XENOBIOTIC-METABOLIZING CYTOCHROME P450 ENZYMES IN HUMAN LUNG

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Abstract

The cytochrome P450 (CYP) enzyme system in human lung is an essential component in the pulmonary carcinogenicity of several inhaled xenobiotic compounds. The aim of this study was to elucidate the expression and regulation of xenobiotic-metabolizing CYP enzymes in human lung.

To evaluate which of the two is a better surrogate cell model for lung tissue, the expression patterns of CYP enzymes in alveolar macrophages and peripheral blood lymphocytes were clarified by reverse transcriptase-polymerase chain reaction (RT-PCR) and compared to the expression in lung tissue. The pattern of CYP expression in alveolar macrophages was found to closely resemble the expression pattern in human lung tissue, while the pattern in lymphocytes was markedly different. The expression of CYP2B6, CYP2C, CYP3A5, and CYP4B1 mRNAs in alveolar macrophages was demonstrated for the first time.

To facilitate mechanistic studies on human pulmonary CYP induction, the A549 lung adenocarcinoma cell line was characterized by RT-PCR, and the CYP expression pattern was found to compare reasonably well to human lung epithelial cells. The induction of CYP1A1 by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) behaved as predicted, and CYP1B1 and CYP3A5 were also inducible by TCDD and dexamethasone, respectively. TCDD elevated the level of CYP1A1 mRNA (56-fold), while the induction of CYP1B1 mRNA was more modest (2.5-fold). The tyrosine kinase inhibitor genistein and the protein kinase C inhibitor staurosporine blocked CYP1A1 induction by TCDD, but did not affect CYP1B1 induction. The serine/threonine protein phosphatase inhibitor calyculin A and okadaic acid enhanced CYP1B1 induction slightly, but did not alter CYP1A1 induction.

The expression of CYP3A forms in human pulmonary tissues was studied with RT-PCR and immunohistochemistry, and both methods established CYP3A5 as the main CYP3A form. CYP3A4 was expressed in only about 20% of the cases. In A549 cells, CYP3A5 was induced about 4-fold by the glucocorticoids budesonide, beclomethasone dipropionate, and dexamethasone. Maximal induction was achieved by concentrations as low as ~100 nM, suggesting that CYP3A5 could be induced *in vivo* in patients using inhaled glucocorticoids. However, there were no differences in CYP3A5 expression in alveolar macrophages in current glucocorticoid users, ex-users, and non-users. Cigarette smoking had a marked decreasing effect on CYP3A5 levels in alveolar macrophages. The presence and possible induction of CYP3A5 by glucocorticoids in human lung could have consequences for the maintenance of physiological steroid hormone balance in lung and the individual susceptibility to lung cancer of patients using glucocorticoids.

Keywords: gene expression, alveolar macrophages, enzyme induction, reverse transcriptase polymerase chain reaction.

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Oulu, December 2000 Janne Hukkanen

Abbreviations

AHH	aryl hydrocarbon hydroxylation
AHR	aryl hydrocarbon receptor
ARNT	aryl hydrocarbon receptor nuclear translocator
B(*a*)P	benzo(*a*)pyrene
bHLH	basic helix-loop-helix
BROD	7-benzyloxyresorufin O-debenzylation
CAR	constitutively active receptor
cDNA	complementary deoxyribonucleic acid
CYP	cytochrome P450
EDHF	endothelium-derived hyperpolarizing factor
EET	epoxyeicosatrienoic acid
ER6	everted repeat element
ECOD	7-ethoxycoumarin O-deethylation
EROD	ethoxyresorufin O-deethylation
GR	glucocorticoid receptor
mRNA	messenger ribonucleic acid
NNK	4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone
PAH	polycyclic aromatic hydrocarbon
PAS	Per-Arnt-Sim homology
PBREM	phenobarbital-responsive enhancer module
PCN	pregnenolone 16α-carbonitrile
PKC	protein kinase C
PM	poor metabolizer
PROD	pentoxyresorufin O-deethylation
PXR	pregnane X receptor
RT-PCR	reverse transcriptase polymerase chain reaction
RXR	retinoid X receptor
SRC-1	steroid receptor coactivator-1
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
XRE	xenobiotic response element

List of original articles

This thesis is based on the following articles, which are referred to in the text by Roman numerals:

I Hukkanen J, Hakkola J, Anttila S, Piipari R, Karjalainen A, Pelkonen O & Raunio H (1997) Detection of mRNA encoding xenobiotic-metabolizing cytochrome P450s in human broncho-alveolar macrophages and peripheral blood lymphocytes. Mol Carcinogenesis, 20: 224-230.

II Anttila S, Hukkanen J, Hakkola J, Stjernvall T, Beaune P, Edwards RJ, Boobis AR, Pelkonen O & Raunio H (1997) Expression and localization of CYP3A4 and CYP3A5 in human lung. Am J Respir Cell Mol Biol 16: 242-249.

III Hukkanen J, Lassila A, Päivärinta K, Valanne S, Sarpo S, Hakkola J, Pelkonen O & Raunio H (2000) Induction and regulation of xenobiotic-metabolizing cytochrome P450s in the human A549 lung adenocarcinoma cell line. Am J Respir Cell Mol Biol 22: 360-366.

IV Hukkanen J, Hakkola J, Lassila A, Piipari R, Anttila S, Pelkonen O & Raunio H (2001) Modulation of CYP3A5 by glucocorticoids and cigarette smoke in human lung-derived cells. Manuscript.

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1 Introduction

The lung constitutes the primary site of entry into the body for a wide variety of inhaled chemicals. Many of these chemical substances, including carcinogens and toxicants, pose a risk to the lung (Gram 1997). As a consequence, the lung is a major target for chemically induced diseases, most notably lung cancer (Hecht 1999). Inhaled chemicals may also cause deleterious effects outside the respiratory system, as exemplified by tobacco smoke and its harmful effects throughout the body. Similarly to liver, another site of entry, human lung has defense mechanisms against substances entering the body. Mechanical, cellular, and enzymatic defense mechanisms act to eliminate hazardous chemicals. In the enzymatic defense reaction, the chemical is first functionalized by phase I enzymes, usually by the cytochrome P450 (CYP) enzyme system, and then conjugated to a more soluble and excretable form by other enzyme systems, such as glutathione S-transferases, sulfotransferases and N-acetyltransferases (Guengerich & Shimada 1991). In general, these enzymatic reactions are beneficial in that they help the pulmonary tissues to eliminate foreign compounds. Sometimes, however, these enzymes transform an otherwise harmless substance into a reactive form (Guengerich 2000). A classic example is the activation of benzo(*a*)pyrene, which is a constituent of tobacco smoke and combustion products, into reactive forms capable of binding to DNA and eventually leading to cancer formation. Activation of this kind is usually accomplished by CYP enzymes (Pelkonen & Raunio 1997).

Hepatic xenobiotic-metabolizing CYP enzymes have been widely studied due to their significance in the metabolism of the majority of clinically used drugs (Ingelman-Sundberg *et al.* 1999). Extrahepatic CYPs are of a minor importance in drug metabolism, but they are of consequence to the toxicity of extrahepatic organs (Raunio *et al.* 1995a). The interest in pulmonary CYP enzymes has been steadily growing because of the significance of CYPs for the formation of lung cancer. This study aimed to elucidate the expression of different CYP forms in human pulmonary tissues and to determine the regulation of certain CYP enzymes in human lung.

2 Review of the literature

2.1 Cytochrome P450 (CYP) enzymes

Cytochrome P450s constitute a superfamily of enzymes crucial for the oxidative, peroxidative, and reductive metabolism of a diverse group of compounds, including endobiotics, such as steroids, bile acids, fatty acids, prostaglandins, and leukotrienes, and xenobiotics, including most of the therapeutic drugs and environmental pollutants (Nelson *et al.* 1996, Bertz & Granneman 1997). The first report on the existence of a CYP enzyme or a "microsomal carbon monoxide-binding pigment", as it was called at that time, was published in 1958 by Klingenberg *et al*. This enzyme gave a unique 450-nm optical absorption peak, and when its hemoprotein nature was recognized, it was given the name cytochrome P450 (Omura 1999).

CYP enzymes are expressed ubiquitously in different life forms: they have been found in animals, plants, fungi, and bacteria (Nelson *et al.* 1996). They seem to be indispensable for eukaryotic species, but not for prokaryotes, since some bacteria lack CYP enzymes (Nelson 1999). Eukaryotics need CYPs for the biosynthesis of sterols, which are constituents of plasma membrane (Omura 1999). Eukaryotic CYP enzymes are membrane-bound, mostly localized to the endoplasmic reticulum, but some CYPs are also present in mitochondrial inner membranes. In order to function, cytochrome P450s require an electron transfer chain. In the endoplasmic reticulum, this source is NADPH-cytochrome P450 reductase, previously called NADPH-cytochrome c reductase (Omura 1999). In mitochondria, electrons are transferred from NADPH by redoxin reductase to redoxin and then to CYP (Gonzalez 1990). Despite their occasionally minimal sequence similarity, all CYPs have a similar structural fold with a highly conserved core (Graham & Peterson 1999).

Humans have been estimated to have at least 53 different CYP genes and 24 pseudogenes (Nelson 1999) (Dr. Nelson homepage: http://drnelson.utmem.edu/CytochromeP450.html). The notable diversity of CYP enzymes has given rise to a systematic classification of individual forms into families and subfamilies. The protein sequences within a given gene family are at least 40% identical (e.g. CYP2A6 and CYP2B6), and the sequences within a given subfamily are > 55% identical (e.g. CYP2A6 and CYP2A7) (Nelson *et al.* 1996). The italicized names refer to genes, e.g. *CYP2A13*.

There are 17 different families currently known in humans. The enzymes in the families 1-3 are mostly active in the metabolism of xenobiotics, whereas the other families have important endogenous functions (Table 1). Inactivating mutations in the CYPs with physiological functions often lead to serious diseases, whereas similar mutations in xenobiotic-metabolizing CYPs rarely do, although they affect the host's drug metabolism and susceptibility to some diseases, without directly causing disease (Nelson 1999).

Table 1. Human CYP families and their main functions. Data adapted from (Gonzalez 1992, Nelson et al. 1996, White et al. 1997, Nelson 1999, Lund et al. 1999).

CYP family	Main functions
CYP1	Xenobiotic metabolism
CYP2	Xenobiotic metabolism Arachidonic acid metabolism
CYP3	Xenobiotic and steroid metabolism
CYP4	Fatty acid hydroxylation
CYP5	Thromboxane synthesis
CYP7	Cholesterol 7α-hydroxylation
CYP8	Prostacyclin synthesis
CYP11	Cholesterol side-chain cleavage Steroid 11β-hydroxylation Aldosterone synthesis
CYP17	Steroid 17α-hydroxylation
CYP19	Androgen aromatization
CYP21	Steroid 21-hydroxylation
CYP24	Steroid 24-hydroxylation
CYP26	Retinoic acid hydroxylation
CYP27	Steroid 27-hydroxylation
CYP39	Unknown
CYP46	Cholesterol 24-hydroxylation
CYP51	Sterol biosynthesis

2.2 Human xenobiotic-metabolizing CYP enzymes

The CYP enzymes in the families 1-3 are active in the metabolism of a wide variety of xenobiotics, but some of them also metabolize endogenous compounds, such as steroid hormones and arachidonic acid (Gonzalez 1992, Capdevila *et al.* 2000). There is one enzyme in these families, CYP2J2, which has not been shown to metabolize foreign compounds (Wu *et al.* 1996). About half of the 53 human CYP forms belong to the families 1-3 (Nelson 1999). The functions and catalytic activities of some of these enzymes are still unknown. Especially the recent progress in the Human Genome Project

has produced new sequences of previously unknown CYPs. It is possible that some of these new enzymes do not metabolize xenobiotics, even though they belong to the families 1-3.

The majority of CYPs are expressed in human liver, but they are also expressed in extrahepatic tissues on a smaller scale (Raunio *et al.* 1995a). A few CYP forms participating in the metabolism of foreign compounds are found only in extrahepatic tissues. The expression is centered on the liver, due to its role as a port of entry for all ingested substances. Other tissues, such as lung and skin, also act as the first lines of defense against exogenous compounds, which partly explains the expression of CYPs in these tissues.

2.2.1 CYP1 family

Three genes, *CYP1A1, CYP1A2* and *CYP1B1,* are members of the CYP1 family. There are no pseudogenes in this family (Nelson *et al.* 1996). All the three genes share the main features of regulation; they are all transcriptionally controlled by the AHR-ARNT (aryl hydrocarbon receptor-aryl hydrocarbon receptor nuclear translocator) pathway (Schmidt & Bradfield 1996). They are also induced by polycyclic aromatic hydrocarbons (PAH), TCDD (Schmidt & Bradfield 1996), and smoking (Willey *et al.* 1997, Zevin & Benowitz 1999). However, there is variation in the extent and cell specificity of their expression and induction. Importantly, they all are active in the metabolism of PAHs into intermediates that can bind to DNA and, if the damage goes unrepaired, may produce mutations involved in neoplasmic transformation (Shimada *et al.* 1996a). Thus, they have been implicated in the formation of chemically caused cancers (Nebert *et al.* 1996). The regulation of the CYP1 family of genes by AHR-ARNT will be discussed in more detail in chapter 2.3.1.

2.2.1.1 CYP1A1

CYP1A1 is a major extrahepatic CYP enzyme (Raunio *et al.* 1995a). It contributes notably to the toxicity of many carcinogens, especially PAHs, since it is the principal enzyme activating them into DNA-binding forms (Shimada *et al.* 1996a). Its level of expression in human liver is very low (Edwards *et al.* 1998). CYP1A1 constitutive expression is also very low in extrahepatic tissues, but it is inducible by AHR ligands in almost every tissue studied, including lung, lymphocytes, mammary gland, and placenta (Raunio *et al.* 1995a). CYP1A1 is highly inducible by PAHs and also by cigarette smoke (Anttila *et al.* 1991). In human primary hepatocytes, CYP1A1 is induced by the AHR agonists 3-methylcholanthrene and omeprazole (Rodríguez-Antona *et al.* 2000, Bowen *et al.* 2000). Because of the significance of CYP1A1 in the activation of procarcinogens, there have been active efforts to link the polymorphisms of the *CYP1A1* gene with the individual susceptibility to chemically induced cancers, especially lung cancer (Raunio *et al.* 1995b). Seven variant alleles have been described, but none of them have been unequivocally shown to correlate with altered catalytic activity of the CYP1A1 protein

(CYP allele nomenclature committee homepage: http://www.imm.ki.se/cypalleles/). The T3801C (*MspI*) and I462V polymorphisms have been studied most. It is concluded that these polymorphisms are associated with a higher risk of lung cancer in the Japanese population, but not in Caucasians (Kawajiri 1999). Recently, CYP1A1 conditional knockout mice were produced. The lack of CYP1A1 protein had no effect on the expression of any other AHR-regulated genes (Dalton *et al.* 2000).

2.2.1.2 CYP1A2

The expression of CYP1A2 is quite strictly liver-specific, since no CYP1A2 protein has been detected in any other tissue (Raunio *et al.* 1995a). CYP1A2 constitutes about 13% of the total hepatic CYP content (Shimada *et al.* 1994, Imaoka *et al.* 1996). It activates PAHs, nitrosamines, aflatoxin B_1, and especially aryl amines into forms that can bind to DNA and produce mutations (Aoyama *et al.* 1990, Shimada *et al.* 1996a, Macé *et al.* 1997, Hammons *et al.* 1997, Hecht 1998). The regulation of CYP1A2 is both AHR-ARNT–dependent and independent (Landi *et al.* 1999). It is induced *in vivo* by cigarette smoke, charbroiled meat, cruciferous vegetables containing indole-3-carbinol, phenytoin, rifampicin, and omeprazole (Landi *et al.* 1999). AHR agonists induce CYP1A2 in human hepatocytes (Morel *et al.* 1990, Xu *et al.* 2000). There are 6 variant alleles of the *CYP1A2* gene, and two of these correlate with increased and decreased induction by smoking (Sachse *et al.* 1999, Nakajima *et al.* 1999). These variants could partially explain the observed high level of interindividual variation in the enzymatic activity measured *in vivo* with caffeine as a probe (Kalow & Tang 1991, Butler *et al.* 1992). CYP1A2 is the main CYP catalyzing the metabolism of several drugs, including clozapine, theophylline, and tacrine (Landi *et al.* 1999). Two strains of CYP1A2-null mice have been produced, which develop normally but have altered drug metabolism (Pineau *et al.* 1995, Liang *et al.* 1996, Gonzalez & Kimura 1999).

2.2.1.3 CYP1B1

Similarly to CYP1A1, CYP1B1 is also mainly an extrahepatic CYP form expressed in almost every tissue, including kidney, prostate, mammary gland, and ovary (Sutter *et al.* 1994, Shimada *et al.* 1996a, Tang *et al.* 1999). In general, CYP1B1 basal expression is higher compared to CYP1A1 (Shimada *et al.* 1996a, Eltom *et al.* 1998). The expression of CYP1B1 in human liver is nonexistent (Sutter *et al.* 1994, Shimada *et al.* 1996a, Hakkola *et al.* 1997, Edwards *et al.* 1998, Tang *et al.* 1999). It has been suggested to be overexpressed in tumors (Murray *et al.* 1997). The induction of CYP1B1 is regulated by the AHR-ARNT pathway (Sutter *et al.* 1994, Savas & Jefcoate 1994), although the responses of CYP1B1 to AHR ligands differ from those of CYP1A1 (Hakkola *et al.* 1997). CYP1B1 catalyzes the metabolism of PAHs and aryl amines (Shimada *et al.* 1996a). The *CYP1B1* gene has several alleles, and interestingly, the functionally impaired alleles have been shown to be linked with human primary congenital glaucoma (Stoilov *et al.* 1997, Stoilov *et al.* 1998). This finding demonstrates that even the CYPs classified

as "xenobiotic-metabolizing" enzymes may have important functions in modulating growth and differentiation. This is in striking contrast to CYP1B1-null mice, which develop normally and have no observable phenotype, demonstrating that CYP1B1 is not required for mouse development (Buters *et al.* 1999). CYP1B1-null mice are protected against 7,12-dimethylbenz[a]anthracene-induced lymphomas (Buters *et al.* 1999). CYP1B1 allelic variants that affect the rate of conversion of estradiol into carcinogenic 4-hydroxyestradiol have been described (Shimada *et al.* 1999, Li *et al.* 2000).

2.2.2 CYP2 family

The human CYP2 family is a heterogeneous group of enzymes. It contains the subfamilies CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, CYP2F, and CYP2J (Nelson *et al.* 1996). CYP2B6, CYP2D6, CYP2E1, CYP2F1, and CYP2J2 are the only functional members in their respective subfamilies, whereas the CYP2A subfamily contains two and CYP2C four functional enzymes. Unlike the CYP1 family, the members of the CYP2 family do not share features of regulation. The substrate and tissue specificities of these enzymes also differ markedly.

2.2.2.1 CYP2A

The human CYP2A subfamily contains three genes i.e. *CYP2A6, CYP2A7,* and *CYP2A13,* and two pseudogenes located in the *CYP2A-2B-2F* gene cluster on chromosome 19 (Hoffman *et al.* 1995). The CYP2A6 protein has been detected in liver (Yun *et al.* 1991), where it constitutes about 4% of total CYP content (Shimada *et al.* 1994, Imaoka *et al.* 1996). There is also evidence of the expression of a CYP2A-related protein in adult and fetal nasal mucosa (Getchell *et al.* 1993, Su *et al.* 1996, Gu *et al.* 2000). There has been a growing interest towards CYP2A6, due to its major role in the metabolism of nicotine *in vitro* (Nakajima *et al.* 1996, Messina *et al.* 1997, Yamazaki *et al.* 1999) and *in vivo* (Kitagawa *et al.* 1999) and in the activation of tobacco-specific nitrosamine NNK (4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (Yamazaki *et al.* 1992, Hecht 1998). Surprisingly, a recent study indicated that CYP2A6 is also involved in the formation of NNK from nicotine (Hecht *et al.* 2000). CYP2A6 catalyzes the metabolism of the gasoline additive MTBE (Hong *et al.* 1999) and some pharmaceuticals, including halothane and losigamone (Raunio *et al.* 1999b).

The genetic polymorphisms of *CYP2A6* have been associated with interindividual differences in smoking behavior (Pianezza *et al.* 1998), although this report has methodological shortcomings (Oscarson *et al.* 1998). Deletions of the *CYP2A6* gene have also been connected to a reduced risk of lung cancer (Miyamoto *et al.* 1999). Coumarin has been used as a probe drug to assess the activity of CYP2A6 *in vivo* (Cholerton *et al.* 1992, Rautio *et al.* 1992, Iscan *et al.* 1994). Very little is known about the mechanisms of regulation of CYP2A6 (Raunio *et al.* 1999b). It is induced *in vivo* by phenobarbital and other antiepileptic drugs (Sotaniemi *et al.* 1995). In human hepatocytes, CYP2A6 is induced by phenobarbital and rifampicin (Dalet-Beluche *et al.* 1992, Rodríguez-Antona *et*

al. 2000). The CYP2A7 protein is non-functional due to its inability to incorporate heme (Yamano *et al.* 1990). Relatively high levels of CYP2A13 mRNA have been detected in human lung and adult and fetal nasal mucosa (Koskela *et al.* 1999, Gu *et al.* 2000, Su *et al.* 2000), and recent results indicate that heterologously expressed CYP2A13 is highly active in the activation of NNK (Su *et al.* 2000).

2.2.2.2 CYP2B6

CYP2B6 is a minor CYP form in human liver, accounting for only 1-2% of total hepatic CYP (Mimura *et al.* 1993, Shimada *et al.* 1994, Imaoka *et al.* 1996). Its expression appears to be regulated tissue-specifically, since in lung and kidney it is expressed as a splicing variant (Czerwinski *et al.* 1994, Nelson *et al.* 1996, Gervot *et al.* 1999). This splicing variant was previously called CYP2B7. The substrates for CYP2B6 include 6-aminochrysene (Mimura *et al.* 1993), methoxychlor (Dehal & Kupfer 1994), NNK (Code *et al.* 1997), and cyclophosphamide (Chang *et al.* 1993). The CYP2B forms in rodents are typically induced by phenobarbital, the classic CYP inducer (Honkakoski & Negishi 1998). Human CYP2B6 is also induced by phenobarbital and rifampicin in primary hepatocytes (Chang *et al.* 1997, Gervot *et al.* 1999, Rodríguez-Antona *et al.* 2000, Pascussi *et al.* 2000). This induction is mediated by nuclear receptor CAR (constitutively active receptor)(Sueyoshi *et al.* 1999) and probably also by PXR (pregnane X receptor) (Pascussi *et al.* 2000) (see chapters 2.3.2. and 2.3.3.). The human CYP2B subfamily also contains the *CYP2B7P* pseudogene (Nelson *et al.* 1996). The *CYP2B* genes are located in the *CYP2A-2B-2F* gene cluster on chromosome 19 (Hoffman *et al.* 1995).

2.2.2.3 CYP2C

The human CYP2C subfamily contains four highly homologous genes: *2C8*, *2C9*, *2C18* and *2C19*, which are located in a cluster on chromosome 10 (Gray *et al.* 1995, Nelson *et al.* 1996). Interestingly, the splicing of CYP2C mRNA transcripts has been shown to produce chimeric mRNAs containing exons from several CYP2C genes (Finta & Zaphiropoulos 2000). The biological function of these mRNAs is unknown. CYP2C accounts for about 20% of the human total liver CYP content (Shimada *et al.* 1994, Imaoka *et al.* 1996). CYP2C9 is the main CYP2C in human liver, followed by CYP2C8 and CYP2C19 (Edwards *et al.* 1998). The CYP2C18 protein is not expressed in liver (Richardson *et al.* 1997). CYP2C mRNA and protein are induced in primary hepatocytes by phenobarbital and rifampicin (Morel *et al.* 1990) (Chang *et al.* 1997). Recent results indicate that PXR and CAR mediate CYP2C8 and CYP2C9 induction in human hepatocytes (Pascussi *et al.* 2000). However, there are also reports showing no induction of CYP2C9 and CYP2C19 mRNAs (Rodríguez-Antona *et al.* 2000) and proteins (Runge *et al.* 2000). Rifampicin and barbiturates can induce CYP2C proteins and related activities *in vivo* (Zilly *et al.* 1977, Perrot *et al.* 1989, Treluyer *et al.* 1997). Recently, it was postulated that CYP2C8 has important physiological functions in the production of endothelium-derived hyperpolarizing factor (EDHF) (Fisslthaler *et al.* 1999).

Pharmaceutical substrates for CYP2C include diazepam, omeprazole, mephenytoin, tolbutamide, and warfarin (Guengerich 1995) as well as many non-steroidal anti-inflammatory drugs (Pelkonen *et al.* 1998). Selective substrates include taxol for CYP2C8, tolbutamide for CYP2C9, and mephenytoin for CYP2C19 (Pelkonen *et al.* 1998). The CYP2C19 poor metabolizer phenotype is detected in 2-4 % of Caucasians and in about 20% of Asians (Ingelman-Sundberg *et al.* 1999). CYP2C9 also has two variants (CYP2C9*2 and CYP2C9*3) with decreased metabolism (Miners & Birkett 1998, Ingelman-Sundberg *et al.* 1999).

2.2.2.4 CYP2D6

The CYP2D subfamily has one gene and four pseudogenes (Nelson *et al.* 1996). The CYP2D6 polymorphism was the first defect in drug metabolism to be specifically associated with altered expression of CYP enzyme (Gonzalez *et al.* 1988). The CYP2D6 poor metabolizer (PM) phenotype is detected in about 6% of Caucasians (Ingelman-Sundberg *et al.* 1999), and it has profound effects on the metabolism of several commonly used pharmaceuticals, including several tricyclic antidepressants, haloperidol, metoprolol, propranolol, codeine, and dextromethorphan (Pelkonen *et al.* 1998). There are at least 30 different defective CYP2D6 alleles, six of which contribute to 95-99% of PM phenotypes (Ingelman-Sundberg *et al.* 1999). Interestingly, duplications of the CYP2D6 gene up to 13 gene copies have been reported (Johansson *et al.* 1993), giving rise to the ultra-rapid metabolizer phenotype. Ultra-rapid metabolizers show increased metabolism and decreased drug effects of CYP2D6 substrates, such as tricyclic antidepressants (Dalen *et al.* 1998). About 4% of Caucasians have multiple *CYP2D6* genes (Ingelman-Sundberg *et al.* 1999). It has been speculated that since CYP2D6 is not inducible, the duplications are a way to adapt to environmental chemical pressures, most likely to alkaloids in the diet (Ingelman-Sundberg *et al.* 1999). CYP2D6 polymorphisms have been linked to altered susceptibility to Parkinson's disease and lung cancer. A meta-analysis did not reveal a link to Parkinson's disease, but PM individuals seem to be somewhat protected (OR = 0.69) against lung cancer (Rostami-Hodjegan *et al.* 1998). CYP2D6 has a minor, but not crucial, role in the activation of tobacco-derived nitrosamine NNK (Crespi *et al.* 1991, Hecht 1998). It has been speculated that the CYP2D6 polymorphism might affect the risk of lung cancer through modulating smoking behavior, since CYP2D6 might be involved in the signal transduction of the dopaminergic pathway in brain (Saarikoski *et al.* 2000). CYP2D6 constitutes about 2% of total hepatic CYP (Shimada *et al.* 1994, Imaoka *et al.* 1996), and the protein is also expressed in duodenum and brain (Pelkonen & Raunio 1997).

2.2.2.5 CYP2E1

CYP2E1 is the only gene in this subfamily (Nelson *et al.* 1996). The CYP2E1 enzyme has been studied extensively due to its role in the metabolism of ethanol and also as an activator of chemical carcinogens (Lieber 1997). CYP2E1 also actives some tobacco-

specific nitrosamines, but not NNK (Yamazaki *et al.* 1992, Kushida *et al.* 2000), the most important nitrosamine in tobacco (Hecht 1999). Most of the over 70 substrates demonstrated are small and hydrophobic compounds (Ronis *et al.* 1996), including only a few pharmaceuticals, such as paracetamol, chlorzoxazone, enflurane, and halothane (Guengerich 1995). Disulfiram is a clinically used inhibitor of CYP2E1 (Guengerich & Shimada 1991). About 7% of the liver CYP content consists of CYP2E1 (Shimada *et al.* 1994, Imaoka *et al.* 1996). It is also expressed in lung and brain (Raunio *et al.* 1995a). The regulation of CYP2E1 is complex, since it is regulated transcriptionally, pretranslationally, translationally, and posttranslationally (Song 1995). Transcriptional regulation seems to play a minor role, in contrast to many other CYP forms. Many substrates of CYP2E1 are also CYP2E1-inducing agents, including acetone, ethanol, pyridine, pyrazole, and isoniazid (Ronis *et al.* 1996). Ethanol intake increases the human CYP2E1 content in liver *in vivo* (Perrot *et al.* 1989), and it is also induced in lymphocytes of poorly controlled insulin-dependent diabetics (Song *et al.* 1990). The regulation of CYP2E1 will be discussed in more detail in chapter 2.3.5. In addition to activating procarcinogens, CYP2E1 also produces free radicals causing tissue injury. These radicals are formed both in the absence and in the presence of substrate (Lieber 1997). Several allelic variants of the *CYP2E1* gene have been detected. Three of these cause amino acid substitutions (Hu *et al.* 1997, Fairbrother *et al.* 1998). One of these alleles (CYP2E1*2) produces a protein with reduced stability (Hu *et al.* 1997), while another (CYP2E1*1D) is associated with increased activity after alcohol exposure and in obese subjects (McCarver *et al.* 1998). CYP2E1 knockout mice develop normally and are protected against paracetamol-induced hepatotoxicity (Lee *et al.* 1996).

2.2.2.6 CYP2F1

The *CYP2F1* gene is located in the *CYP2A-2B-2F* gene cluster in chromosome 19 (Hoffman *et al.* 1995). Interestingly, it has two full-length copies in addition to one pseudogene, but it is not known whether both of them produce functional transcripts (Hoffman *et al.* 1995). CYP2F1 mRNA has been identified in human lung (Nhamburo *et al.* 1990) and placenta (Hakkola *et al.* 1996), but not in liver (Hakkola *et al.* 1994). No expression of CYP2F1 protein has been demonstrated in any human tissue. Recombinant CYP2F1 enzyme is capable of activating pulmonary toxicants 3-methylindole and naphthalene (Lanza *et al.* 1999).

2.2.2.7 CYP2J2

CYP2J2 is expressed mainly extrahepatically in heart, kidney (Wu *et al.* 1996), lung (Zeldin *et al.* 1996), pancreas (Zeldin *et al.* 1997b), and gastrointestinal tract (Zeldin *et al.* 1997a). CYP2J2 is involved in the metabolism of arachidonic acid into epoxyeicosatrienoic acids (EETs), which have physiological functions (Wu *et al.* 1996, Zeldin *et al.* 1997a, Capdevila *et al.* 2000). CYP2J2 has not been demonstrated to exhibit activity towards xenobiotics.

2.2.3 CYP3 family

The human CYP3 family contains only one subfamily (Nelson *et al.* 1996). CYP3A includes four genes, *CYP3A4*, *CYP3A5*, *CYP3A7*, and the recently identified *CYP3A43*. The CYP3A enzymes have overlapping catalytic specificities. Their tissue expression patterns differ, however, as CYP3A4 is mainly expressed in liver, CYP3A5 in extrahepatic tissues, and CYP3A7 in fetal liver (Thummel & Wilkinson 1998). CYP3A4 and CYP3A7 are regulated by PXR, whereas CYP3A5 is controlled by glucocorticoid receptor.

2.2.3.1 CYP3A4

The CYP3A4 enzyme is the most important drug-metabolizing CYP in human liver. About 30-40% of the total hepatic CYP content consists of CYP3A4 (Shimada *et al.* 1994, Imaoka *et al.* 1996) and it is also present in small intestine (Kolars *et al.* 1992). It has been estimated that about 50% of the drugs metabolized by CYPs are metabolized by CYP3A4 (Bertz & Granneman 1997). The substrates for this enzyme include drugs, such as quinidine, nifedipine, diltiazem, lidocaine, lovastatin, erythromycin, cyclosporin, triazolam, and midazolam, and endogenous substances, including testosterone, progesterone, and androstenedione (Pelkonen *et al.* 1998, Guengerich 1999). Midazolam and erythromycin have been used as *in vivo* probes for CYP3A4 activity (Thummel & Wilkinson 1998). CYP3A4 also activates procarcinogens, including aflatoxin B_1 (Aoyama *et al.* 1990), PAHs, NNK (Hecht 1999), and 6-aminochrysene (Yamazaki *et al.* 1995). CYP3A4 is induced in human hepatocytes by rifampicin (Morel *et al.* 1990, Schuetz *et al.* 1993), dexamethasone (Pichard *et al.* 1992, Schuetz *et al.* 1993, Kocarek *et al.* 1995), and phenobarbital (Schuetz *et al.* 1993, Kocarek *et al.* 1995) among others. CYP3A4 is induced *in vivo* by rifampicin and barbiturates in liver (Perrot *et al.* 1989, Ged *et al.* 1989) and by rifampicin in small intestine (Kolars *et al.* 1992). The induction of CYP3A4 is mainly regulated by the novel orphan receptor PXR (Lehmann *et al.* 1998, Moore *et al.* 2000a), but also other receptors, including CAR and, indirectly, the glucocorticoid receptor are involved (Sueyoshi *et al.* 1999, Pascussi *et al.* 2000, Moore *et al.* 2000a)(See chapters 2.3.2. and 2.3.3.). Three variant alleles have been detected for the *CYP3A4* gene. A 5'-flanking allelic variant has been associated with prostate cancer (Rebbeck *et al.* 1998) and leukemia (Felix *et al.* 1998). However, the functional significance of this alteration is uncertain (Westlind *et al.* 1999). Recently, two other amino acid alterations have been discovered, one of which encodes an enzyme with reduced activity (Sata *et al.* 2000).

2.2.3.2 CYP3A5

CYP3A5 is expressed polymorphically in human liver (Wrighton *et al.* 1989), but consistently in lung (Kivistö *et al.* 1996), colon (Gervot *et al.* 1996), kidney (Schuetz *et al.* 1992, Haehner *et al.* 1996), oesophagus (Lechevrel *et al.* 1999), and anterior pituitary gland (Murray *et al.* 1995), demonstrating CYP3A5 to be a more extrahepatic CYP3A form. About 20-25% of livers have substantial levels of CYP3A5 protein (Aoyama *et al.* 1989, Wrighton *et al.* 1989). However, more sensitive protein or mRNA detection methods reveal CYP3A5 in almost every liver sample (Jounaidi *et al.* 1996, Edwards *et al.* 1998). There are some variant alleles of the *CYP3A5* gene (Jounaidi *et al.* 1996, Paulussen *et al.* 2000). An allelic variant with linked –45A>G and –369T>G sequence mutations has been shown to lead to increased expression of CYP3A5 protein in liver (Paulussen *et al.* 2000). There are also two *CYP3A5* pseudogenes (Nelson *et al.* 1996). In comparison to CYP3A4, CYP3A5 shows roughly the same substrate preference pattern, but the turnover rates are usually lower (Aoyama *et al.* 1989, Wrighton *et al.* 1990, Yamazaki *et al.* 1995). However, CYP3A5 is unable to metabolize some CYP3A4 substrates, including erythromycin and quinidine (Wrighton *et al.* 1990). CYP3A5 is generally thought to be uninducible in human liver and primary hepatocytes (Wrighton *et al.* 1989, Schuetz *et al.* 1993, Chang *et al.* 1997), but there is some evidence for the induction of CYP3A5 in colon carcinoma cell lines by reserpine and clotrimazole (Schuetz *et al.* 1996). Surprisingly, in a recent study, CYP3A5 mRNA was induced in human primary hepatocytes by rifampicin and phenobarbital (Rodríguez-Antona *et al.* 2000). The promoter region of the *CYP3A5* gene has been shown to contain functional glucocorticoid-responsive element half-sites that mediate induction by dexamethasone in the HepG2 cell line (Schuetz *et al.* 1996). Unlike the other CYP3A enzymes, CYP3A5 is probably not regulated by PXR (Blumberg *et al.* 1998).

2.2.3.3 CYP3A7

CYP3A7 is mainly expressed in human fetal liver, where it is the major CYP form (Kitada & Kamataki 1994). Low levels of CYP3A7 mRNA have also been detected in adult liver (Hakkola *et al.* 1994, Schuetz *et al.* 1994). CYP3A7 has similar catalytic properties compared with other CYP3A enzymes, including testosterone 6β-hydroxylation (Kitada *et al.* 1985, Kitada *et al.* 1987, Kitada *et al.* 1991). CYP3A7 is induced in adult human primary hepatocytes by rifampicin (Greuet *et al.* 1996), and this induction is mediated by the PXR pathway (Pascussi *et al.* 1999). Whether or not CYP3A7 is induced in fetal liver is unknown.

2.2.4 CYP4B1

CYP4B1 is the only CYP4 family member with activity towards xenobiotics. It was isolated from a lung cDNA library, and the mRNA was found to be expressed in human lung, but not in liver (Nhamburo *et al.* 1989). mRNA expression has also been demonstrated in human colon (McKinnon *et al.* 1994) and placenta (Yokotani *et al.* 1990, Hakkola *et al.* 1996). Human CYP4B1 protein expression was demonstrated recently in bladder (Imaoka *et al.* 2000). Heterologously expressed CYP4B1 catalyzes 6β-hydroxylation of testosterone, a typical CYP3A reaction, but not 2-aminofluorene or 4-ipomeanol, which are typical CYP4B1 reactions in animals (Nhamburo *et al.* 1989, Waxman *et al.* 1991, Czerwinski *et al.* 1991). However, one study showed that CYP4B1 is not functional due to its inability to incorporate heme, and it was speculated that the original human CYP4B1 expression vector had been contaminated by CYP3A5, suggesting that the earlier results on expressed CYP4B1 are erroneous (Zheng *et al.* 1998). In contradiction to this, it was recently shown that human kidney microsomes catalyzed 2-aminofluorene and this reaction was inhibited by CYP4B1 antibody, suggesting that human CYP4B1 could be functional and also catalyze 2-aminofluorene (Imaoka *et al.* 2000).

2.2.5 Novel CYPs

The Human Genome Project has revealed new CYP genes not previously discovered. The Cytochrome P450 homepage provided by Dr. David R. Nelson (drnelson.utmem.edu/ CytochromeP450.html) lists four new genes (CYP2R1, CYP2S1, CYP2U1 and CYP3A43) as well as several new pseudogenes in the families 1-3. As most of these genes are from a draft sequence, their existence and classification may change. Almost nothing is known about these new CYPs yet. Preliminary results show that CYP3A43 mRNA is expressed in liver, small intestine, and fetal liver (Westlind *et al.* 2000).

2.3 Regulation of human xenobiotic-metabolizing CYP expression

2.3.1 Aryl hydrocarbon receptor (AHR)

Aryl hydrocarbon receptor (AHR) is a basic helix-loop-helix (bHLH) protein belonging to the Per-Arnt-Sim (PAS) family of transcription factors. It transcriptionally induces expression of human CYP1A1, CYP1A2, and CYP1B1 (Quattrochi *et al.* 1994, Tang *et al.* 1996, Whitlock 1999), as well as several other genes, including some phase II metabolizing enzymes (Schmidt & Bradfield 1996). AHR ligands include PAHs and TCDD (Whitlock 1999). The unliganded AHR is maintained in cytoplasm in a complex containing chaperon proteins, such as a dimer of HSP90 (heat shock protein 90), ARA9 (also called AIP1 and XAP2) and p23 (Figure 1). These other proteins are involved in the

correct folding and stabilization of AHR (Gu *et al.* 2000). Upon ligand binding, AHR sheds the chaperon proteins and translocates to the nucleus, where it forms a heterodimer with the AHR nuclear translocator (ARNT) (Hoffman *et al.* 1991). This heterodimer binds to the xenobiotic response elements (XRE) of CYP genes activating transcription (Hankinson 1994). ARNT also belongs to the bHLH/PAS family. A novel PAS protein called AHR repressor inhibits AHR signal transduction by competing with AHR for ARNT and also by binding to XRE. The AHR repressor is induced by AHR, thus forming a negative feedback loop for the regulation of AHR (Mimura *et al.* 1999, Gu *et al.* 2000). Protein kinase C and tyrosine kinase are involved in AHR signal transduction, as inhibitors of these kinases block the induction of target genes (Carrier *et al.* 1992, Berghard *et al.* 1993, Gradin *et al.* 1994, Kikuchi *et al.* 1998). Three AHR knockout mice have been generated, which exhibit decreased liver size, hepatic fibrosis, decreased constitutive expression of CYP1A2, and resistance to TCDD-elicited CYP1A1 induction (Fernandez-Salguero *et al.* 1995, Schmidt *et al.* 1996, Mimura *et al.* 1997, Lahvis & Bradfield 1998). Two strains of ARNT-null mice have also been generated, but these mice die *in utero* (Kozak *et al.* 1997, Maltepe *et al.* 1997).

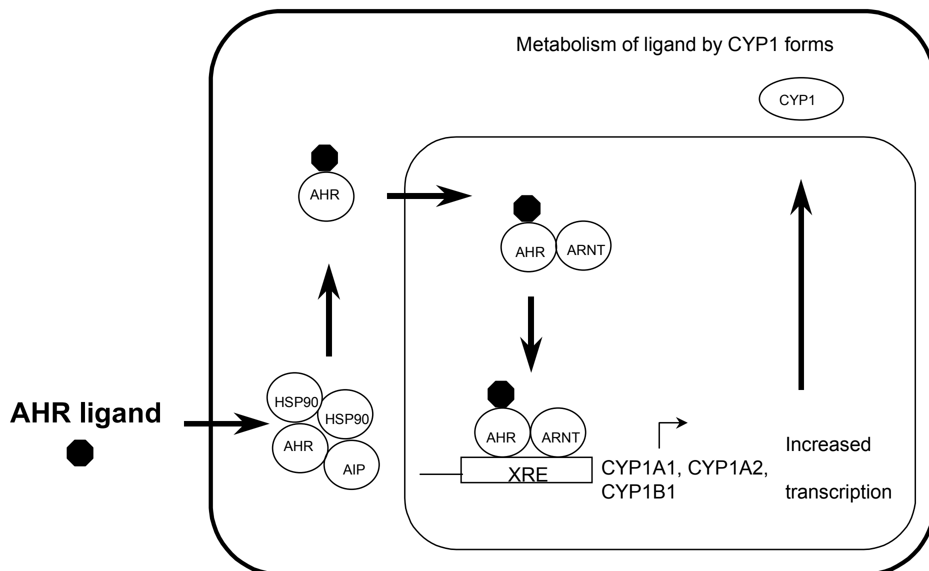

Fig. 1. Schematic presentation of the mechanism of AHR-mediated CYP1 induction.

2.3.2 Pregnane X receptor (PXR)

The pregnane X receptor (PXR, also called SXR or PAR) is a recently identified orphan nuclear receptor (Kliewer *et al.* 1998). It mediates the induction of CYP3A4 (Bertilsson *et al.* 1998, Blumberg *et al.* 1998, Lehmann *et al.* 1998) and CYP3A7 (Pascussi *et al.* 1999) as well as the human carboxylesterases HCE-1 and HCE-2 (Zhu *et al.* 2000).

Recent results indicate that CYP2C8 and CYP2C9 are also regulated by PXR (Pascussi *et al.* 2000). PXR, similarly to its principal target gene CYP3A4, is mainly expressed in liver, small intestine, and colon (Bertilsson *et al.* 1998, Blumberg *et al.* 1998, Lehmann *et al.* 1998). Its ligands include a wide variety of structurally diverse, low-affinity exogenous and endogenous chemicals, e.g. steroid hormones and steroid metabolites, such as progesterone, estrogen, corticosterone, 5β-pregnane, and androstanol (Blumberg *et al.* 1998, Moore *et al.* 2000a), and dietary compounds, such as coumestrol (Blumberg *et al.* 1998) and carotenoids (Pichard-Garcia *et al.* 2000). Recently, it was shown that hyperforin, a constituent of St. John's wort, a herbal remedy for depression, is the most potent PXR activator reported with EC_{50} of 23 nM (Moore *et al.* 2000b). Pharmaceuticals activating PXR include rifampicin, phenobarbital, nifedipine, clotrimazole, RU486 (mifepristone), and metyrapone (Harvey *et al.* 2000, Moore *et al.* 2000a). Many of the PXR ligands are also shared by CAR (Moore *et al.* 2000a) (discussed in chapter 2.3.3.). Upon ligand binding, PXR forms a heterodimer with the retinoid X receptor-α (RXRα) and transactivates ER6 (everted repeat with a 6 bp spacer) elements upstream of the CYP genes (Figure 2) (Waxman 1999). RXR serves as a common heterodimerization partner for many orphan nuclear receptors, including CAR. The binding of PXR/RXR to ER6 is followed by recruitment of coactivator proteins, e.g. SRC-1 (steroid receptor coactivator-1), and transcriptional activation of the respective gene (Savas *et al.* 1999). A recent report provided evidence for a second binding site for PXR in the ~ 7800 bp upstream 5'-flanking region of the *CYP3A4* gene having ER6-like binding sites (Goodwin *et al.* 1999). PXR and RXRα are induced by glucocorticoid receptor (GR) (Pascussi *et al.* 2000). Thus, the activation of GR by glucocorticoids, such as dexamethasone, leads to the induction of PXR/RXR and to the increase of CYP3A4 induction by endogenous and exogenous compounds. PXR, and also CAR, expression is down-regulated by the inflammatory cytokine interleukin-6 (Pascussi *et al.* 2000). This could partly explain the observed repression of several CYPs by cytokines (Abdel-Razzak *et al.* 1993, Muntane-Relat *et al.* 1995). PXR-null mice were recently produced showing no induction by typical mouse CYP3A inducers (Xie *et al.* 2000). The loss of PXR did not alter the basal CYP3A expression in mice. Transgenic mice containing human PXR were also produced showing induction by human specific inducers, such as rifampicin.

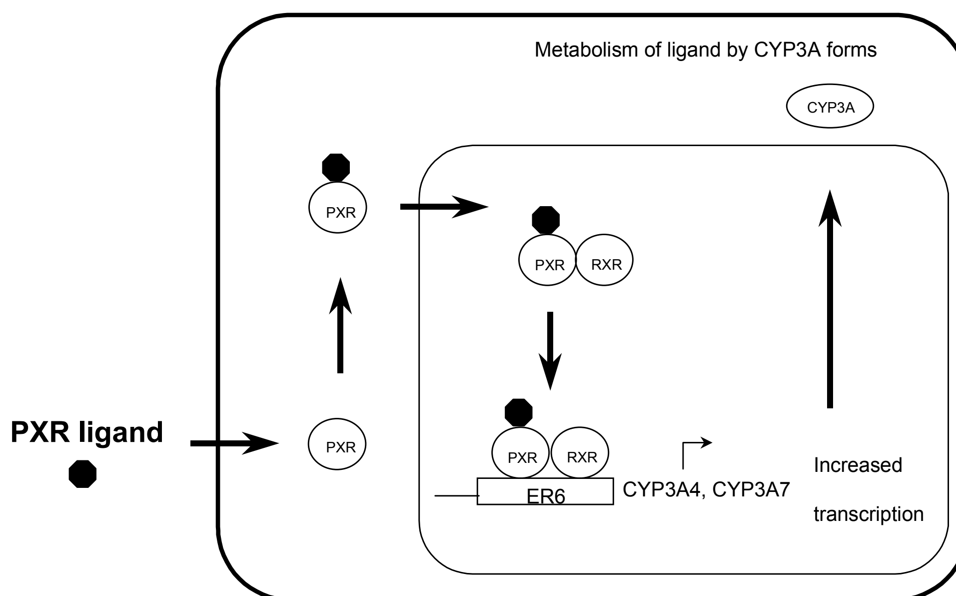

Fig. 2. Schematic presentation of the mechanism of PXR-mediated CYP3A induction.

2.3.3 Constitutively active receptor (CAR)

Constitutively active receptor (CAR, also called constitutive androstane receptor) is a novel orphan nuclear receptor, which was originally characterized as a constitutive activator of retinoid acid response elements (RARE). It is called “constitutive” because of its ability to transactivate RAREs and other response elements without being bound to ligand (Baes *et al.* 1994, Tzameli *et al.* 2000). CAR is predominantly expressed in liver (Baes *et al.* 1994), and it mediates the induction of CYP2B6 and, to a lesser extent, CYP3A4 (Sueyoshi *et al.* 1999, Tzameli *et al.* 2000). Recent results indicate that CYP2C8 and CYP2C9 are also regulated by CAR (Pascussi *et al.* 2000). CAR is down-regulated by the inflammatory cytokine interleukin-6, which could explain the repression of CYPs by inflammatory mediators (Abdel-Razzak *et al.* 1993, Muntane-Relat *et al.* 1995). Importantly, CAR was recently shown to mediate the widely studied induction of CYP2B genes by phenobarbital, the classic inducer of xenobiotic metabolism (discussed below) (Honkakoski *et al.* 1998a). However, the only activator shown to bind to human CAR is 5β-pregnane. Phenobarbital is not a CAR ligand (Moore *et al.* 2000a). Deactivators or inverse agonists, such as androstanol and clotrimazole, also bind to human CAR (Forman *et al.* 1998, Moore *et al.* 2000a). CAR acts differently than the more traditional receptors: as mentioned above, CAR is constitutively active without ligand. Upon binding an inverse agonist, CAR is deactivated through the release of the co-activator SRC-1 from the ligand-binding domain (Forman *et al.* 1998, Moore *et al.* 2000a). In contrast, agonist binding to CAR results in a further increase in the basal binding of CAR to SRC-1 (Moore *et al.* 2000a). Similarly to PXR, CAR requires the

heterodimerization partner RXR to enable binding to DNA. CAR/RXR heterodimers bind to a conserved 51-base pair element called PBREM (phenobarbital-responsive enhancer module) in the 5'-flanking region of the *CYP2B* genes and to the ER6 element of the *CYP3A4* gene (Honkakoski *et al.* 1998a, Sueyoshi *et al.* 1999). PBREM has been shown to mediate the induction by phenobarbital and phenobarbital-like inducers (Honkakoski *et al.* 1998b). It has been proposed that CAR is deactivated *in vivo* by endogenous inverse agonist steroids related to androstanol, thus suppressing CYP2B6 transcription (Figure 3). This suppression is overcome by agonist binding to CAR, which abolishes the inhibitory inverse agonists from CAR leading to the induction of CYP2B6 (Waxman 1999).

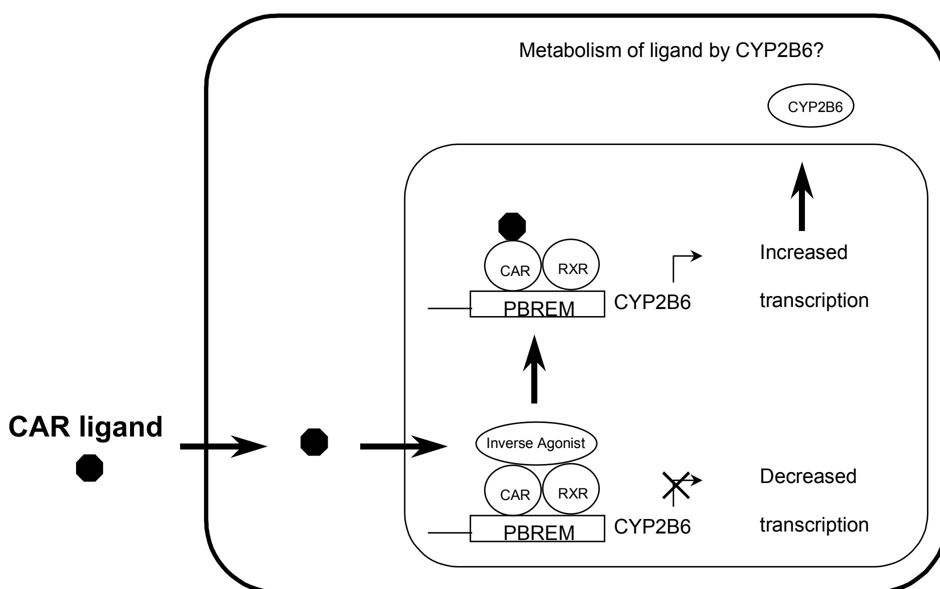

Fig. 3. Schematic presentation of the proposed mechanism of CAR-mediated CYP2B6 induction.

Phenobarbital is the classic archetype of a large number of structurally diverse compounds inducing numerous xenobiotic-metabolizing enzymes as well as affecting various other cellular processes. This group includes organic solvents, pesticides, polychlorinated biphenyls, and certain drugs (Honkakoski & Negishi 1998). The xenobiotic-metabolizing genes induced by phenobarbital include CYPs in the subfamilies 2A, 2B, 2C and 3A. The most effectively induced genes are members of the CYP2B family (Denison & Whitlock 1995), CYP2B6 in humans (Chang *et al.* 1997). As mentioned above, phenobarbital induction of CYP2B6 is mediated by CAR, even though phenobarbital is not a ligand of CAR (Honkakoski & Negishi 1998, Moore *et al.* 2000a). The exact mechanism of phenobarbital induction is still unclear, but recent unpublished results suggest that phenobarbital not only facilitates the translocation of CAR to the nucleus, but also activates CAR in the nucleus (Figure 4). These steps are dependent on phosphorylation, since translocation and activation are inhibited by protein phosphatase (PP) and CaM kinase (CK) inhibitors, respectively (Negishi 2000). This model is

supported by the finding that, in mouse primary hepatocytes, CAR is located in the cytoplasm and is only translocated to the nucleus after inducer treatment (Kawamoto *et al.* 1999). Thus, the regulation of CAR function would be dependent not only on the repression and derepression of constitutive activity, but also on the nuclear translocation and activation of CAR (Honkakoski & Negishi 2000, Tzameli *et al.* 2000). Recently, CAR-null mice were produced showing no induction of CYP2B by phenobarbital (Wei *et al.* 2000). Also, basal expression of CYP2B was decreased demonstrating that CAR does have constitutive activity.

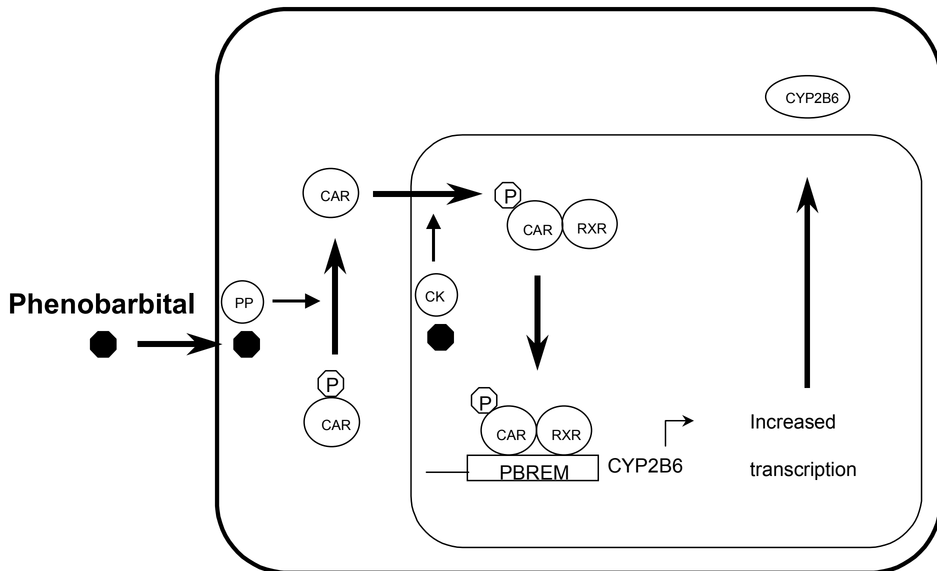

Fig. 4. Schematic presentation of the possible role of phenobarbital on CYP2B6 induction.

2.3.4 Glucocorticoid receptor (GR)

Glucocorticoids, of which dexamethasone is the most widely studied, influence several aspects of CYP induction. However, most of these effects are not dependent on GR binding to CYP genes, but rather on complex protein-protein interplay between GR and various other receptors (Honkakoski & Negishi 2000). For example, dexamethasone has been shown to potentiate CYP1A1 induction by TCDD (Celander *et al.* 1997). Dexamethasone induces PXR and RXR expression, leading to an increase in CYP3A4 induction by PXR agonists (Pascussi *et al.* 2000). This explains the results on the dexamethasone-elicited induction of CYP3A4 in human hepatocytes (Pichard *et al.* 1992, Schuetz *et al.* 1993). The only human CYP gene induced directly by GR is *CYP3A5* (Figure 5). There is no consensus glucocorticoid responsive element in the *CYP3A5* gene, but instead GR binds to the glucocorticoid responsive element half-sites in the 5'-flanking region of *CYP3A5* (Schuetz *et al.* 1996).

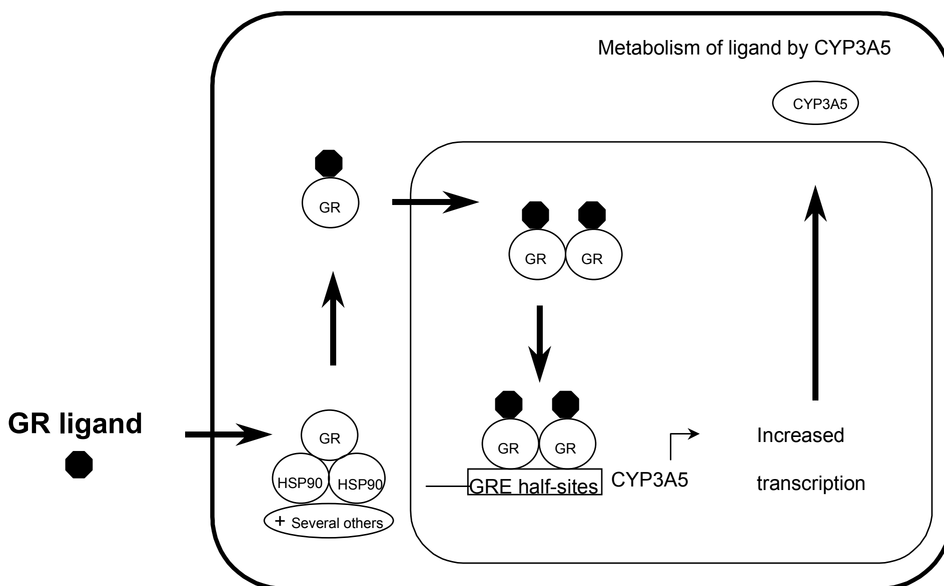

Fig. 5. Schematic presentation of the mechanism of GR-mediated CYP3A5 induction.

2.3.5 Other regulatory mechanisms for xenobiotic-metabolizing CYPs

The regulation of CYP2A6 expression is mostly unknown. CYP2A6 has been shown to be induced by phenobarbital and rifampicin (Dalet-Beluche *et al.* 1992, Sotaniemi *et al.* 1995), which points to the influence of CAR and/or PXR. However, there is no direct evidence to confirm this. A related CYP2A gene in mice, CYP2A5, is induced by cAMP-elevating agents and several hepatotoxic compounds (Raunio *et al.* 1999b). At least mRNA stabilization is involved in the regulation (Aida & Negishi 1991, Tilloy-Ellul *et al.* 1999). The significance of these observations for CYP2A6 induction is unknown.

CYP2E1 is regulated in a complex manner, since it is regulated transcriptionally, pretranslationally, translationally, and posttranslationally (Song 1995). The most important steps are probably the stabilization of mRNA and protein (Ronis *et al.* 1996). Transcriptional regulation seems to play a minor role in contrast to many other CYPs. However, starvation and chronic ethanol intake are thought to increase transcription as well as CYP2E1 protein stability (Ronis *et al.* 1996). Hepatocyte nuclear factor 1α has been shown to activate rat hepatic *CYP2E1* gene expression (Liu & Gonzalez 1995). Unlike other cytokines, interleukin-4 induces human CYP2E1 in primary hepatocytes (Abdel-Razzak *et al.* 1993). Several other cytokines, including interleukin-1β, interleukin-6, tumor necrosis factor-α, and interferon-γ, down-regulate CYP1A2, CYP2C, CYP2E1, and CYP3A (Abdel-Razzak *et al.* 1993, Muntane-Relat *et al.* 1995, Pascussi *et al.* 2000).

Hepatocyte nuclear factor 4 participates in liver-specific basal expression of several CYP enzymes in the subfamilies 2A, 2C, 2D and 3A (Honkakoski & Negishi 2000), although its role in the regulation of human CYPs has been less thoroughly characterized. It binds to the HPF1 motif in the promoter regions of *CYP* genes, including human *CYP2C9* (Ibeanu & Goldstein 1995). BTEB (basic transcription element binding factor) has also been shown to bind to BTE (basic transcription element) in the 5'-flanking regions of the *CYP3A4* and *CYP3A7* genes (Hashimoto *et al.* 1993). A sequence variation in BTE affects the basal expression of the *CYP3A5* gene (Paulussen *et al.* 2000).

2.4 CYP enzymes in human lung

2.4.1 General aspects of the metabolic capacity of human lung

There is still great controversy regarding the significance of pulmonary metabolism of xenobiotics due to the low levels of enzymes present in human lung. It is possible that the liver, having by far the greatest metabolic capacity of all organs, converts ingested and inhaled toxicants into proximate or ultimate reactive metabolites, which are then transported via the bloodstream into lung cells. In this case, intermediates capable of interacting with cellular targets would have to cross several cell membranes and escape numerous scavenger systems. Considering the highly reactive nature of these intermediates, this is unlikely at the low concentrations of toxicants that enter the human body. In contrast, if the reactive intermediates were formed within (or in near proximity of) the actual target cells, such as lung epithelial cells, they would need to cross only intracellular membranes (or a few cell membranes) to reach their targets (DNA and protein) (Raunio *et al.* 1999a). It must be taken into consideration that the majority of lung toxicants enter the body through the respiratory tract, exposing pulmonary epithelium to higher concentrations than liver cells, thus enabling even restricted local metabolism to contribute significantly. It is also notable that many inhaled, highly lipophilic compounds, such as most PAHs, have longer retention times and higher local doses in pulmonary epithelium than less lipophilic compounds, indicating that at least these lipophilic substances are primarily site-of-entry toxicants (Dahl & Lewis 1993, Gerde *et al.* 1998). On the other hand, the capability of even the most reactive ultimate carcinogen of benzo(a)pyrene, stereoisomer of 7,8,9,10-tetrahydro-7,8-diol-9,10-epoxide, known as diol-epoxide 2, to cause pulmonary adenomas after intraperitoneal injection in mice shows that even highly reactive intermediates may have carcinogenic effects outside the site-of-entry, although the doses used were well above the relevant human doses (Kapitulnik *et al.* 1978).

Thus, a key question concerning organ-specific chemical toxicity and carcinogenicity is whether the actual target tissue has the capacity to activate (or efficiently inactivate) chemicals. Animal models show that, in the case of pulmonary toxicity, several target cells in the lung have the capacity to convert chemicals into reactive forms as well as to detoxify them (Gerde *et al.* 1998). Finding evidence for this in humans is more difficult, but various lines of research have established that whole lung tissue as well as several pulmonary cell types possess metabolic capacity towards numerous xenobiotics (Hecht

1999). Although the lung contains several enzymatic pathways capable of xenobiotic metabolism, it is generally agreed that the CYP superfamily is the main system catalyzing the oxidative metabolism and metabolic activation of most toxicants. However, from the susceptibility point of view, it is equally important to measure variations in phase II detoxifying enzymes and pro-oxidant and antioxidant systems, although these aspects are less well characterized. Tobacco smoke being the most widely studied pulmonary toxicant and carcinogen, it is of utmost importance that both NNK and benzo(a)pyrene, the most important tobacco carcinogens, are activated by CYPs (Hecht 1999), and that these activation reactions do take place in human lung (Autrup 1990).

Compared to the widespread interest in local pulmonary activation and inactivation of carcinogens and toxicants, the role of pulmonary metabolism in the systemic clearance of foreign chemicals has received less attention (Roth & Vinegar 1990). Although the pulmonary metabolic capacity is far lower than that in liver, it cannot be completely ignored for several reasons: 1) The total cardiac output of blood passes through the lung, in contrast to any other organ in human body apart from the heart, thus exposing the whole quantity of circulating xenobiotics to pulmonary enzymes. 2) Any xenobiotic given intravenously, intramuscularly, subcutaneously, or topically is circulated through the lung before reaching other organs, including liver. In these cases, pulmonary metabolism can be regarded as first-pass metabolism. 3) Due to its great metabolic capability, the liver converts many harmless compounds into more toxic and carcinogenic forms. A small portion of these are probably able to leave the liver for blood and on to the next downstream capillary bed of the lung. For these compounds the lung can be considered the second line of defense. 4) Certain basic amino compounds are accumulated in the lungs. These include such drugs as imipramine, amiodarone, and amide type local anesthetics (Foth 1995). 5) Pulmonary veins have very large vascular surface area, exposing the circulating foreign compounds efficiently to pulmonary enzymes. These points make it a feasible assumption that CYP enzymes of the lung could also have some toxicological and clinical significance in the systemic clearance of chemical compounds.

2.4.2 Expression of xenobiotic-metabolizing CYP enzymes in human pulmonary tissues

The detection of individual CYP forms in human lung has been difficult by conventional methods, such as protein purification, catalytic activity studies, and Western immunoblotting, due to the low abundance of CYPs in lung (Wheeler & Guenthner 1991, Raunio *et al.* 1995a). Depending on the procedure used, spectroscopic quantitation of CYPs in human lung has revealed that the amount of microsomal CYP protein is at a level of about 2-10 pmol/mg protein (Wheeler & Guenthner 1990, Shimada *et al.* 1992). Also, the complex nature of the lung as an organ with 40 different cell types with varying amounts of CYP enzymes have complicated the research. With the advent of the RT-PCR technology, it has become possible to detect minute amounts of mRNA in tissue samples. RT-PCR is extremely sensitive and the results obtained with it cannot be regarded as a direct indication of the existence of corresponding proteins. Rather, RT-PCR is valuable as a screening method, revealing mRNA that can potentially be translated into functional

protein in a given tissue. Conversely, the absence of mRNA in RT-PCR analysis is a strong indication of the lack of a corresponding protein product at biologically meaningful levels. The expression of various CYP enzymes at mRNA and protein levels in human lung is summarized in Table 2.

Table 2. Summary of expression of CYPs in human lung. -- moderate negative evidence, - weak negative evidence, +/- conflicting evidence, + weak positive evidence, ++ moderate positive evidence, +++ strong positive evidence. For references, see chapter 2.4.2.

CYP	mRNA	Protein	Comments
1A1	+++	+++	Smokers
	+/-	--	Non-smokers
1A2	+/-	+/-	
1B1	++	+/-	Protein in alveolar macrophages
2A6	++	+/-	
2B6	+++	+++	
2C	+	+/-	Protein in serous cells of bronchial glands
2D6	+/-	+/-	
2E1	+++	+++	
2F1	+++		
2J2	+	++	
3A4	+/-		
3A5	++	+	
3A7	+/-		
4B1	+++	+	

CYP1A1 is by far the most actively studied human pulmonary CYP enzyme due to its importance in PAH metabolism (Shou *et al.* 1994). The first report on the expression of CYP1A1 mRNA in human lung was published by Omiecinski *et al.* in 1990 (Omiecinski *et al.* 1990). Soon after that, the induction of CYP1A1 mRNA by tobacco smoking (McLemore *et al.* 1990), the expression (Wheeler *et al.* 1990) and purification (Shimada *et al.* 1992) of CYP1A1 protein in human lung, and the localization and induction of CYP1A1 protein by tobacco smoke (Anttila *et al.* 1991) were reported. CYP1A1 protein is only detected in smokers (Anttila *et al.* 1991), and CYP1A1 expression correlates positively with the aryl hydrocarbon hydroxylation (AHH) and ethoxyresorufin O-deethylation (EROD) activities in human lung tissue (McLemore *et al.* 1990, Wheeler *et al.* 1990, Anttila *et al.* 1991, Anttila *et al.* 1992, Bartsch *et al.* 1992). CYP1A1 mRNA expression in the lung is more abundant in female than male smokers (Mollerup *et al.* 1999). AHH activity and CYP1A1 mRNA expression decrease to the basal levels within 2 months after the cessation of smoking (Petruzzelli *et al.* 1988, McLemore *et al.* 1990). Recently, the expressions of pulmonary CYP1A1 mRNA and protein were shown to correlate positively with the aromatic/hydrophobic DNA adduct levels in human lung tissue (Mollerup *et al.* 1999, Cheng *et al.* 2000).

Another PAH-metabolizing CYP, CYP1A2, was detected recently by RT-PCR and Western blot in peripheral lung (Macé *et al.* 1998), but other reports do not corroborate this finding (Wheeler *et al.* 1990, Shimada *et al.* 1992, Shimada *et al.* 1996b, Kelly *et al.* 1997, Thum & Borlak 2000). The expression of CYP1B1 protein in lung tissue is also controversial. There are two reports on the expression of CYP1B1 protein with conflicting results, probably due to differences in the sensitivity of the antibodies used (Murray *et al.* 1997, Tang *et al.* 1999). CYP1B1 mRNA is expressed in human lung (Sutter *et al.* 1994, Willey *et al.* 1996, Shimada *et al.* 1996a), and it is inducible by smoking in bronchial epithelium (Willey *et al.* 1997). CYP1B1 is also induced by smoking in alveolar macrophages (Piipari *et al.* 2000). CYP1B1 is highly active in the activation of PAHs (Shimada *et al.* 1996a), and it could play a significant role in the activation of PAHs in such cell types as alveolar macrophages, where CYP1A1 is not expressed (Piipari *et al.* 2000).

Expression of CYP2A6 mRNA is detected in bronchial epithelium (Macé *et al.* 1998, Crawford *et al.* 1998), but two reports on the expression of the protein are contradictory (Shimada *et al.* 1996b, Macé *et al.* 1998). Our group and others were unable to demonstrate CYP2A6 mRNA in whole lung tissue homogenate, perhaps due to dilution of bronchus-specific mRNA expression with other cell types of the lung (Koskela *et al.* 1999, Thum & Borlak 2000). The expression of pulmonary CYP2A6 protein would be of utmost interest because CYP2A6 has a crucial role in the activation of NNK, the tobacco-specific procarcinogen (Hecht 1998). Surprisingly, a novel finding indicates that CYP2A6 is also involved in the formation of NNK from nicotine (Hecht *et al.* 2000). A Japanese study showed a protective effect of deletion of the *CYP2A6* gene against lung cancer (Miyamoto *et al.* 1999). Importantly, a recent study showed relatively high levels of CYP2A13 mRNA in human lung, and heterologously expressed CYP2A13 was even more active in the activation of NNK than CYP2A6 (Su *et al.* 2000).

The *CYP2B6* gene is expressed in human lung as a splicing variant (previously called CYP2B7) (Gonzalez *et al.* 1992, Czerwinski *et al.* 1994, Willey *et al.* 1997, Thum & Borlak 2000), and the corresponding protein is expressed as well (Mori *et al.* 1996, Macé *et al.* 1998, Gervot *et al.* 1999). It might play a role in the activation of NNK (Hecht 1998).

A recent immunohistochemistry study suggested a cell-specific expression of CYP2C proteins only in the serous cells of bronchial glands (Yokose *et al.* 1999). Of the four earlier Western blot studies, two support (Nakajima *et al.* 1994, Shimada *et al.* 1996b) and two fail to support the expression of CYP2C proteins in human lung (Wheeler *et al.* 1992, Toussaint *et al.* 1993). CYP2C8 and CYP2C18 mRNAs have been detected in lung (Macé *et al.* 1998). CYP2C8 has been implicated in the production of the endothelium-derived hyperpolarizing factor (EDHF), probably 11,12-epoxyeicosatrienoic acid, which affects vascular tone (Fisslthaler *et al.* 1999). If CYP2C8 is expressed in certain cell types of lung, it could have some physiological functions.

There has been widespread interest in studying the expression of pulmonary CYP2D6 due to its alleged role in the activation of NNK (Hecht 1998). However, the findings on the expression of CYP2D6 in human lung are inconsistent despite numerous studies (Shimada *et al.* 1996b, Kivistö *et al.* 1997, Huang *et al.* 1997, Guidice *et al.* 1997, Macé *et al.* 1998, Thum & Borlak 2000). An immunohistochemical study with CYP2D6 antibody revealed neither uniform nor cell-specific CYP2D6 protein expression in lung

(Kivistö *et al.* 1997), suggesting that the possible low-level expression of CYP2D6 indicated in some studies is probably biologically meaningless. CYP2D6-catalyzed bufuralol hydroxylation has not been detected in human lung (Shimada *et al.* 1996b, Kivistö *et al.* 1997).

The expression of pulmonary CYP2E1 mRNA and protein has been established in several studies (Wheeler *et al.* 1992, Botto *et al.* 1994, Kivistö *et al.* 1995a, Shimada *et al.* 1996b, Macé *et al.* 1998, Crawford *et al.* 1998, Thum & Borlak 2000). CYP2E1 is an interesting CYP form, because it is the CYP enzyme that most actively forms oxygen radicals causing tissue injury (Ronis *et al.* 1996). CYP2E1 is also active in the activation of some tobacco-specific nitrosamines, but not NNK (Yamazaki *et al.* 1992, Kushida *et al.* 2000). CYP2E1-related catalytic activities, namely oxidation of butadiene, demethylation of N-nitrosodimethylamine, and hydroxylation of p-nitrophenol, are detected in lung microsomes (Csanady *et al.* 1992, Botto *et al.* 1994, Forkert *et al.* 2000).

Expression of CYP2F1 has been detected at the mRNA level (Nhamburo *et al.* 1990, Willey *et al.* 1996, Thum & Borlak 2000), but there are no published results on the expression of CYP2F1 protein. Recombinant CYP2F1 is capable of activating the pulmonary toxicant 3-methylindole (Lanza *et al.* 1999).

CYP2J2 mRNA and protein are expressed in human lung (Wu *et al.* 1996, Zeldin *et al.* 1996). CYP2J2 is not active towards xenobiotics, but it has been shown to form EETs from arachidonic acid (Wu *et al.* 1996). EETs affect vascular and bronchial smooth muscle tone (Zeldin *et al.* 1995, Capdevila *et al.* 2000), and CYP2J2 might thus play an important physiological role in the pulmonary regulation of both vascular and bronchial tone.

Several studies demonstrate the expression of CYP3A protein in human lung (Wheeler *et al.* 1992, Kivistö *et al.* 1995b, Kivistö *et al.* 1996, Shimada *et al.* 1996b, Kelly *et al.* 1997, Macé *et al.* 1998), and RT-PCR studies show that the main pulmonary CYP3A form is CYP3A5 (Kivistö *et al.* 1996, Macé *et al.* 1998). CYP3A-catalyzed nifedipine oxidase and testosterone 6β-hydroxylation activities are detected in lung microsomes (Shimada *et al.* 1989, Shimada *et al.* 1996b, Kelly *et al.* 1997). There are two immunohistochemical studies showing no CYP3A protein in human lung (Murray *et al.* 1988, Yokose *et al.* 1999). The antibodies used were probably reactive with CYP3A4, but not with CYP3A5. CYP3A5 could play some role in the pulmonary activation of both NNK and especially benzo(a)pyrene (Hecht 1998, Hecht 1999), at least when the CYP1A1 level is low. In human pulmonary microsomes, the last step of benzo(a)pyrene activation is stimulated by α-naphthoflavone, a CYP3A activator (Shimada *et al.* 1989, Shimada *et al.* 1992). PAH-DNA adduct levels correlate positively with the amount of CYP3A5 in alveolar macrophages of smokers (Piipari *et al.* 2000).

There is only one preliminary report on the expression of CYP4B1 protein in human lung (Imaoka *et al.* 1999), but at least CYP4B1 mRNA is expressed in human lung (Nhamburo *et al.* 1989, Czerwinski *et al.* 1994, Willey *et al.* 1996, Thum & Borlak 2000). It has been speculated that the human CYP4B1 enzyme is not functional due to its inability to incorporate heme (Zheng *et al.* 1998).

In conclusion, at least the CYPs 1A1 (in smokers), 2B6, 2E1, 2J2, and 3A5 proteins are expressed in human lung. Even other CYP forms are likely to be expressed, but their expression is probably very low or restricted to specific cell types or individuals. The studies on the expression of certain CYP forms at protein level (CYP2F1 and CYP4B1)

are still missing or preliminary. In alveolar macrophages, i.e. the most easily accessible pulmonary cell type, at least CYP1B1, CYP2E1, and CYP3A5 proteins are expressed, while CYP1A1 is not (Botto *et al.* 1994, Piipari *et al.* 2000).

2.4.3 Localization of individual CYP forms in human lung

The cell-specific localization of individual CYP enzymes in the lung is still poorly known, since there are only a handful of good immunohistochemical studies about CYP forms in human lung. For a better understanding of cell-specific carcinogenicity and toxicity, it would be of great benefit to have a comprehensive picture of the localization of different CYP forms. The overall distribution of CYP enzymes can be estimated from the immunohistochemical distribution of NADPH-cytochrome P450 reductase, which is detected in bronchial and bronchiolar epithelium, Clara cells, alveolar lining cells and alveolar macrophages (Hall *et al.* 1989).

According to immunohistochemistry analyses, CYP1A1 is mainly expressed in the epithelium of the peripheral airways, i.e. bronchiolar, terminal bronchiolar and alveolar epithelium. CYP1A1 expression is not present in the epithelium of bronchi larger than 1 mm in diameter, and it is only seen in the lung of smokers (Anttila *et al.* 1991). Alveolar macrophages do not express CYP1A1 regardless of smoking status (Anttila *et al.* 1991, Piipari *et al.* 2000). In some cases, endothelium expresses CYP1A1 (Anttila *et al.* 1991). Induced CYP1A1 may be a precondition for the development of peripheral lung cancer in smokers, as not a single case of this disease with non-inducible CYP1A1 in the lung was found, and furthermore, CYP1A1 was localized in the part of the airways where peripheral cancers arise (Anttila *et al.* 1991, Anttila *et al.* 1994). A study of the localization of CYP1A1 mRNA by *in situ* hybridization corresponded well to the protein findings (Saarikoski *et al.* 1998). CYP1A1 mRNA has also been detected in bronchial and peripheral samples by RT-PCR (Willey *et al.* 1997, Macé *et al.* 1998).

Alveolar macrophages are the only pulmonary cell type in which the expression of CYP1B1 protein has been established (Piipari *et al.* 2000). CYP1B1 is induced at mRNA and protein levels by tobacco smoking in these cells.

Immunohistochemical studies with CYP3A antibody show that CYP3A protein is present in all lung samples studied, and it is localized to bronchial, bronchiolar and alveolar epithelium as well as alveolar macrophages (Kivistö *et al.* 1995b, Kivistö *et al.* 1996). The results on CYP3A mRNA expression in peripheral and bronchial samples establish CYP3A5 as the main CYP3A form (Kivistö *et al.* 1996, Macé *et al.* 1998).

In an immunohistochemical study CYP2J2 was detected throughout the epithelium of the pulmonary airway from the trachea to alveoli and in alveolar macrophages (Zeldin *et al.* 1996). Surprisingly, bronchial smooth muscle cells and vascular endothelium also showed strong expression. In epithelium, this expression was most intense in ciliated cells and less intense in alveoli.

CYP2E1 is also localized to human bronchial, bronchiolar and alveolar epithelium, and alveolar macrophages (Botto *et al.* 1994, Kivistö *et al.* 1995a, Mori *et al.* 1996). Endothelial cells have some expression (Botto *et al.* 1994). The localization of maximum expression cannot be determined, since none of these studies examined both bronchial

and peripheral lung. CYP2E1 mRNA has been detected by RT-PCR in bronchial and peripheral samples (Willey *et al.* 1996, Macé *et al.* 1998, Crawford *et al.* 1998). One immunohistochemical report localizes CYP2B6 to human Clara cells (Mori *et al.* 1996), and CYP2B6 mRNA is expressed in bronchial and peripheral lung (Willey *et al.* 1997, Macé *et al.* 1998). A recent study by Yokose *et al*. revealed CYP2C protein in serous cells of bronchial glands, but not in any other lung cell type (Yokose *et al.* 1999). mRNAs of CYP2C8 and CYP2C18 have also been detected in both bronchial and peripheral tissue samples (Macé *et al.* 1998).

It can be concluded that the expression of CYPs in human lung in general is more widely distributed than in pulmonary tissues of laboratory animals, where the expression concentrates mainly in Clara cells, type II alveolar cells, and alveolar macrophages (Wheeler & Guenthner 1991, Kikkawa 1992). Perhaps the most obvious difference between the localization of CYP enzymes in man and rodents is the weaker expression of CYPs in human Clara cells.

2.5 Regulation of human pulmonary xenobiotic-metabolizing CYP expression

Knowledge about the regulation of CYPs in human lung is rather limited. Virtually nothing is known about the tissue-specific expression of certain pulmonary CYP enzymes, such as CYP4B1 and CYP2F1, which are quite strictly lung-specific. Tobacco smoking does not induce CYP4B1 (Czerwinski *et al.* 1994).

By far the greatest amount of information has been gathered concerning the induction of CYP1A1 and CYP1A1-related enzyme activities AHH and EROD (Wheeler & Guenthner 1991). Both AHR and ARNT are expressed in human lung (Roberts *et al.* 1986, Hayashi *et al.* 1994). CYP1A1 protein is only detected in smokers (Anttila *et al.* 1991), and CYP1A1 expression correlates positively with the AHH and EROD activities in human lung tissue (McLemore *et al.* 1990, Wheeler *et al.* 1990, Anttila *et al.* 1991, Anttila *et al.* 1992, Bartsch *et al.* 1992). AHH activity decreases to the basal level within 2 months and CYP1A1 mRNA expression within 6 weeks after cessation of smoking (Petruzzelli *et al.* 1988, McLemore *et al.* 1990). CYP1A1 mRNA expression in the lung is more than 2-fold higher in female smokers compared with male smokers (Mollerup *et al.* 1999). It has been speculated that the complex interactions between the estrogen receptor and AHR pathways could explain this difference (Mollerup *et al.* 1999). CYP1B1, which is controlled by AHR/ARNT, is also induced by tobacco smoking in bronchial epithelium and alveolar macrophages (Willey *et al.* 1997, Piipari *et al.* 2000). AHH activity in alveolar macrophages is increased by smoking (Cantrell *et al.* 1973), and since CYP1A1 is neither expressed nor induced in these cells (Anttila *et al.* 1991, Piipari *et al.* 2000), the induced CYP form with AHH activity is probably CYP1B1. CYP3A5 expression is repressed by tobacco smoking in alveolar macrophages (Piipari *et al.* 2000). The mechanism of this repression is unknown.

No expression of PXR has been detected in human lung tissue by Northern blot analysis (Bertilsson *et al.* 1998, Blumberg *et al.* 1998, Lehmann *et al.* 1998). This does not exclude the possibility of expression in some specific pulmonary cell types.

Extremely low levels of CAR mRNA are detected in pulmonary tissue by Northern blot after long exposure times (Baes *et al.* 1994). It would be of interest to clarify the possible cell specificity. The CYP inducer rifampicin induces 7-ethoxycoumarin O-deethylation (ECOD) activity in human lung microsomes of patients with tuberculosis more than 2-fold (Ohnhaus & Bluhm 1987). ECOD is catalyzed by many CYP enzymes, including CYP1A1, CYP2A6, CYP2B6, and CYP2E1, and also CYP3A to a lesser extent (Waxman *et al.* 1991, Yamazaki *et al.* 1996). It is not known which, if any, of these enzymes is induced in human lung by rifampicin.

Induction of CYP1A1 has been studied quite extensively in various human pulmonary cell lines, the most popular ones being the adenocarcinoma line A549 and the bronchioloalveolar carcinoma line NCI-H322. In general, CYP1A1 is induced by AHR agonists in pulmonary cancer cell lines (McLemore *et al.* 1989, Stanley *et al.* 1992, Vogel *et al.* 1994, Döhr *et al.* 1997, Foster *et al.* 1998, Urani *et al.* 1998, Ueng *et al.* 2000). In the human immortalized bronchial cell lines BEAS-2B and BEP2D, CYP1A1 mRNA is also induced by PAHs (Offord *et al.* 1995, Willey *et al.* 1996). McLemore *et al*. tested 24 human lung cancer cell lines for the benz(a)anthracene induction of CYP1A1 mRNA (McLemore *et al.* 1989). Eleven cell lines showed low-level basal CYP1A1 mRNA expression, and 16 were induced after benz(a)anthracene treatment. AHH activity correlated positively with the CYP1A1 mRNA levels. In A549 cells, transforming growth factor-β_1 repressed both basal and induced expression of CYP1A1 and CYP1B1 and basal expression of AHR, while ARNT expression was not affected (Vogel *et al.* 1994, Döhr *et al.* 1997). These repressions are dependent on protein synthesis, since the protein synthesis inhibitor cycloheximide abolished the effect of transforming growth factor-β_1 (Vogel *et al.* 1994, Döhr *et al.* 1997). In NCI-H322 cells, interferon γ decreased both basal and induced CYP1A1-related EROD activity, while interleukin-1β, tumor necrosis factor, interferon α, and interferon β were without effect (Stanley *et al.* 1992).

In A549 cells, CYP2B6 protein and the related PROD (pentoxyresorufin O-deethylation) activity were detected. PROD activity was not induced by phenobarbital (Foster *et al.* 1998). In a study with NCI-H322 cells (Stanley *et al.* 1992), CYP2B-, CYP2C-, and CYP3A-related 7-benzyloxyresorufin O-debenzylation (BROD) activity (Waxman *et al.* 1991, Gervot *et al.* 1999) was increased by Aroclor 1254, a mixture of polychlorinated biphenyls containing both AHR ligands and phenobarbital-like inducers (Ngui & Bandiera 1999). CYP2B6, CYP2C8, and CYP3A proteins were not detected even after Aroclor 1254 induction (Stanley *et al.* 1992). BROD activity was also induced by β-naphthoflavone and phenobarbital in human cultured lung slices and bronchial epithelial cells, respectively (Hoet *et al.* 1997, El Adlouni *et al.* 2000). CYP2B6 mRNA has been detected in BEAS-2B cells and CYP2E1 mRNA in BEP2D cells (Willey *et al.* 1996, Macé *et al.* 1998).

In conclusion, CYP1A1 induction in lung tissue and CYP1B1 induction in alveolar macrophages are probably mediated by the AHR/ARNT pathway, and these inductions are seen at the mRNA, protein, and catalytic activity levels. The regulation of other CYP enzymes in human lung tissue remains to be characterized.

3 Aims of the present study

Since the CYP enzyme system is an essential component in the pulmonary toxicity and carcinogenicity of several xenobiotic compounds, the present study aimed to elucidate the expression and regulation of xenobiotic-metabolizing CYP enzymes in human lung.

The specific aims were:

1. To determine and compare the patterns of expression of CYP enzymes in human lung tissue, alveolar macrophages, and peripheral blood lymphocytes, in order to establish the better surrogate cells for lung tissue.
2. To find out which form is the main CYP3A enzyme expressed in human lung tissue and to confirm the localization of the CYP3A enzymes in human lung.
3. To characterize the CYP enzyme expression pattern of the A549 cell line and to verify its suitability for mechanistic studies on pulmonary CYP induction.
4. To find out if the induction of CYP1A1 by TCDD in A549 cells is regulated by phosphorylation in a similar fashion compared to other human cell models, and to elucidate the differences in the pulmonary regulation of CYP1A1 and CYP1B1 induction by TCDD.
5. To determine the mechanism and dose-response of CYP3A5 induction by glucocorticoids in A549 cells and to find out if this induction occurs in alveolar macrophages of patients with respiratory diseases.

4 Materials and methods

4.1 Human tissue samples

Human liver, lung, alveolar macrophage, and lymphocyte samples were used in this study. In article I, seven bronchoalveolar lavage samples (4 smokers and 3 non-smokers) obtained during standard diagnostic procedure and eight blood samples (8 non-smokers) for the isolation of peripheral blood lymphocytes were used. Pooled lung samples from 18 patients obtained from surgery of lung tumors were used as a reference. Four of the lung samples were from smokers, four from non-smokers, and 10 from ex-smokers. In article II, lung samples for immunohistochemistry were obtained during surgery of tumorous pulmonary lesions from 27 patients, including 12 smokers, 14 ex-smokers, and one non-smoker. In addition, RT-PCR analysis was performed for eight of the 27 patients. The lung tissue samples used were macroscopically normal (I, II). Human liver samples were used as positive controls in RT-PCR in the articles I-IV. The samples were from rejected liver transplants. Alveolar macrophages from 32 bronchoalveolar lavage samples (12 non-smokers, 10 ex-smokers, and 10 smokers) were used to quantitate CYP3A5 expression in article IV. The samples were obtained during standard diagnostic procedures. Six patients were current glucocorticoid users, five had stopped their glucocorticoid use three days to three weeks before the procedure, and 21 patients had never used glucocorticoids. Additional details about the gender and diagnoses of the patients are given in the articles I and II.

4.2 Cell culture

The human A549 adenocarcinoma cell line derived from human type II pneumocyte was used in the present study (Lieber *et al.* 1976). The cell line was originally obtained from American Type Culture Collection (Rockville, MD, USA). The cells were cultured in Ham's F12 medium with L-glutamine (GIBCO BRL, Grand Island, NY, USA)

supplemented with 10% fetal bovine serum (GIBCO BRL) and 10 µg/ml gentamicin (GIBCO BRL). The cell culture conditions are described in detail in the articles III and IV.

4.3 Chemicals

The following chemicals from the following suppliers were used in this study: TCDD (National Cancer Institute Chemical Carcinogen Repository, Bethesda, MD, USA), rifampicin, dexamethasone, phenobarbital, pregnenolone 16α-carbonitrile (PCN), clotrimazole, beclomethasone dipropionate, budesonide, nifedipine (Sigma, St Louis, MO, USA), okadaic acid, calyculin A, staurosporine and genistein (Calbiochem, La Jolla, CA, USA).

4.4 Extraction of mRNA, mRNA blot analysis and cDNA synthesis

The mRNAs of cultured cells and human tissues were extracted with the QuickPrep Micro mRNA Purification Kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The amount and integrity of mRNA was verified with conventional RNA (Northern) blot analysis and hybridization with ^{32}P-labeled beta-actin cDNA probe in articles III and IV. cDNA was synthesized with the First-Strand Synthesis Kit (Amersham Pharmacia Biotech, Uppsala, Sweden).

4.5 Qualitative RT-PCR

The PCR primers were designed to be gene-specific, with the exception of the CYP2C primers, which detected all known human CYP2C forms (i.e. 2C8, 2C9, 2C18 and 2C19). To exclude the possibility of cross-hybridization with other sequences, each primer was compared with the EMBL human gene bank using the FASTA program (Genetics Computer Group, Madison, WI, USA). The primers were also designed to amplify regions containing at least one intron in the gene, to exclude contamination of cDNA with genomic DNA. Because the genomic structure of CYP1B1 was not available at the time of primer design, we were unable to target the CYP1B1 primers to separate exons. Therefore, DNase treatment to destroy genomic DNA was performed in article III. The primers, their locations, the sizes of the PCR products, and the details of the PCR reactions were described in the articles I-IV and the references therein.

4.6 Quantitative RT-PCR

Competitive PCR controls for CYP1A1, CYP1B1, CYP3A4, and CYP3A5 were prepared according to Jin et al. (1994) (III-IV). The basis of this method is PCR amplification of a part of the target sequence with the same 3' primer and a recombinant 5' primer to produce a shortened template that can be amplified by the original primer pair. The obtained PCR products, which contained a small 50-70 bp deletion but were otherwise identical in sequence to the target templates, were then cloned into plasmids, which can be produced in bacteria.

Quantitation was performed by adding a constant amount of the studied cDNA into multiple PCR tubes with serial dilutions of control. An aliquot of the reaction mixture was electrophoresed in ethidium bromide stained agarose gel, and the bands were measured directly from the agarose gels with the ImageMaster VDS densitometer and calculated with the ImageMaster 1D software (Amersham Pharmacia Biotech). The amount of studied mRNA was quantitated by determining graphically the point at which the amount of control molecules was equal to the target template. The results were correlated with the amount of beta-actin mRNA in each sample quantitated by Northern blot.

4.7 Immunohistochemistry

The expression of CYP3A protein in human lung was studied with three different CYP3A antibodies in article II. Polyclonal CYP3A antibody recognized both CYP3A4 and CYP3A5 (Belloc *et al.* 1996). Antipeptide CYP3A4 antibody detected both CYP3A4 and CYP3A7, and antipeptide CYP3A5 antibody bound only to CYP3A5 (Edwards *et al.* 1995). Both paraffin and frozen sections were studied with the avidin-biotin complex method as described in detail in article II.

4.8 Statistical analysis

The correlations of the staining intensities of every cell type between different slides from the same individual were tested with Spearman's rank correlation and the differences in staining intensity between the peripheral and central parts of the lung with the Mann-Whitney *U* test (II). In article III, the statistical significance of the differences between the treatments was tested with Student's *t* test. In article IV, the differences in CYP3A5 induction by different inducers were tested with Student's *t* test. The data on the dose-response curves were analyzed with one-way ANOVA followed by Student-Newman-Keuls *post hoc* test. The results on the effects of glucocorticoid use and smoking on CYP3A5 mRNA levels in macrophages were analyzed with two-way ANOVA followed by Bonferroni's *post hoc* test.

5 Results

5.1 Expression of xenobiotic-metabolizing CYP mRNAs in human lung tissues

5.1.1 Whole lung tissue

To obtain a reference CYP expression pattern in whole lung, specimens from lung tissue were combined into three pools according to the smoking histories of the individual patients (I). In ethidium bromide-stained agarose gels, the expression of CYP1A1 was detected in the current smoker pool, but not in the non-smoker and ex-smoker pools. Products of CYP2B6, CYP2E1, CYP2F1, CYP3A5, and CYP4B1 were found in all the three pools, whereas CYP1A2, CYP2C8-19, and CYP2D6 products were not detected. The CYP expression data in whole lung are shown compiled in Table 3.

5.1.2 Alveolar macrophages

The expression of CYP2B6, CYP2C8-19, CYP2E1, CYP2F1, CYP3A5, and CYP4B1 was detected in each of the alveolar macrophage samples (n = 7), while CYP1A1, CYP1A2, CYP3A4, and CYP3A7 were not detected in any of them (Table 3) (I). Amplification with CYP2D6 primers yielded multiple-size products, possibly reflecting the expression of pseudogenes and aberrantly spliced mRNAs (Nelson *et al.* 1996). The patients' smoking status did not have any apparent effect on the expression pattern.

5.1.3 Lymphocytes

In peripheral blood lymphocytes, only CYP2E1 mRNA was consistently detectable in every sample (n = 8) (Table 3) (I). Low levels of CYP2B6 expression were detected in most samples, and CYP2C8-19, CYP3A5 and CYP4B1 mRNAs were found in only a

minority of them. As with the alveolar macrophage samples, CYP2D6 amplification produced multiple bands. CYP1A1, CYP1A2, CYP2F1, CYP3A4, and CYP3A7 products were not detected in any of the samples.

Table 3. Expression of CYP mRNAs in lung tissue, alveolar macrophages, peripheral lymphocytes, and A549 cells (articles I and III).

CYP	Lung tissue	Alveolar macrophages	Lymphocytes	A549 cells
1A1	+ *	-	-	+
1A2	-	-	-	-
1B1				+
2A6-13				-
2B6/7	+	+	+/-	+
2C8-19	-	+	+/-	+
2D6	-	-**	- **	- **
2E1	+	+	+	+
2F1	+	+	-	-
3A4	-	-	-	-
3A5	+	+	+/-	+
3A7	-	-	-	+
4B1	+	+	+/-	-

* CYP1A1 was detected only in current smoker pool in lung tissue. ** CYP2D6 amplification produced multiple bands of different sizes in lymphocytes, macrophages, and A549 cells

5.1.4 A549 cells

In A549 cells, correct-sized amplification products were seen for CYP1A1, CYP1B1, CYP2B6, CYP2C8-19, CYP2E1, CYP3A5, and CYP3A7 (Table 3) (III). CYP3A5 was the most abundantly expressed member of the CYP3A family, while CYP3A7 was observed in notably lower quantities and CYP3A4 mRNA was missing. CYP1A2, CYP2A6, CYP2A7, CYP2A13, CYP2F1, and CYP4B1 mRNAs were not detected. Precisely as in the macrophage and lymphocyte samples, CYP2D6 amplification yielded multiple bands of different sizes.

5.2 Expression of CYP3A4 and CYP3A5 in human lung

To study the expression of *CYP3A* genes in human lung tissue, gene-specific RT-PCR was performed with primers detecting CYP3A4, CYP3A5, and CYP3A7 in eight individual lung samples (II). CYP3A5 mRNA was found in all samples. CYP3A4 mRNA was found in one sample and CYP3A7 in none of the samples. Three different anti-CYP3A antibodies were used in the immunohistochemical analysis performed to

determine the identity and distribution of CYP3A proteins in the lung (II). Polyclonal anti-CYP3A antibody (detecting all the three CYP3A forms) and anti-peptide CYP3A5 antibody yielded positive immunostaining in one or several lung cell types in all the 27 cases studied. Epithelial staining with the specific anti-peptide CYP3A4 antibody was observed in 5 out of 27 cases. The sample showing CYP3A4 mRNA expression in RT-PCR also showed CYP3A4 protein by immunostaining with anti-peptide CYP3A4 antibody.

5.3 Localization of CYP3A4 and CYP3A5 in human lung

Similar cell type localization of positive immunostaining was observed with the polyclonal anti-CYP3A and anti-peptide CYP3A5 antibodies (II). Also, a good correlation in the staining intensities of different cell types and individuals between the two antibodies was observed (Spearman's rank correlation, for epithelial cells according to the highest grade of staining among different epithelial cell types $r=0.70$, for endothelium $r=0.66$, and for macrophages $r=0.65$), suggesting that the antibodies detect the same CYP3A5 protein. CYP3A5 protein was localized in ciliated and mucous cells of the bronchial wall, bronchial glands, bronchiolar ciliated and terminal cuboidal epithelium, type I and type II alveolar epithelium, vascular and capillary endothelium, and alveolar macrophages. The localization was similar in frozen and paraffin sections. Not every cell type was immunostained in every individual, and occasionally only a few cells were positive. Usually, a minority of a given cell type was stained. Immunostaining was mainly cytoplasmic and nuclear in bronchial epithelium and glands, and mainly apical in bronchiolar and alveolar epithelial cells.

In a few cases, positive immunostaining occurred with the anti-peptide CYP3A4 antibody in the apical part of epithelial cells of bronchial glands, bronchiolar ciliated and terminal cuboidal epithelial cells and type II alveolar epithelial cells. Alveolar macrophages were stained occasionally.

The interindividual variation in the staining intensity of cell types between slides was tested statistically. The staining intensity of every cell type tested correlated between two different samples from the same individual (Spearman's rank correlation, $r=0.81$ for endothelial cells, $r=0.64$ for pneumocytes, and $r=0.55$ for alveolar macrophages). The staining intensity was stronger in the samples from the central than in those from the peripheral parts of the lung. The difference was more significant in alveolar macrophages (Mann-Whitney U-test, $U=80.5$, $p<0.001$) and pneumocytes ($U=165.0$, $p<0.05$) than in endothelial cells ($U=194.0$, nonsignificant).

5.4 Induction of CYP1 and CYP3A mRNAs in A549 cells

To determine the induction capacity of individual CYP forms in the families CYP1 and CYP3A by xenobiotics, the levels of CYP1A1, CYP1B1, CYP3A4, and CYP3A5 mRNAs were measured by quantitative RT-PCR after exposing the A549 cells to well-defined chemical inducers (III). A 24-h exposure to TCDD (10 nM) caused a 56-fold

increase in the amount of CYP1A1, while a similar treatment produced only a 2.5-fold induction of CYP1B1 mRNA. CYP1A1 and CYP1B1 mRNAs were not affected by treatment with 10 µM rifampicin, 10 µM dexamethasone, or 1.5 mM phenobarbital. CYP3A4 mRNA was not expressed constitutively, and its amount did not increase to detectable levels after exposure to any of the inducers studied (PCN, clotrimazole, rifampicin, phenobarbital, dexamethasone, TCDD). Interestingly, the amount of CYP3A5 mRNA was induced 8-fold and 11-fold by dexamethasone and phenobarbital, respectively, while RU486 (mifepristone), nifedipine, PCN, clotrimazole, rifampicin, and TCDD did not cause any induction (articles III and IV).

5.5 Modulation of TCDD-induced CYP1A1 and CYP1B1 in A549 cells

The role of intracellular protein phosphorylation in CYP1A1 and CYP1B1 induction was assessed by culturing A549 cells in the presence of TCDD and protein kinase or protein phosphatase inhibitors. 50 nM staurosporine (a protein kinase C inhibitor) and 50 µM genistein (a tyrosine kinase inhibitor) blocked 79 % and 65 % of TCDD-elicited induction of CYP1A1, respectively. The protein phosphatase inhibitors calyculin A (3 nM) and okadaic acid (10 nM) had minor effects on CYP1A1 induction, but slightly enhanced the induction of CYP1B1 mRNA. Genistein and staurosporine had negligible effects on CYP1B1 induction by TCDD.

5.6 Characterization of the mechanism of CYP3A5 induction by glucocorticoids in A549 cells

To further characterize the induction of CYP3A5 mRNA by glucocorticoid dexamethasone, A549 cells were exposed for 24 h to budesonide and beclomethasone dipropionate, which are inhaled glucocorticoids used in the treatment of asthma, and mRNA was measured by quantitative RT-PCR. 10 µM concentrations produced 3- to 4-fold inductions with all the three compounds used. Induction by glucocorticoids was further studied by dose-response analysis, which showed that ~100 nM concentrations were sufficient for maximal induction. This induction was completely blocked by the glucocorticoid receptor antagonist RU486 (mifepristone). Due to the marked variation, only the budesonide dose-response and the blocking of beclomethasone dipropionate induction by RU486 reached statistical significance ($P < 0.05$). Qualitative RT-PCRs with PXR, CAR, and GR primers were performed to detect the expression of mRNAs of receptors regulating CYP3A. Only GR mRNA was present in A549 cells. A 24-h exposure to dexamethasone did not induce PXR mRNA.

5.7 Effect of inhaled glucocorticoids and smoking on the alveolar macrophage CYP3A5 mRNA levels

To test the hypothesis that inhaled glucocorticoids affect the level of CYP3A5 mRNA of alveolar macrophages in patients with respiratory diseases *in vivo*, CYP3A5 mRNA was measured with quantitative RT-PCR. The differences of CYP3A5 expression in current glucocorticoidusers, ex-users, and non-users were minimal and statistically non-significant (P=0.809). The use of glucocorticoids elevated the CYP3A5 level by 45% in non-smokers (P=0.438). However, the patients' smoking status had profound consequences for the CYP3A5 levels. CYP3A5 expression in current smokers was only 7 % of the CYP3A5 level in non-smokers ($P < 0.05$). In ex-smokers the expression was slightly reduced (88 %) compared to non-smokers ($P < 0.106$). Sex and age were tested as confounding factors, but they did not influence the results.

6 Discussion

CYP enzymes in human lung have been studied clearly less than CYPs in pulmonary tissue of animals, mainly due to difficulties in obtaining sufficient amounts of human tissue. The relative lack of CYP enzymes in human lung compared to rodent lung has also hampered this research. The detection of individual CYP forms in human lung has been difficult by conventional methods, and the complexity of human lung with its different cell types and varying amounts of CYP enzymes have created additional difficulties. Since the adoption of both RT-PCR methods and immunohistochemistry with the sensitive detection and specific antibodies, the knowledge about human pulmonary CYP enzymes has been accumulating steadily.

Due to the scarcity of human tissue, there has been a constant search to find a good surrogate tissue substituting for human pulmonary tissue. The most widely used surrogate has been peripheral blood lymphocytes, since they are relatively easy to obtain. There have been major efforts to establish lymphocytes as a reliable surrogate with studies measuring and comparing AHH activity and/or DNA adducts in lymphocytes and lung tissues (Law 1990), but the results have been inconclusive. In the present study (I), the expression of CYP enzymes in lymphocytes and alveolar macrophages was compared to expression in whole lung tissue. The pattern of CYP expression in alveolar macrophages was found to closely resemble the expression pattern in human lung tissue, while the pattern in lymphocytes was markedly different. This result indicates that alveolar macrophages are a better surrogate for lung tissue than peripheral blood lymphocytes. The expression of CYP2B6, CYP2C, CYP3A5, and CYP4B1 in human alveolar macrophages was demonstrated for the first time. There is a previous study on the expression of CYPs in alveolar macrophages, showing an almost total lack of CYP mRNA expression (Willey *et al.* 1996). The reason for this discrepancy is unknown. Another previous study demonstrated some expression of CYP2E1 protein in alveolar macrophages (Botto *et al.* 1994). Subsequently, the expression of CYP3A5 protein and the lack of CYP1A1 protein in alveolar macrophages were detected, confirming our mRNA results (Piipari *et al.* 2000). Also, CYP1B1 mRNA and protein were observed to be expressed in macrophages (Hakkola *et al.* 1997, Piipari *et al.* 2000).

Mechanistic studies on pulmonary CYP regulation have been infrequent, due to a lack of good models. Animal models may give implications for human regulation, but they must always be interpreted with caution. Their natural benefit is the information they give on CYP regulation in a whole organism. Human primary pulmonary cells would probably be the best alternative, but they are hard to obtain and culture. Recently, human primary bronchial epithelial cells were made commercially available, but at considerable cost. Human papillomavirus and simian virus 40 immortalized bronchial epithelial cells are also available. Both primary and immortalized cell lines are expensive to culture and have a finite lifespan. The most widely used human cell models are the cancer cell lines A549 and NCI-H322, which are inexpensive and relatively easy to culture. All cancer cell lines naturally have their typical drawbacks as models, i.e. they have undergone changes in genetic structure, growth, and differentiation not characteristic of the original cell type. In the current study (III), the A549 adenocarcinoma cell line derived from type II pneumocyte was characterized and the CYP expression pattern and behavior were found to correlate reasonably well with human lung epithelial cells. The induction of enzymes in the families CYP1 and CYP3A was studied due to their importance in the activation of tobacco-derived procarcinogens. The induction of CYP1A1 behaved as predicted, and CYP1B1 and CYP3A5 were also inducible. The induction of CYP1A1 and CYP1B1 has been demonstrated previously in this cell line (Döhr & Abel 1997). The most obvious difference compared to lung tissue was the lack of CYP2F1 and CYP4B1 expression. Also, the expression of the nuclear receptors CAR and PXR was missing (IV), as in human lung tissue (Baes *et al.* 1994, Kliewer *et al.* 1998). This characterization of an easily cultured and inexpensive cell model will facilitate future studies on human pulmonary CYP regulation, but as mentioned above, fail to resolve the problems inherently involved in the use of cancer cell lines.

In A549 cells, TCDD, a potent AHR ligand, clearly elevated the level of CYP1A1 mRNA (56-fold), while the increase in the amount of CYP1B1 mRNA was much more modest (2.5-fold) (III), corroborating the earlier findings on the same cell line (Döhr & Abel 1997). The role of protein phosphorylation on pulmonary TCDD-induced CYP1A1 and CYP1B1 was determined using specific protein kinase and phosphatase inhibitors. The tyrosine kinase inhibitor genistein and the protein kinase C inhibitor staurosporine blocked CYP1A1 induction by TCDD, corroborating the earlier studies with human primary keratinocytes and the HepG2 hepatoma cell line (Berghard *et al.* 1993, Gradin *et al.* 1994, Chen & Tukey 1996, Kikuchi *et al.* 1998). These results showed that CYP1A1 induction is regulated by protein kinases in human lung in a similar fashion as in other human tissues. In contrast, kinase inhibitors did not affect CYP1B1 induction. The lack of effect on CYP1B1 induction indicates that its induction by TCDD may be less dependent on AHR. The serine/threonine protein phosphatase inhibitors calyculin A and okadaic acid enhanced CYP1B1 induction slightly, but did not alter CYP1A1 induction. Altogether, these findings provided information, for the first time, on the regulation of CYP1 induction by protein kinases and phosphatases in human lung.

The expression of CYP3A forms in pulmonary tissues was studied with RT-PCR analysis and immunohistochemistry with specific anti-peptide antibodies (II). Both methods established CYP3A5 as the main CYP3A form in human lung, CYP3A4 being expressed in only about 20% of cases. There was a previous report on the expression of CYP3A5 in human lung using a non-specific antibody (Kivistö *et al.* 1996). In that study,

the identity of the protein was established with gene-specific RT-PCR. The present study provided new insight into the differential expression of CYP3A4 and CYP3A5 in lung. CYP3A5 was shown to be expressed in a large area within the airways, being present in the ciliated and mucous cells of bronchi, bronchial glands, bronchiolar ciliated and terminal epithelium, alveolar type I and type II epithelium, endothelium and alveolar macrophages. The highest CYP3A5 level was detected in bronchial lung.

CYP3A5 was induced about 4-fold by the glucocorticoids budesonide, beclomethasone, and dexamethasone in A549 cells (IV). Induction was completely blocked by the glucocorticoid receptor antagonist RU486 (mifepristone). Only the glucocorticoid receptor, but neither PXR nor CAR, was expressed in these cells, indicating that the induction is mediated by glucocorticoid receptor. Maximal induction was achieved at concentrations as low as ~100 nM, suggesting that CYP3A5 could be induced *in vivo* in patients using inhaled glucocorticoids. Due to the marked variation, only the budesonide dose-response and the blocking of beclomethasone dipropionate induction by RU486 reached statistical significance. Previously, the promoter region of the *CYP3A5* gene has been shown to contain functional glucocorticoid-responsive element half-sites mediating induction by dexamethasone via glucocorticoid receptor in HepG2 cells (Schuetz *et al.* 1996). We tested the hypothesis that CYP3A5 would be induced by inhaled glucocorticoids in alveolar macrophages of patients with respiratory diseases. However, the differences in CYP3A5 expression in alveolar macrophages in current glucocorticoid users, ex-users, and non-users were minimal and statistically non-significant. The observed 45% increase in the CYP3A5 level in non-smoking glucocorticoid users was statistically non-significant due to small sample size and would hardly have any clinical significance. This minimal induction could be due to several possible reasons. 1.) The observed induction of CYP3A5 in A549 cells does not occur *in vivo*. 2.) Glucocorticoids do not induce CYP3A5 in alveolar macrophages. 3.) Inhaled doses of glucocorticoids do not reach alveolar macrophages in sufficient amounts, since the target organ for inhaled glucocorticoids is bronchial epithelium, and the majority of alveolar macrophages resides in alveoli. A study with bronchial epithelial samples from glucocorticoid users and controls would be needed to find out if CYP3A5 is induced in human lung *in vivo*.

Cigarette smoking had a marked decreasing effect on CYP3A5 levels in alveolar macrophages (IV). CYP3A5 expression in current smokers was only 7 % of the CYP3A5 level in non-smokers. This is in agreement with a recent study which demonstrated that smoking decreases CYP3A5 protein levels in alveolar macrophages (Piipari *et al.* 2000). Importantly, it was also shown that CYP3A5 protein levels correlate positively with PAH-DNA adducts in alveolar macrophages of smokers. This apparent contradiction can be explained as follows: in *smokers* the CYP3A5 level correlates positively with adducts, although their CYP3A5 level is decreased by smoking when compared to non-smokers. The repression of CYP enzyme by smoking is a novel phenomenon, since no other CYP is known to be down-regulated by tobacco smoke. The mechanism of this repression is unknown.

The presence and possible induction of CYP3A5 in human lung has several implications. The induction of CYP3A5 by glucocorticoids suggests that CYP3A5 could have some physiological roles in maintaining the steroid hormone balance in lung, since CYP3A5 is active in the metabolism of steroid hormones (Waxman *et al.* 1991), including

the endogenous glucocorticoid cortisol (Wrighton *et al.* 1990). Thus, the concentration of endogenous glucocorticoids might be regulated by negative feedback involving induction of CYP3A5. The inhaled glucocorticoid budesonide is also metabolized by CYP3A (Jonsson *et al.* 1995), indicating that budesonide probably autoinduces its own metabolism in human lung. CYP3A forms are of importance for the metabolism of inhaled xenobiotics, including tobacco-derived procarcinogens, such as PAHs and NNK (Hecht 1999). Both CYP3A4 and CYP3A5 are active in the metabolism of benzo(a)pyrene and especially its proximate carcinogen benzo(a)pyrene-7,8-diol (Roberts-Thomson *et al.* 1993, Shou *et al.* 1994). This is reflected in the positive correlation between PAH-DNA adducts and CYP3A5 levels in alveolar macrophages (Piipari *et al.* 2000). Also, in human pulmonary microsomes the last step of benzo(a)pyrene activation is stimulated by α-naphthoflavone, a CYP3A activator (Shimada *et al.* 1989, Shimada *et al.* 1992). Inhaled and systemic glucocorticoids are in common clinical use in such diseases as asthma and rheumatic diseases. If CYP3A5 is induced in human lung *in vivo* by glucocorticoids, it could have a modulating effect on the individual susceptibility to lung cancer in patients using glucocorticoids. This is an interesting topic for future research.

7 Conclusions

1. Due to the similarity of the CYP expression patterns, alveolar macrophages are better surrogate cells for lung tissue than peripheral blood lymphocytes. Alveolar macrophages expressed mRNAs of CYP2B6, CYP2C, CYP3A5, and CYP4B1, which have not been demonstrated before.
2. CYP3A5 is the main CYP3A form in human lung. It was expressed in every sample, while CYP3A4 was expressed in about 20% of cases. CYP3A5 was expressed in bronchial, bronchiolar, and alveolar epithelium as well as in endothelium and alveolar macrophages.
3. Both the expression pattern and the induction profile of CYPs in the A549 cell line were found to be reasonably similar to those seen in human lung tissue. These findings establish this cell line as a good model for mechanistic studies on pulmonary CYP enzymes.
4. CYP1A1 induction by TCDD was regulated by phosphorylation similarly in A549 cells as in other human cell models. The regulation of TCDD-elicited induction of CYP1B1 was different compared to CYP1A1.
5. CYP3A5 induction by inhaled glucocorticoids was mediated by glucocorticoid receptor, and induction was achieved at low nanomolar concentrations, which makes it feasible for this induction to occur in the bronchial epithelium of patients using inhaled glucocorticoids. However, inhaled glucocorticoids were not found to induce CYP3A5 in human alveolar macrophages *in vivo*.
6. Smoking decreased the CYP3A5 mRNA level in alveolar macrophages.

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