawamura *et al.*, 1993). BBT and *o*MB do not inhibit P450 4Bx, an orthologue of rabbit P450 4B1 in guinea pig lung, whereas ABT inactivates this pulmonary isozyme (Woodcroft *et al.*, 1990; Chapter 4). P450 2C2, isolated from rabbit kidney catalyzes the epoxidation of AA (Laethem *et al.*, 1992). A variety of P450 isozymes purified from rat liver convert AA to (16-20)-OH-AA and/or EETs (Oliw *et al.*, 1982, Capdevila *et al.*, 1990a; Falck *et al.*, 1990; Tanaka *et al.*, 1990; Laethem *et al.*, 1993). It is not known which, if any orthologues of these isozymes are expressed in guinea pig kidney. In guinea pig liver and lung P450 2Bx efficiently converts AA to EETs (Chapter 7). It is extremely unlikely that this isozyme contributes to the formation of EETs in guinea pig kidney because BBT and *o*MB are very potent inactivators of this isozyme (Woodcroft *et al.*, 1990; Woodcroft and Bend, 1990). PRF activity is virtually undetectable in guinea pig kidney microsomes (<1 pmol/min/mg protein), and P450 2B immunoreactive protein is not detectable in guinea pig kidney microsomes (Yamada *et al.*, 1992).

In guinea pig liver the formation of (16-20)-OH-AA and EETs was induced by treatment with β-NF. α-NF, a inhibitor highly selective for the P450 1A family, partially inhibited the formation of these two classes of metabolites in microsomes from β-NF-induced guinea pigs ($\sim$50% and $\sim$35% inhibition, respectively, at 3×10^{-7}M). Because P450 1A1 does not contribute to P450-

dependent AA metabolism in either kidney or lung of guinea pig, it is likely that P450 1A2, a member of the 1A subfamily that is present in liver but not extrahepatic tissues (Liem *et al.*, 1980; Goldstein and Linko, 1984), contributes to the formation of these metabolites in guinea pig liver microsomes. ABP activity, associated primarily with 1A2 in liver, was also induced by β-NF-treatment and inhibited by α-NF. Purified 1A2 from rat liver metabolizes AA to (16-20)-OH-AA and EETs (ratio of 2:1) in reconstituted systems (Capdevila *et al.*, 1990a; Falck *et al.*, 1990; Tanaka *et al.*, 1990). At each concentration of α-NF studied the formation of the P450 metabolites of AA was inhibited less than monooxygenase activities (ERF and ABP) highly selective for P450 1A indicating that other isozyme(s) must also contribute to total P450-dependent AA metabolism in guinea pig liver. Many other P450 isozymes purified from liver of other species, including members of the 2B, 2C, 2E and 4A subfamilies, have been reported to metabolize AA (Section 1.2.1). In chick embryo liver a β-NF-inducible isozyme that does not belong to the P450 1A family, β-NF_{AA}, oxidizes AA to form EETs and a small amount of (16-20)-OH-AA (Nakai *et al.*, 1992).

In summary, this study demonstrated that P450 1A1 does not bioactivate AA in guinea pig kidney. Consequently, P450 1A2 is likely the isozyme induced by β-NF and inhibited by α-NF that converts AA to both (16-20)-OH-AA and EETs in guinea pig liver. In addition, BBT and αMB were shown to selectively inactivate P450 1A1 in comparison to the isozymes that metabolize AA in guinea pig kidney.

CHAPTER 8

CONCLUDING REMARKS AND FUTURE WORK

8.1 CONCLUDING REMARKS

The research findings presented in this thesis represent a major contribution to the field of tissue specific, (in this case, lung), P450 isozyme-selective mechanism-based inhibition of P450. We have shown that BBT and *o*MB, at appropriate low doses, are isozyme-selective/specific (P450 2Bx) and lung-specific mechanism-based inhibitors of the P450 monooxygenase system in guinea pig *in vivo* when administered i.v. To our knowledge these are the only compounds which have been shown to be specific (at appropriate doses) for the inactivation of pulmonary (vs hepatic) P450 *in vivo*. Although characterization of the *in vivo* effects of BBT and *o*MB was confined to the guinea pig in this thesis, these inhibitors may also be effective tissue specific inhibitors in other species. *In vitro*, these compounds have also been shown to inactivate P450 in an isozyme-selective manner in pulmonary microsomes from rabbit (Mathews and Bend, 1986) and goat (Huijzer *et al.*, 1989). Also, they are effective inactivators of rabbit pulmonary P450 2B4 *in vivo* although the lowest dose examined also caused considerable inactivation of the hepatic P450 system (Mathews and Bend, 1993). It should be noted that while these inhibitors are effective across multiple species, their potency, both *in vitro* and *in vivo*, may vary amongst species (eg. they are more potent inactivators of P450 2B in pulmonary microsomes from guinea pig than rabbit; Mathews and

Bend, 1986; Woodcroft *et al.*, 1990) and therefore it is necessary to determine the appropriate concentration/dose for the species of interest.

The isozyme-selectivity of these inhibitors in lung makes them extremely useful probes for examining the ability of pulmonary P450 2Bx to metabolize both endogenous and xenobiotic compounds. In microsomal systems they can be used to complement studies with inhibiting antibodies to P450 isozymes or NADPH-P450 reductase. Whereas studies with antibodies are limited to disrupted cells, BBT and *a*MB also inactivate P450 in systems with intact cellular structure and therefore can also be used in isolated cells, tissues (perfused organs) and *in vivo*. The combined isozyme- and tissue-selectivity of these inhibitors *in vivo* makes them extremely useful for determining the contribution of pulmonary P450 2Bx to the *in vivo* metabolism (both bioactivation and detoxication) of chemicals. For example, *a*MB inhibits the conversion of 3-methylindole to its toxic form in pulmonary microsomes from goat *in vitro* (Huijzer *et al.*, 1989). It is believed that the pulmonary toxicity caused by 3-methylindole *in vivo* is due to pulmonary (vs hepatic) bioactivation although this has not yet been conclusively demonstrated. Using *a*MB *in vivo*, it should be possible to determine the contribution of pulmonary (vs hepatic) P450 to 3-methylindole mediated pulmonary toxicity. Also, if a single dose of *a*MB or BBT can inactivate P450 2B for 12-24 hr without causing toxicity it may be useful for prophylactic treatment against this pulmonary toxin.

It is now becoming increasing apparent that the "xenobiotic metabolizing P450s" (ie. P450 families 1-4) are important not only in the metabolism of

exogenous chemicals, but also of endogenous compounds. Data presented in this thesis demonstrate that guinea pig pulmonary microsomes oxidize AA, an endogenous compound, to two classes of P450-dependent metabolites, EETs, and (16-20)-OH-AA. We have used BBT and *o*MB, in combination with other methodology, to demonstrate that P450 2Bx is solely responsible for the formation of EETs, the more abundant metabolite class. A *β*-NF-inducible isozyme, probably P450 2Ax, forms (16-20)-OH-AA in guinea pig lung. Characterization of P450-dependent AA metabolism in lung at the isozyme level has not previously been reported for any species. This is also, to our knowledge, the first endogenous substrate reported for pulmonary P450 2B in any species.

P450-dependent AA metabolites have a wide variety of biological effects and have been implicated to be important in physiology and pathophysiology (Section 1.2). The effects of these metabolites on the pulmonary system are currently unknown (Section 1.2.3). Because BBT and *α*-MB inactivate P450 2Bx in systems with intact cellular structure they may be useful probes for determining the physiological and/or pathobiological roles of EETs in the lung, especially after conditions such as oxidant stress which result in AA release in lung. It is possible *in vivo* that EETs produced in the lung may enter the blood stream and reach the heart. 11,12-EET, the most abundant regioisomer formed by guinea pig pulmonary microsomes, as well as 5,6-EET, have been shown to delay the recovery of contractile force in reperfused isolated guinea pig heart following low-flow ischemia (Moffat *et al.*, 1993). In isolated guinea

pig myocytes these EETs also increased intracellular Ca^{2+} and cell shortening (Moffat *et al.*, 1993). All of the EETs regioisomers have been reported to dilate preconstricted canine coronary arteries *in vitro* (Rosolowsky *et al.*, 1990).

Finally, we have shown in both guinea pig lung and kidney that P450 1A1 does not metabolize AA. Therefore, it is apparent that guinea pig is an inappropriate species for studying the bioactivation of AA by P450 1A1 and the possible role of this metabolic activation in physiology or pathobiology although it should make as excellent "negative control" in this context.

8.2 FUTURE WORK

Some future areas of study stemming from the research represented in this thesis are as follows:

1) To examine the time-course of inactivation of pulmonary P450 2Bx at low doses of BBT and/or *α*MB and to determine if rebound induction occurs.

2) To determine if the inactivation of pulmonary P450 by BBT and *α*MB can be maintained long-term by multiple injections of these compounds.

3) To determine the specific mechanism(s) of inactivation of P450 2Bx by *α*-MB and BBT.

4) To examine the inactivation of microheterogenous forms of P450 2B by BBT and *α*MB.

5) To examine the inhibition of pulmonary P450 2Ax by ABT, BBT and *α*MB.

6) To use BBT and *o*MB as *in vitro* and *in vivo* probes.

7) To identify the EETs produced by pulmonary microsomes at both the regioisomeric and stereoisomeric level. Once identified, the effects of these particular EETs on lung and heart can be investigated using isolated lung muscle or heart cells, or isolated perfused lung or heart preparations.

8) To determine if the *β*-NF-inducible P450 2Ax isozyme is responsible for the formation of (16-20)-OH-AA in guinea pig lung microsomes. Also, to investigate the metabolism of arachidonic acid by mouse P450 2A4 and 2A5 (expressed in yeast).

9) To determine the effects of BBT and *o*MB on the cyclooxygenase and lipooxygenase pathways of arachidonic acid metabolism.

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