Formation and metabolism of the tryptophan-derived 6-formylindolo[3,2-\textit{b}]carbazole - a light-induced Ah-receptor ligand

Linda Bergander
ABSTRACT

The aryl hydrocarbon receptor (AhR) is a ligand dependent transcription factor ubiquitously expressed in mammalian cells. It is a genetically ancient protein mostly known for binding the extremely toxic contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). Binding to the AhR explains the variety of toxic responses of TCDD as well as the induction of several drug metabolizing enzymes. Induction of cytochrome P4501A1 (CYP1A1) is the most well characterized of the AhR regulated responses. The physiological functions of AhR and the endogenous ligand(s) for the receptor are under investigation but are not yet unraveled.

Several tryptophan (TRP) derived indol-containing compounds have been reported to possess AhR affinity/CYP1A1 inducing capacity and TRP mediates CYP1A1 induction by UV light. The TRP photoproduct, 6-formylindolo[3,2-b]carbazole (FICZ) has the highest AhR affinity described so far and it causes a rapid and transient induction of the CYP1A1 gene in human cells. A number of reports on constitutive CYP1A1 activity in cultured cells is therefore most likely explained by the presence of TRP-derived AhR ligands in cell culture media.

The aims of the studies were to investigate the impact of FICZ and FICZ metabolism on CYP1A1 gene regulation, to explore the metabolic fate of FICZ and to identify whether normal laboratory light could lead to formation of FICZ and thereby contribute to earlier observed CYP1A1 inducing effects by cell culture media.

Metabolic studies using fractions of Aroclor-induced and non-induced rat liver and human liver as well as heterologously expressed enzymes revealed that FICZ can be efficiently metabolized by the CYP enzymes 1A1 and 1A2 and by an unknown cytosolic enzyme, to a number of hydroxylated and other oxidized metabolites. All of the hitherto identified 11 hydroxylated metabolites of FICZ are prone to conjugation reactions by glucuronosyltransferases and sulfotransferases. The metabolites formed by human enzymes are primarily sulfated. Thus, the sulfated metabolites of FICZ will be crucial in the future analyzes of FICZ formation in vivo. FICZ was identified to be formed, not only by UV illumination, but also by normal laboratory light. The constitutive CYP1A1 activity was significantly induced through the formation of several TRP related photoproducts in light-exposed medium. One of these photoproducts was identified as FICZ. Thus, the TRP photoproduct, FICZ, fits into a model in which FICZ auto-regulates the expression of induced enzymes. It is hypothesized that FICZ might function as a chemical messenger that activates AhR in response to light and might be one of several possible endogenous AhR ligands.
Till mina kära,
Henrik och Tilde
# TABLE OF CONTENTS

**ABSTRACT** ii  
**LIST OF PUBLICATIONS** vi  
**LIST OF ABBREVIATIONS** vii  

**INTRODUCTION** 1  

**THE ARYL HYDROCARBON RECEPTOR** 1  
- bHLH/PAS proteins 1  
- Receptor activation 2  
- The AhR gene battery 2  

**REGULATION OF AhR FUNCTION** 4  
- Feed back regulation 4  
- AhR turnover 5  

**AhR LIGANDS** 6  
- Synthetic ligands 6  
- Naturally occurring “non-classical” ligands 7  
- Indole containing molecules as endogenous AhR ligands and CYP1A1 inducers 8  

**6-FORMYLINDOLO[3,2-b]CARBAZOLE** 12  
- Formation 12  
- Properties 13  

**EVIDENCE FOR AN ENDOGENOUS FUNCTION OF THE AhR** 13  

**METABOLISM** 14  

**DISTRIBUTION AND TRANSPORT THROUGH CELL MEMBRANES** 15  

**PHASE I** 16  
- Cytochrome P450 17  
  - CY1A1/1A2 18  
  - CYP1B1 20  
- Epoxide hydrolase 20  

**PHASE II** 21  
- UDP-Glycuronosyltransferase 21  
- Sulfotransferase 22
THE PRESENT STUDY

AIMS

COMMENTS ON METHODOLOGY
METABOLIC SYSTEMS
Different liver fractions
Cell lines
Heterologously expressed enzymes
INSTRUMENTATION USED FOR DETECTION AND STRUCTURE ASSIGNMENT OF METABOLITES
High performance liquid chromatography (HPLC)
Mass spectrometry (MS) and nuclear magnetic resonance (NMR)
MEASUREMENTS OF CYP1A1 INDUCTION
Gene expression analysis by RT-PCR
EROD-assay
INHIBITION OF METABOLIZING ENZYMES

RESULTS AND DISCUSSION

TRANSIENT INDUCTION OF CYP1A1 BY FICZ (Paper I)
CHARACTERIZATION OF PRIMARY METABOLITES OF FICZ (Papers I, III, IV)
STRUCTURE IDENTIFICATION OF THE PRIMARY METABOLITES OF FICZ (Papers II, IV)
CHARACTERIZATION OF ENZYMES CRITICAL FOR METABOLIC TRANSFORMATION OF FICZ (Papers I, III, IV)
IMPACT OF TRYPTOPHAN IN CELL CULTURE MEDIUM (Papers I, V)

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

ACKNOWLEDGEMENTS

REFERENCES
LIST OF PUBLICATIONS

This thesis is based on the following papers referred to in the text by their roman numerals:


*Both authors contributed equally

Papers I, II, III are reproduced by permission of the publishers.
LIST OF ABBREVIATIONS

AHH  aryl hydrocarbon hydroxylase
AhR  aryl hydrocarbon receptor
Arnt aryl hydrocarbon receptor nuclear translocating protein
AhRR aryl hydrocarbon receptor repressor
BaP  benzo[a]pyrene
bHLH/PAS basic-helix-loop-helix/Per-Arnt-Sim
CICZ indolo[3,2-b]carbazole-6-carboxylic acid
CYP  cytochrome P450
DIM  3,3'-diindolylmethane
EH   epoxide hydrolase
EROD 7-ethoxyresorufin-O-deethylase
3EP  3-ethynylphenanthrene
FICZ 6-formylindolo[3,2-b]carbazole
HAH halogenated aromatic hydrocarbons
IAA  indole-3-acetic acid
IAld indole-3-acetaldehyde
I3C  indole-3-carbinol
ICZ  indolo[3,2-b]carbazole
LC-MS liquid chromatography mass spectrometry
3MC  3-methylcholanthrene
NADP nicotinamide adenine dinucleotide phosphate
NMR nuclear magnetic resonance spectroscopy
NOE nuclear Overhauser effect
2-OH-FICZ 2-hydroxyindolo[3,2-b]carbazole-6-carboxaldehyde
8-OH-FICZ 8-hydroxyindolo[3,2-b]carbazole-6-carboxaldehyde
2,10-dOH-FICZ 2,10-dihydroxyindolo[3,2-b]carbazole-6-carboxaldehyde
4,8-dOH-FICZ 4,8-dihydroxyindolo[3,2-b]carbazole-6-carboxaldehyde
2,8-dOH-FICZ 2,8-dihydroxyindolo[3,2-b]carbazole-6-carboxaldehyde
PAH polycyclic aromatic hydrocarbons
PAPS 3'′-phosphoadenosine-5′-phosphosulfate
11PP 1-(1-proponyl)pyrene
21PP 2-(1-proponyl)-phenanthrene
SULT sulfotransferase
TA  tryptamine
TCDD 2,3,7,8-tetrachlorodibenzo-p-dioxin
TRP tryptophan
UDPGA uridine diphosphate-glucuronic acid
UGT UDP-glucuronosyltransferase
XRE xenobiotic responsive element
INTRODUCTION

THE ARYL HYDROCARBON RECEPTOR

The Aryl hydrocarbon receptor (AhR) is a ligand dependent cytosolic transcription factor found in most tissues and cells. The protein is responsible for binding of the extremely toxic contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), first shown by Poland and co-workers in 1976 (Poland, et al., 1976). Dioxins belong to a large group of aromatic substances that bind to the AhR (Poland and Knutson, 1982; Safe, 1986) and consequently, AhR has been referred to as the dioxin receptor. AhR is a genetically ancient protein which evolved 400-500-million years ago, and has both vertebrate and invertebrate homologs (Hahn, et al., 1997). Why would an ancient protein, that in particular binds a recent environmental contaminant and thereby initiates a variety of biochemical and toxic responses such as induction of metabolic enzymes, chloracne, reproductive and developmental toxicity, carcinogenesis, immune suppression, wasting syndrome, be ubiquitously expressed in mammalian cells? The AhR most likely has physiological functions implicating that an endogenous ligand(s) has existed during evolution and may still exist.

BHLH/PAS proteins

The AhR and the AhR nuclear translocating protein (Arnt) belong to a group of transcriptional regulatory proteins called the basic-helix-loop-helix/PAS proteins (bHLH/PAS). The PAS domain was originally recognized for its homology to the Drosophila circadian protein Per, the AhR dimerization partner Arnt and the Drosophila CNS development protein Sim (Gu, et al., 2000). In addition to the ligand binding and protein interacting PAS domain, the nearby basic domain of bHLH contributes to DNA binding and the HLH is a region responsible for interacting with proteins like Arnt and the heat shock protein 90 (Hsp90) (Reyes, et al., 1992; Mason, et al., 1994; Crews and Fan, 1999). The bHLH/PAS proteins have important roles in developmental processes (Nambu, et al., 1991), control of circadian rhythm (Sassone-Corsi, 1997) and response to hypoxia (Wang, et al., 1995).
Receptor activation

The AhR is localized to the cytosol as part of a protein complex, including two molecules of Hsp90, an immunophilin chaperone referred to as either the Ah receptor-interacting protein (AIP) (Ma and Whitlock, 1997), ARA9 (Carver and Bradfield, 1997) or XAP2 (Meyer, et al., 1998), the co-chaperone p23 (Kazlauskas, et al., 1999) as well as c-Src tyrosine kinase (Enan and Matsumura, 1996). The two Hsp90 molecules facilitate ligand binding to the AhR by preserving the ligand-binding conformation of the receptor in the cytosol (Carver, et al., 1994). The exact roles of AIP/ARA9/XAP2 and p23 are not fully established, however, XAP2 has been reported to be important for determining the stability of the AhR protein by protection from ubiquitination (Meyer and Perdew, 1999; Kazlauskas, et al., 2000; Ma and Baldwin, 2000) and the p23 co-chaperone is involved in enhancement of AhR signaling (Cox and Miller, 2002) as well as modulating ligand responsiveness in the activation process of the AhR (Kazlauskas, et al., 1999).

After ligand binding to the AhR, it undergoes a transformation process in which the interacting proteins dissociate from the AhR-ligand complex, and the liganded AhR translocates into the nucleus and forms a heterodimer with the helix-loop-helix type nuclear transcription factor Arnt (Reyes, et al., 1992; Whitelaw, et al., 1993). The AhR/Arnt heterodimer binds to specific enhancer sequences termed xenobiotic responsive elements (XRE) in DNA and thereby activates the expression of the AhR gene battery. The ligand plays an essential role in the AhR activation process, the transformation, translocation as well as preservation of the structural integrity of the receptor protein. The mechanisms of AhR activation and inactivation (discussed below) are illustrated in Fig 1.

The AhR gene battery

The AhR activates the transcription of a battery of genes. This battery includes drug metabolizing enzymes such as the cytochrome P450 (CYP) family members CYP1A1/1A2 and 1B1, UDP-glucuronosyltransferase (UGT1A6), NADPH quinone oxidoreductase (NQO1), aldehyde dehydrogenase-3 (ALDH3A1) and glutathione-S-transferase (GST Ya).
Furthermore, a large number of genes involved in growth, differentiation and cellular homeostasis have been shown to be upregulated by TCDD-induced AhR activation, for instance, plasminogen activator inhibitor-2, transforming growth factors (TGF-\(\alpha\) and TGF-\(\beta\)), c-fos and c-jun (Hankinson, 1995; Safe, 1995; Nebert, et al., 2000). Puga and coworkers characterized the transcriptional response of human hepatoma HepG2 cells after treatment with TCDD using a high-density microarray screening approach.
technique. Out of 310 genes involved in calcium regulation, receptor-associated kinases, cell cycle regulation, differentiation, apoptosis, development, protein trafficking and metabolism, 114 were up-regulated by TCDD and 196 were down-regulated (Puga, et al., 2000). Recently, a novel AhR response element, XRE-II, was identified to which the AhR/Arnt heterodimer binds via a yet unidentified protein (Sogawa, et al., 2004). A total of 36 genes were reported to contain the XRE-II motif, of which 13 were significantly changed by treatment of rats with TCDD (Boutros, et al., 2004). Among these induced genes were ALDH3A2, Cyclin L and TGFβ3. The best understood example of a gene under the control of the AhR is CYP1A1 measured as 7-ethoxyresorufin-O-deethylase (EROD) activity.

Most metabolic enzymes of the AhR gene battery prefer substrates that either bind to the AhR and thereby induce the expression of these enzymes or are the corresponding phase I metabolites of AhR agonists. Thus, the purpose of the drug metabolizing enzymes encoded by the AhR gene battery is most likely to facilitate the elimination of the inducers and their metabolites (Nebert, 1991; Denison and Whitlock, 1995; Schrenk, 1998).

There are several reported interactions between AhR and other important signaling molecules as reviewed by Carlson and Perdew (2002). In this review direct interactions with AhR (AIP, the estrogen receptor (ER), NfkB and Hsp90) or direct interactions with the AhR complex proteins (HIF-1α, AhRR and CBP) and finally indirect interactions (cross talk) with AhR (hypoxia, PKC, ER and c-myc) are presented. For instance, as shown by Ohtake and colleagues, the ligand activated AhR/Arnt heterodimer directly associates with estrogen receptors ER-α and ER-β, leading to activation of transcription and estrogenic effects (Ohtake, et al., 2003).

REGULATION OF AhR FUNCTION

Feedback regulation

The Ah receptor repressor (AhRR) belongs to the growing family of bHLH/PAS transcription factors. This protein represses the function of AhR by competing with AhR in forming a heterodimer with Arnt and subsequently binding to the XRE elements (Fig 1). Interestingly, the
AhR/Arnt heterodimer activates the transcription of the AhRR gene by binding to XRE sequences upstream of the AhRR gene and thus, the AhRR protein inhibits the function of AhR. In this way, the AhR is feed-back regulated by the AhRR (Mimura, et al., 1999; Watanabe, et al., 2001; Karchner, et al., 2002). The methods of regulation may not apply to all tissues, however. Human AhRR displays a tissue-specific expression and is specifically expressed in testis and ovary. A high expression of AhRR would, in the feedback regulated system, coincide with high CYP1A1 levels. However, the AhRR displays an opposite expression to CYP1A1 which points to a constitutive AhRR expression in testis and ovary (Yamamoto, et al., 2004).

Feed-back regulation of the AhR signal is moreover efficiently achieved by the transcription of metabolizing enzymes expressed by means of the AhR pathway (Fig 1). This is an important mechanism in maintaining homeostasis in those biological systems, where a signal is presumably down-regulated by the subsequent metabolism of the inducing ligand (Nebert, 1991). For example, mice lacking a functional CYP1A1 activity shows elevated expression levels of the AhR gene battery, suggesting that a ligand is accumulated in the cells due to a non-functional metabolizing enzyme which leads to constitutive expression by the AhR (Chang and Puga, 1998).

AhR turnover

The AhR is also regulated by degradation at the protein level. Several reports have suggested that the AhR is downregulated subsequent to ligand binding to the protein, as reviewed in Pollenz (2002). Treatment of cells with the metabolically stable ligand, TCDD, reduces the half-life of AhR from 28 to 3 hours and causes a depletion of AhR levels. The turnover of AhR has been shown to involve ubiquination of AhR, mediated by both the 26S proteosome and AhR degradation promoting factor (Ma and Baldwin, 2000). However, exposure of cells to metabolically unstable ligands like β-naphtoflavone (β-NF) and 3-methylcholanthrene (3MC) results in a more rapid return of the AhR protein to basal levels over time (Swanson and Perdew, 1993; Giannone, et al., 1998).
AHR LIGANDS

AhR ligands can be separated into two categories, synthetic or natural. Structure-activity relationship studies of polycyclic aromatic hydrocarbons (PAH), halogenated aromatic hydrocarbons (HAH) and indolo[3,2-b]carbazoles, which all are aromatic compounds, indicate that the ability of compounds to form planar structures is a requirement for binding to the AhR binding pocket and that the possible maximal dimensions of a ligand are 14 Å x 12 Å x 5 Å (Waller and McKinney, 1995). It has been speculated that two different recognition sites of either the HAH or PAH types of AhR ligands are central for binding (Rannug, et al., 1991). In the case of PAH, the most activating fragment was found to be the ‘bay’ region, previously found to be related to carcinogenicity (Wood, et al., 1977). In addition, high affinity ligand binding appears to be dependent upon key electronic and thermodynamic characteristics of the ligand (Gillner, et al., 1993; Waller and McKinney, 1995).

Synthetic ligands

The majority of known AhR ligands are synthetic, including the most potent ligands for the receptor. There are two large groups of synthetic ligands, the planar HAH and the PAH (Fig 2). The members of HAH include polychlorinated dibenzodioxins (PCDD), dibenzofuranes (PCDF) and biphenyles (PCB). Each of these members has a large number of congeners depending on the number and positions of the chlorine groups – PCDD has 75, PCDF 135 and PCB 209. TCDD, a congener of PCDD is one of the most potent and toxic AhR ligands known, and is not produced for any commercial purpose, but formed as a byproduct during incomplete combustion processes (Fiedler, 1996). Most HAH are biologically persistent and resistant to metabolic degradation. There is a concentration-dependent interindvidual variability of TCDD elimination efficiency in humans, with a half-life ranging from 3 to over 10 years (Aylward, et al., 2004). Due to the resistance of TCDD to metabolic degradation, the induction of CYP1A1 mRNA and enzyme by TCDD is sustained in character (Riddick, et al., 1994) and the exposure to TCDD can result a variety of toxic effects (as mentioned above).
The members of the PAH include compounds such as benzo[a]pyrene (BaP), 3MC, benzantracenes and benzoalavones (Denison and Nagy, 2003). The PAH have a somewhat lower binding affinity than HAH towards AhR (nM to µM compared to pM to nM range, respectively), perhaps due to a better fit to the binding pocket for HAH. 3MC was the first CYP1A1 inducer to be discovered (Conney, et al., 1956). It nearly equals TCDD in AhR binding affinity but contrary to TCDD, it is metabolically degraded, resulting in a transient induction of the CYP1A1 enzyme (Riddick, et al., 1994).

Figure 2. Synthetic AhR ligands belonging to the groups halogenated aromatic hydrocarbons (HAH) and polycyclic aromatic hydrocarbons (PAH).

Naturally occurring “non-classical” ligands

Reports of agonists of the AhR with structures different than the classical AhR ligands have recently been emerging (Fig 3). Lipoxin A4 is a degradation product of arachidonic acid, a reaction catalyzed by lipoxygenase. Lipoxin A4 has been shown to bind to the AhR and to transiently induce CYP1A1 (Schaldach, et al., 1999). Several prostaglandins, also metabolites of arachidonic acid, have been reported to activate the AhR signaling pathway although at rather high concentrations (100 µM) (Seidel, et
al., 2001). Denison and Nagy (2003) raised the possibility that prostaglandins may be more prone to bind to the receptor \textit{in vivo} due to the fact that they are charged at physiological pH and that the CYP1A1 induction measured in cell suspensions in methylcellulose (Sadek and Allen-Hoffmann, 1994) could be a result of release of arachidonic acid metabolites in membranes, i.e. lipoxin A4 and some prostaglandins. Bilirubin is the primary degradation product of hemoglobin and a weak but competitive ligand for the AhR. Additionally, biliverdin also appears to induce CYP1A1, but this is probably only indirectly, through its role as a precursor of bilirubin (Sinal and Bend, 1997; Phelan, \textit{et al.}, 1998). It has been demonstrated that congenitally jaundiced Gunn rats, defective in the functional conjugating enzyme UGT*01, have high plasma levels of bilirubin and exhibit constitutively elevated expression levels of CYP1A1 and CYP1A2 (Kapitulnik and Gonzalez, 1993).

\textbf{Figure 3. Structures of "non-classical" naturally occurring AhR ligands and CYP1A1 inducers.}

\textbf{Indole-containing molecules as endogenous AhR ligands and CYP1A1 inducers}

Several indole-containing substances are derived from the essential amino acid tryptophan (TRP) (Fig 4). It is one of the most strongly near-UV absorbing amino acids and can act as an important endogenous chromophore. TRP is involved in many photo-oxidation processes (Creed,
1983) and is a key amino acid in a variety of functions in animals and humans.

The indole family includes a variety of substances such as indole-3-carbinol (I3C), 3,3'-diindolylmethane (DIM), indole-3-acetic acid (IAA), tryptamin (TA), indolo[3,2-b]carbazole (ICZ) and 6-formylindolo[3,2-b]carbazole (FICZ) that all exhibit AhR affinity and/or CYP1A1 inducing capacity (Loub, et al., 1975; Gillner, et al., 1985; Bradfield and Bjeldanes, 1987b; Bjeldanes, et al., 1991; Jellinck, et al., 1993; Miller, 1997; Heath-Pagliuso, et al., 1998; Wei, et al., 1998). However, the majority of these compounds (I3C, TA, IAA and TRP) display a low AhR affinity and/or AhR-dependent gene expression.

Figure 4. Formation of indole-containing derivatives of tryptophan that possess AhR affinity and/or CYP1A1 inducing capacity.
TA is a decarboxylated metabolite of TRP (Juorio and Durden, 1984) and a trace amine found in low concentrations in the mammalian CNS (Jones, 1982) and it has been speculated that it plays a role in schizophrenia (Sullivan, et al., 1980). TA is further metabolized by human monoamine oxidase enzymes (MAO) to indole-3-acetaldehyde (IAld) (Yu, et al., 2003), which in turn can be converted to the plant growth hormone indole-3-acetic acid (IAA, auxin) (Helander and Tottmar, 1987; Kawaguchi and Syono, 1996; Bartel, 1997). The conversion to IAA makes it difficult to measure tissue levels of TA. However the CNS concentration of TA has been reported to be as high as 120 ng/g in the presence of MAO inhibitors (van Nguyen, et al., 1989).

I3C is an autolysis product of glucobrassicin, a compound found in vegetables of the Brassica family (cabbage, brussel sprouts, etc) (McDanell, et al., 1988). I3C is highly reactive under acidic conditions and can under these circumstances form biologically active compounds with high AhR binding affinity (Perdew and Babbs, 1991). Bjeldanes and Bradfield reported that the induced CYP1A1 activity detected in liver of rodents after ingestion of cruciferous vegetables cannot, however, be totally explained by I3C (Bradfield and Bjeldanes, 1987a) and that I3C was only capable of inducing EROD activity when administered orally and not via intraperitoneal injection (Bradfield and Bjeldanes, 1987b). Interestingly, gastrointestinal bacteria in combination with oxygen are able to convert TRP to AhR ligands (Perdew and Babbs, 1991) and both ICZ and DIM have been detected in the digestive tract of animals fed on Savoy cabbage or intubated with I3C (Bjeldanes, et al., 1991; Perdew and Babbs, 1991; Kwon, et al., 1994). ICZ displays a high AhR affinity (0.2-3.6 nM) and is a potent inducer of AhR dependent gene expression in humans and animals (Gillner, et al., 1985; Bjeldanes, et al., 1991; Perdew and Babbs, 1991; Kwon, et al., 1994; Chen, et al., 1995). A complete endogenous mechanism for a possible formation of I3C has not yet been presented. However, by compiling reports dealing with different parts of the pathway such as i) the conversion of TRP to 3-methylindole (skatole), a result of fermentation and decarboxylation by microorganisms in bovine rumen [Yokoyama, 1974 #268], ii) the detection of skatole in human faeces [Dehnhard, 1991 #93] and iii) the conversion of skatole to I3C by several cytochrome P450 enzymes including, CYP1A1, 1A2 and 1B1 (Thornton-Manning, et al., 1996; Lanza and Yost, 2001), an endogenous mechanism for I3C formation seems quit conceivable.
Yeast of the genus *Malessezia*, found in for example, human skin, can convert TRP to malassezin, a CYP1A1 inducer that does not fulfill the structural requirements of an AhR agonist but, on the other hand, easily cyclizes in vitro to give ICZ (Wille, et al., 2001). Tryptanthrin is also formed from TRP by a family of yeast, *Candida lipolytica*, found in human food and has been reported to interact with the AhR and induce CYP1A1 (Schrenk, et al., 1997).

The color pigments indigo and indirubin are produced by fermentation of plant material (Balfour-Paul, 1998) and both substances have been reported to be equipotent AhR ligands to TCDD in a yeast AhR signaling assay (Adachi, et al., 2001). However, in mammalian systems indigo and indirubin are described to be less potent as AhR ligands (Guengerich, et al., 2004). These substances have been detected in human urine (Sapira, et al., 1971; Adachi, et al., 2001) and indirubin displays a transient induction of CYP1A1 and 1B1 in human breast cancer cells (Spink, et al., 2003). Indigo is produced commercially by means of bacterial systems where microbial oxygenases oxidize indole, to indoxyl, which in turn rapidly oxidizes in air and dimerizes to form indigo and indirubin (Russel and Kaupp, 1969). Furthermore, it has been reported that indigo and indirubin are products of human cytochrome P450-catalyzed metabolism of indole (Gillam, et al., 2000).

Recently, an indole containing AhR agonist was isolated from porcine lung and identified as 2-(1’H-indole-3´-carbonyl)-thiazole-4-carboxylic acid methyl ester, although the origin has not been established (Song, et al., 2002).

As mentioned above, UV irradiation of TRP produces several photoproducts of which some show AhR affinity. Studies by Paine established that aryl hydrocarbon hydroxylase activity (AHH) in rat liver cells treated with UV-illuminated cell culture media is dependent on the amino acid concentration as well as on the formation of a stable inducing intermediate in the media (Paine, 1976a, 1976b). In 1980 Paine and Francis postulated that histidin was the oxidized amino acid responsible for the observed induction, and that TRP was needed for the response (Paine and Francis, 1980). In the 1980s two research groups published observations showing that exposure of mice and rats to UV light resulted in induced AHH activity (Goerz, et al., 1983; Mukhtar, et al., 1986; Goerz, et al., 1996). Photolysis studies revealed that TRP could give rise to photoproducts that
are capable of activating the AhR and induce AHH activity (Rannug, et al., 1987; Helferich and Denison, 1991; Sindhu, et al., 1996). Several photoproducots have been chemically identified (Creed, 1983; Borkman, et al., 1986; Rannug, et al., 1995). One of these TRP-derived substances is the ICZ derivative, 6-formylindolo[3,2-b]carbazole (discussed below).

6-FORMYLINDOLO[3,2-b]CARBAZOLE

In a line of studies aiming at determining the optimal AhR ligand, TRP photoproducots were produced and tested by Rannug and co-workers. The irradiation of TRP solutions by UV led to the detection of three photoproducots with very high AhR affinity, formed in a time-dependent manner (Rannug, et al., 1987). In 1995, two of these photoproducots were identified as 6-formylindolo[3,2-b]carbazole (FICZ) and 6,12-diformylindolo[3,2-b]carbazole, respectively (Rannug, et al., 1995).

Formation

The mechanism of FICZ formation from TRP is not known in detail, although indole-3-acetaldehyde (IAld) is regarded as the precursor of FICZ (Fig 5). It has been speculated that IAld is formed as an intermediate upon irradiation of TRP (Saito, et al., 1984). However, attempts to detect IAld from irradiated TRP have been unsuccessful due to the instability of the substance. Oxidation of IAld would lead to the formation of α-hydroxyacetaldehyde, which could readily undergo acid-catalysed condensation to yield FICZ.

The formation of FICZ by heat treatment of IAld supports the hypothesized mechanism of formation (Rannug, et al., 1995). A chemical synthesis of FICZ was developed by Tholander and Bergman in 1999 (Tholander and Bergman, 1999).

Properties

As mentioned earlier FICZ binds to the AhR with very high affinity. It demonstrates a 4-7 times stronger binding affinity towards the AhR than
TCDD itself under the same conditions, having a $K_d$ of 0.07 nM compared to 0.48 nM for TCDD (Rannug, et al., 1987). FICZ induces AHH activity in rat hepatoma cells (Rannug, et al., 1987; Rannug and Rannug, 1989) and shows a transient induction of CYP1A1 mRNA in a human keratinocyte cell line (Wei, et al., 1998).

Figure 5. The likely mechanism of formation of FICZ from tryptophan.

The induction of CYP1A1 mRNA by FICZ is efficient in short time incubations, with an effect observed at 10-100 pM concentrations (Wei, et al., 1998). In this study, the transient induction of CYP1A1 mRNA by FICZ in addition to a transient EROD activity emphasized that CYP1A1 was involved in FICZ metabolism. A likely formation of TRP photoproducts in mammalian cells upon UV-irradiation was revealed when cells pre-treated with TRP before UV irradiation showed an induction of CYP1A1 mRNA in an AHR-dependent manner (Wei, et al., 1999).

FICZ also shows antimutagenic properties in the Salmonella typhimurium strain TA100 in the presence of a rat liver S9 metabolic system and BaP (Rannug, et al., 1992). This effect is likely to be explained by the inhibitory effect of FICZ on EROD activity, which has been documented with rat hepatocytes and with the cDNA expressed human cytochrome P4501A1 enzyme (Rannug, et al., 1992; Wei, et al., 1998).
EVIDENCE FOR AN ENDOGENOUS FUNCTION OF THE AhR

Numerous studies of the Ah receptor imply that an endogenous ligand is present during AhR signaling. This is based on the observations that AhR activation and AhR dependent responses exist in the absence of an exogenous ligand. Under conditions of stress such as hyperoxia (Okamoto, et al., 1993; Hazinski, et al., 1995; Couroucli, et al., 2002) and hydrodynamic shear (Mufti, et al., 1995; Mufti and Shuler, 1996) CYP1A1 is induced. In addition, studies point to the involvement of AhR in cell progression and normal development. Cells defective in AhR display a changed morphology, decreased albumin synthesis and slowed growth rate due to prolongation of the G1 phase (Ma and Whitlock, 1996). In addition a constitutively active AhR expressed in transgenic mice has implicated a role for AhR in the control of growth and proliferation due to observed development of severe tumours in the stomach (Andersson, et al., 2002). Knock out of the mouse AhR gene affects normal development of the liver, causing hepatic defects, including reduced liver weight and slowed early growth, as well as a disturbed immune system (Fernandez-Salguero, et al., 1995; Schmidt, et al., 1996). Moreover, activation of the nuclear AhR complex in cell culture in the absence of an exogenous ligand has been reported (Singh, et al., 1996). This selected evidence is a possible indication that the AhR has an endogenous function and thus, requires an endogenous ligand for its performances.

METABOLISM

Biotransformation of xenobiotics and endogenous molecules is an essential mechanism for maintaining homeostasis in cells. There are four major principles in the biotransformation process of xenobiotica (foreign substances), namely:

1. absorption mainly through the gastrointestinal tract, lung and skin
2. distribution by means of lymph and blood
3. metabolism in liver as well as extrahepatic tissues
4. excretion through bile or urine.
Fig 6. The concept of xenobiotic disposition in the body.

The metabolism of xenobiotics as well as endobiotics (natural in the body) occurs by means of two main steps introduced by Williams (1971), that is, phase I and phase II. During phase I, oxygen is introduced into the molecule in a NADPH-dependent process which is discussed in detail below. Phase II, generally involves conjugation reactions, which transform the substrate to even more water soluble molecules prior to excretion. Phase II is generally preceded by phase I reactions although there are cases of direct phase II metabolism (1,2-dichloroethane, bilirubin, paracetamol and steroid hormones) (Rannug, et al., 1978; de Wildt, et al., 1999; You, 2004). Fig 6 illustrates a model of xenobiotic fates in the body (further discussed below).

DISTRIBUTION AND TRANSPORT THROUGH CELL MEMBRANES

The distribution of a chemical is mainly determined by the blood flow and whether a chemical, after oral administration, is susceptible to biotransformation in the gastrointestinal tract. The removal of chemical before the entry to the general circulation is referred to as the first pass effect. The
distribution is largely dependent on its ability to pass cell membranes and on its affinity to various body compartments. Additionally, some chemicals are able to accumulate in various parts of body as a result of binding to, for instance, plasma proteins or fat (Klaassen, 1996). Numerous studies have shown that chemicals bound to plasma proteins cannot cross body membranes and that the lipid/water partition of a chemical is essential for the rate of which it enters human red blood cells (Schanker, 1961).

Depending on where a biotic exerts its action, it usually passes through a number of cell membranes either by passive or active transport and critical qualities for the membrane permeability are determined by hydrophobicity (i.e. lipophilic groups, degree of ionization and size) and protein binding capacity of the chemical.

PHASE I

The reactions of the phase I biotransformation system include hydrolysis, reduction and oxidation and lead to the introduction of a functional group into the molecule (–OH, -SH, -NH₂ or –COOH) and subsequently a small increase in water solubility. Several enzymes are involved in the phase I reactions, although only a few examples will be discussed. The oxidation reactions are mainly carried out by the cytochrome P450 (CYP) family of enzymes and are generally the primary enzymatic defence against foreign compounds; however CYP activities are also involved in the biotransformation of endogenous molecules. In line with the oxidation reactions there are several reducing pathways depending on functional group of the substrate. NADPH-quinone oxidoreductase or DT-diaphorase (Ernster, 1987) reduces quinones by a two electron reduction to hydroquinones without oxygen consumption; thereby the pathway is essentially nontoxic and not associated with oxidative stress. Hydrolysis reactions are catalyzed by carboxylesterases (Satoh, 1987) and epoxide hydrolase (EH) in microsomes and cytosol and peptidases in blood and lysozomes. EH plays an important role in detoxifying electrophilic epoxides (discussed below).

Phase I reactions can additionally be described as an activation of the molecule since a formation of reactive electrophilic intermediates is an occasional consequence of the enzymatic reaction of CYP enzymes, and these
intermediates may be more toxic than the parent molecule (Guengerich, 2000). If these reactive molecules are not further metabolized by phase II conjugation, they may cause damage to proteins and DNA within the cell.

Cytochrome P450

The cytochrome P450 superfamily of enzymes is a large family found in bacteria, plants, insects and vertebrates. These enzymes play an important role in the oxidative metabolism of both endogenous and exogenous substrates. To date, the family comprises 57 functional human CYP genes that have been identified and classified into subfamilies based on amino acid sequence homology (Nelson, 2004).

The CYP enzymes are heme-containing enzymes with iron protoporphyrin IX as the prosthetic group and the classification of a CYP enzyme is based on its ability to absorb light at 450 nm when reduced and bound to carbon monoxide. The basic reaction catalyzed by CYP is:

\[
\text{Substrate (RH)} + \text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow \text{Product (R-OH)} + \text{NADP}^+ + \text{H}_2\text{O}
\]

One atom of molecular oxygen is incorporated into the substrate and the other oxygen is reduced to water. In the endoplasmic reticulum the CYP enzyme receives electrons from NADPH via the NADPH-cytochrome P450 reductase enzyme and following binding of the substrate to the enzyme, the heme iron is reduced from the ferric (Fe\textsuperscript{3+}) to the ferrous state (Fe\textsuperscript{2+}).

The CYP superfamily can be divided into two groups based on the nature of the substrates. One group is involved in the biosynthesis and metabolism of endogenous, physiologically important substances such as steroids, fatty acids, vitamins, thromboxane and bile acids (e.g. CYP11, CYP4, CYP24, CYP5 and CYP27 respectively) (Rendic and DiCarlo, 1997). The other group of CYP enzymes is thought to preferentially metabolize xenobiotics like drugs and environmental pollutants (CYP1, CYP2 and CYP3) (Lewis, 2003). Another classification of the CYP superfamily based on localization of the enzyme is also possible. The highest concentration of CYP enzymes are found in liver, bound to the endoplasmic reticulum, but there are also mitochondrial CYPs, known to be mainly responsible for steroid biosynthesis. However,
xenobiotica-inducible CYPs have also been reported to occur in the mitochondrial fractions of rat liver, lung and brain as well as human brain mitochondrial fractions (Addya, et al., 1997; Anandatheerthavarada, et al., 1997; Morse, et al., 1998; Bhagwat, et al., 1999; Zhang, et al., 2001).

It is known that many clinically relevant diseases are a consequence of mutations in many genes that cause inborn errors of metabolism. The clinical importance of the CYP enzymes has been reviewed by Nebert and Russel (2002).

The CYP1 family consists of three enzymes, CYP1A1, 1A2 and 1B1, all transcriptionally controlled by the AhR signaling pathway. These enzymes are discussed below. Unlike the CYP1 family, the members of the CYP2 family do not share features of regulation. The CYP2 family is the largest P450 family in mammals known to metabolize more than half of the frequently prescribed drugs. The CYP3 family contains only four members all of which are regulated by the pregnane X receptor and the constitutive androstane receptor.

CYP1A1/CYP1A2

The cytochrome P450 1A1 enzyme is one of the most extensively studied enzymes concerning the inducibility by xenobiotics like PAH and dioxins. The induction of CYP1A1 by TCDD is a very sensitive response where increased CYP1A1 levels are observed at dosages as low as 0.1 ng/kg body wt (ED50) (Kedderis, et al., 1991). The AHH activity, measured as the ability of PAH to enhance their own metabolism was previously used to reflect the CYP1A1 activity (Nebert and Gelboin, 1968b, 1968a) but today the activities of CYP1A1/1A2 are usually measured as the dealkylation of 7-ethoxy and 7-methoxyresorufin, respectively (EROD, MROD) (Burke and Mayer, 1974, 1983). The CYP enzymes 1A1 and 1A2 are known to have overlapping substrate specificities (Shou, et al., 1996) even though CYP1A1 metabolizes a variety of relatively planar polycyclic aromatic hydrocarbons (Doehmer, et al., 1995) and CYP1A2 is identified to preferentially cause N-hydroxylations (Butler, et al., 1989).
CYP1A2 constitutes approximately 13% of the total CYP enzyme content in human liver (Shimada, et al., 1994) but unlike most CYPs, microsomal CYP1A1 is not constitutively expressed in the liver at the protein level, although it has a high capacity for transcriptional up-regulation via the AhR in liver as well as in other organs like kidney, intestine, lung, skin, stomach, colon, duodenum and brain (Dey, et al., 1999; Wei, et al., 2001). The mitochondrial CYP1A1 consists of a N-terminal truncated microsomal CYP1A1 with different substrate specificity compared to the intact protein (Addya, et al., 1997; Anandatheerthavarada, et al., 1998). The mitochondrial CYP1A1 content in uninduced rat lung has been reported to be 1.5-fold higher than the microsomal CYP1A1 (Bhagwat, et al., 1999) and human brain CYP1A1 mRNA is mainly localized in the mitochondria (Zhang, et al., 2001). The role of CYP1A1 in the central nervous system has been questioned by Nebert and Dieter since the blood-brain barrier would prevent the typical pharmaceutical agent from entering the brain and thus drug detoxification in brain is not necessary. Enzymes like CYP1A1 therefore seem to have a distinct role in this organ (Nebert and Dieter, 2000). In addition, the constitutive CYP1A1 mRNA levels in mouse ovum are strikingly increased shortly after fertilization, the purpose which might be prevention of AhR activation by a rapid degradation of any endogenous or exogenous ligand (Dey and Nebert, 1998). It has been suggested that CYP1A1 metabolizes an endogenous substrate postulated to be an endogenous ligand for the AhR (Hankinson, et al., 1985; Chang and Puga, 1998).

Interestingly, as shown by Uno and coworkers, after induction of the CYP1A1 gene, which has been considered a potential risk factor for toxicity and cancer, the CYP1A1 (+/+) wild type mice survive longer than CYP1A1 (-/-) knockout mice after administration of a daily dose of BaP. Furthermore, mice with a functional CYP1A1 gene were more protected from immunotoxicity, myelotoxicity and wasting than the CYP1A1 (-/-) knockout mouse after oral treatment of BaP. By this means, CYP1A1 seems to be much more important in detoxification than in metabolic activation (Uno, et al., 2004a). In contrast to the CYP1A1 protection against BaP-induced toxicity, CYP1A1 (-/-) knockout male mice were protected against high-dose TCDD-induced lethality, wasting syndrome and uroporphyria (Uno, et al., 2004b). The protection provided by a loss of the CYP1A1 gene was small in comparison to the protection seen in AhR deficient mice (Fernandez-Salguero, et al., 1996).
CYP1B1

CYP1B1 was identified in 1994 as a new member of the CYP1 gene family. It was cloned from TCDD-treated human keratinocytes (Sutter, et al., 1994). Human CYP1B1 mRNA is mainly observed in extrahepatic tissues including kidney, intestine, eye and brain (Sutter, et al., 1994; Shimada, et al., 1996; Rieder, et al., 1998; Stoilov, et al., 2004). However, native protein has not been isolated from normal human tissue, suggesting a very low constitutive expression of CYP1B1 protein (Murray, et al., 2001). The CYP1B1 enzyme, similar to CYP1A1, is not constitutively expressed in rat liver but is inducible by dioxin (Walker, et al., 1998). It is, like CYP1A1/1A2, able to metabolize a variety of xenobiotics, such as PAH and many N-heterocyclic amines, arylamines and others (Shimada, et al., 1996; Shimada, et al., 1997) as well as endogenous compounds like 17β-estradiol (Hayes, et al., 1996).

CYP1B1 may play an important role in the metabolic activation of carcinogens since CYP1B1 null mice are resistant to the cytotoxicity caused by the prototype carcinogen, 7,12-dimethylbenz[a]antracene. In addition, the enzyme shows increased expression in a wide range of human tumors (Murray, et al., 1997). CYP1B1 mutations in humans are associated with early onset of glaucoma, (Bejjani, et al., 1998) and thus, Nebert and colleagues have discussed these effects as a result of a deficient metabolism of an important endogenous substrate of CYP1B1, required for the development of the anterior chamber of the eye (Nebert and Russell, 2002).

Epoxide hydrolase

There are five classes of mammalian epoxide hydrolase (EH) enzymes, of which one class, the microsomal EH (Skoda, et al., 1988), exhibits a broad range of substrate specificity and is primarily associated with the metabolism of xenobiotics and certain steroids. Microsomal EH (mEH) is implicated in detoxification reactions where various epoxides are hydrated to the corresponding dihydrodiols (Seidegard and DePierre, 1983). mEH has been found in a wide variety of organs (Oesch, et al., 1977) and the distribution in liver is similar to the distribution of CYPs. Both enzymes are found in the endoplasmic reticulum in the centrlobular region of the liver (Wolf, et al., 1984). There are implications that both mEH and the phase II enzyme UDP-
glycuronosyl transferase (discussed below) interact with CYP1A1 as a way to facilitate series of multi step drug metabolic conversions (Taura, et al., 2000).

mEH is an important enzyme since there are several oxidizing systems apart from CYP that lead to peroxidations of lipids, cholesterol and plasmalogen, forming epoxides and potentially causing toxic effects (Sevanian and Peterson, 1986; Spiteller, 1993). Although most epoxides are highly reactive and toxic, the Vitamin K epoxide is a non-toxic stable epoxide, which is formed and consumed in the liver (Olson, 1984). It should be emphasized that, apart from EH, other enzymes like glutathione transferase and epoxide reductase have the ability to deactivate epoxides. Epoxides may also be deactivated by a non-enzymatic reaction with water, forming diols and phenols. The pathway depends on localization and structure of the epoxide.

PHASE II

The phase II reactions in the biotransformation of xenobiotics (mainly preceded by the CYP enzyme reactions) result in a large increase in hydrophilicity of a chemical and thus facilitate its excretion from the body. These reactions include glucuronidation, sulfation, acetylation or conjugation to amino acids or glutathione. The phase II reactions are generally faster than the phase I reaction, thereby the rate of multi-step biotransformation reactions are normally defined by the rate of the CYP reactions (Klaassen, 1996). The enzymes catalyzing the phase II reactions that will be discussed are uridine diphospho-glucuronosylglucuronosyl transferases (UGT) and sulfotransferases (SULT).

UDP-Glycuronosyl transferase

Glucuronide formation in biotransformation reactions represents an important process in which several endogenous and exogenous substances are converted to water soluble products and prepared for excretion. Glucuronidation requires a “high-energy” cofactor, the uridine diphosphate-glucuronic acid (UDPGA), synthesized from UDP-glucose. The reaction is catalyzed by UGTs that are present in various tissues, including liver, kidney,
skin and brain (Burchell and Coughtrie, 1989). Two vertebrate gene families have been identified, named UGT1 and UGT2, with about 15 members and cDNA expression experiments have identified over 350 individual compounds that serve as substrates for these enzymes (Tukey and Strassburg, 2000).

The endogenous substrates of UGT include bilirubin, steroid hormones and thyroxin. The exogenous substrates include, among others, paracetamol, morphine and PAH. PAH cause a transcriptional up-regulation of especially the UGT1A6 isoform (Bock, et al., 1998). Chemicals with an ability to be glucuronidated generally have electron rich nucleophilic heteroatoms (N, O or S), like alcohols and phenols, carboxylic acids, amines and thiols. The glucuronidation reactions form O-glucuronide ethers, O-glucuronid esters, N-glucuronides and S-glucuronides (Klaassen, 1996). UGT is localized to the lumen side of the endoplasmic reticulum and thereby the microsomal UGT exhibits a latency of activity, which can be overcome by detergents.

Substrates conjugated to glucuronic acid have the ability to be substrates of the intestinal enzyme β-glucuronidase. Cleavage by β-glucuronidase releases the aglycone (the original substance), which can be reabsorbed and enter a cycle called enterohepatic recirculation and thereby delay the excretion of a xenobiotic (Roberts, et al., 2002).

Sulfotransferase

All of the xenobiotic UGT substrates, except for carboxylic acids, that undergo O-glucuronidation can also undergo sulfate conjugations and the resulting products are water soluble sulfuric acid esters (Klaassen, 1996). The SULT enzymes located in the cytosol are responsible for conjugation of exogenous and small endogenous compounds (like neurotransmitters and hormones). There are presently 10 different human SULTs known and classified into the subfamilies, 1A, 1B, 1C, 1E, 2A, 2B, and 4A. Almost every SULT shows a unique tissue distribution and the substrate specificities are different but overlapping (Glatt, et al., 2001).

SULTs utilize 3’-phosphoadenosine-5’-phosphosulfate (PAPS) as the donor of sulfate to be transferred to the substrate. The nucleophilic oxygen or
nitrogen of the substrate attacks the electrophilic sulfur atom of PAPS with a subsequent cleavage of the phosphosulfate bond. The formation of PAPS in vivo is a two-step process from inorganic sulfate and ATP (Falany, 1997). The formed sulfate conjugates of xenobiotics are mainly excreted in urine but sulfate conjugates excreted into the bile have the ability to enter the enterohepatic recirculation, like the glucuronides. However the hydrolysis of sulfate conjugates is catalyzed by aryl sulfatase, present in gut microflora (Goldin, 1990).

In general sulfation reactions follow a low capacity but high affinity xenobiotic metabolism pathway whereas glucuronidation is a high capacity and low affinity pathway. Thereby the concentration of substrate is a limiting factor in the choice of pathway.
**THE PRESENT STUDY**

**AIMS**

The natural ligand(s) of the AhR and the endogenous function of AhR are still not known although several observations point to the existence of an endogenous ligand. Earlier observations by our group have demonstrated that the tryptophan photoproduct, FICZ, is an efficient AhR ligand and CYP1A1 inducer at low concentrations (Rannug, *et al.*, 1987; Wei, *et al.*, 1998). It was also reported that UV-light causes an AhR dependent induction of CYP1A1 mRNA, which was enhanced by tryptophan (Wei, *et al.*, 1999). These observations indicate that FICZ might be an endogenous AhR ligand. The overall goal is to detect FICZ *in vivo* and for this purpose stable metabolites of FICZ need to be identified. The specific aims of the present study have been to:

- Investigate the impact of FICZ and FICZ metabolism in CYP1A1 gene regulation.

- Characterize and chemically identify metabolites formed from FICZ by the phase I enzymes.

- Determine the role of the AhR-regulated CYPs in the metabolic transformation of FICZ.

- Study the impact of glucuronidation and sulfation in the metabolism of FICZ.

- To elucidate the influence of normal laboratory light and tryptophan on CYP1A1 induction and subsequently identify formed photoproducts.
COMMENTS ON METHODOLOGY

METABOLIC SYSTEMS

Different liver fractions

Liver fractions from untreated and Aroclor 1254 pretreated Sprague-Dawley male rats and liver fractions from humans have been utilized as metabolizing systems in the studies of FICZ (Papers I - V). The 9000 x g preparation (S9 fraction) of pooled liver homogenates contains both cytosolic and microsomal proteins. Thus the S9 fraction contains the majority of phase I and phase II enzymes. To retain the activity of the phase I and phase II enzymes the S9 fraction has to be supplemented with the required cofactors. The CYP enzymes require NADPH. An NADPH generating system is achieved by the addition of glucose-6-phosphate and NADP+ according to the following stoichiometry:

\[
\text{Glucose-6-P} + \text{NADP}^+ \rightarrow 6\text{-phosphogluconate} + \text{NADPH}
\]

The reaction is catalyzed by the cytosolic enzyme glucose-6-phosphate dehydrogenase. Thus, this enzyme needs to be added when microsomes are used. To allow reactions to proceed without any limitations it is necessary to consider the critical cofactors for the phase II enzymes as well. For this study PAPS and UDPGA, which are essential for sulfation and glucuronidation reactions, respectively, were added. The microsomal fraction is prepared by differential centrifugation and only includes endoplasmic reticulum bound enzymes. This system normally contains a higher Phase I enzyme activity per unit compared to the S9 fraction.

Cell lines

Mouse hepatoma Hepa-1 cells were employed in the present work in order to characterize the FICZ dependent transcriptional regulation of the CYP1A1 gene as well as to investigate the significance of the CYP1A1 enzyme in the metabolism of FICZ (Paper I). Rat hepatoma MH1C1 cells were utilized for EROD activity measurements in the study of how normal laboratory light influences the constitutive aryl hydrocarbon receptor activity (Paper V). A
number of AHH deficient mutants that are resistant to BaP have been isolated from the wild-type (wt) hepa-1 cells (Hankinson, 1983). One of these, the c37 clone, was used in this study together with the wt cell line. The c37 line is deficient in CYP1A1 activity due to two point mutations in the CYP1A1 structural gene, leading to amino acid substitutions. The two mutations together totally abrogate the AH activity (Kimura, et al., 1987). The CYP1A1 content was increased for the metabolism experiments by treatment of the cells with β-naphtoflavone for 24 hours before the S9 fractions were prepared.

Based on the knowledge that TRP can be converted to high affinity AhR ligands and since several groups have been reported that light-exposed cell medium transiently induces CYP1A1 gene expression and activity (Paine, 1976b; Paine and Francis, 1980; Kocarek, et al., 1993; Harvey, et al., 1998; Feng, et al., 2002) the cell medium was protected from light in all experiments unless otherwise stated.

Heterologously expressed enzymes

Human cytochrome P450 enzymes expressed in the yeast *Saccharomyces cerevisiae* were utilized to characterize the substrate specificity of CYP1A1 and 1A2 in the formation of FICZ metabolites as well as to determine the kinetics of the enzyme reactions.

Cytosolic preparations of *Salmonella typhimurium* TA1538 expressing the human sulphotransferases, 1A1, 1A2, 1A3, 1B1, 1E1 and 2A1 were used to measure the formation of sulfate conjugates from the mono- and dihydroxylated metabolites of FICZ. The formation of conjugates was measured over time using an HPLC and a fluorescence detector.

**INSTRUMENTATION USED FOR DETECTION AND STRUCTURE ASSIGNMENT OF METABOLITES**

High performance liquid chromatography (HPLC)

HPLC is a widely used analytical technique for separation and detection of chemical substances. Detection by UV can be performed by either
monitoring the absorption at a specific wavelength or by scanning between two wavelengths by using a diode array detector. A diode array detector has two major advantages. It allows for the best wavelength to be selected for the actual analysis and it facilitates the detection of impurities. Aromatic compounds, like FICZ, contain conjugated phi-electrons exhibit the greatest fluorescence intensity. Other compounds also fluoresce, but usually to a lesser degree (Kemp, 1991).

![Absorbance vs Wavelength](image)

Figure 7. HPLC UV spectra of 6-formylindolo[3,2-b]carbazole in a gradient of 0.1% triflouracetic acid in acetonitrile and water.

Analyses of FICZ were performed by both UV detection and fluorescence detection. The specific UV spectrum of FICZ (Fig 7) with an absorption peak at 386 nm made it possible to analyze complex samples at 386 nm instead of the commonly used 254 nm. Comparing the UV spectra of the HPLC peaks has been useful in determining FICZ and its metabolites. The fluorescence properties of FICZ (Rannug, et al., 1995) are characterized by a maximum excitation/emission at 390 nm and 525 nm, respectively. The HPLC system was employed for the detection of FICZ and its formed metabolites as well as for the fractionation of complex samples (Papers I-V).
Mass spectrometry (MS) and nuclear magnetic resonance (NMR)

Mass spectrometers are used for a wide variety of studies e.g. mass measurements of different molecules. The result of ionization, ion separation and detection is a mass spectrum providing information about molecular weight as well as structural properties (Johnstone and Rose, 1996). Examination of FICZ metabolites by MS in this study was performed by utilizing LC-MS with negative electrospray ionization (-ESI) and a triple quadrupole as the analyzer (Paper II, IV, V).

Nuclear magnetic resonance spectroscopy is a theoretically complex but powerful analytical tool in which one utilizes a property of atomic nuclei, i.e. the behaviour of the protons as tiny spinning bar magnets. Introduction of a compound to a magnetic field makes it possible to record differences in the magnetic properties of various magnetic nuclei and to deduce their positions within a molecule. As not all atoms possess an overall spin, $^{12}$C and $^{16}$O are invisible to NMR in contrast to $^{13}$C and $^1$H. Two different techniques of 2-dimensional NMR spectra, COSY and NOESY, have been applied in this study to deduce the environment of an atom by measuring coupling between protons. COSY stands for correlation spectroscopy and is a way of measuring interactions of protons through chemical bonds and NOESY (nuclear Overhauser effect spectroscopy) gives information about interactions between protons that are spatially close (Kemp, 1991).

In this study, NMR was utilized to identify the chemical structures of the components in the three major fractions of FICZ metabolites (Paper II).

MEASUREMENTS OF CYP1A1 INDUCTION

Gene expression analysis by RT-PCR

Reverse transcriptase-polymerase chain reaction (RT-PCR) is a method based on amplification of mRNA to facilitate the analysis of small amounts of mRNA in cells and tissues. In this study RT-PCR was employed to
investigate the effects of TRP and FICZ on the expression of CYP1A1 in Hepa-1 cells (Paper I).

EROD-assay

The O-dealkylations of ethoxyresorufin and methoxyresorufin, EROD and MROD, are widely used methods for determining the activity of the cytochrome P450 enzymes CYP1A1 and CYP1A2, respectively. For the purpose of this study, the EROD activity was measured in the Hepa-1 cells (Paper I) and MH1C1 cells (Paper V) using 96-well multiple plates using slightly modified published methods (Pohl and Fouts, 1980; Kennedy, et al., 1993). Since CYP1A1, 1A2 and 1B1 are known to have overlapping substrate specificities, the measured EROD activity may, to some extent, reflect the activity of all three enzymes. However, mouse Hepa-1 cells have no known CYP1B1 activity (Zhang, et al., 1998).

INHIBITION OF METABOLIZING ENZYMES

A number of cytochrome P450 inhibitors have been developed that exhibit a relatively high degree of specificity for certain CYP enzymes. In combination with other metabolizing enzyme inhibitors these inhibitors are useful in the elucidation of the contribution of specific enzymes in metabolism studies.

In the present study, rat and human hepatic S9 fractions were employed together with specific enzyme inhibitors to study the participation of the CYP1 family of enzymes and microsomal EH in the metabolism of FICZ. Three different polycyclic aromatic acetylene compounds were employed as selective CYP inhibitors. 3-ethynylphenanthrene (3EP), 2-(1-propanyl)phenanthrene (21PP) and 1-(1-propanyl)pyrene (11PP) synthesized by Foroozesh et al., (Foroozesh, et al., 1997) were selected for their ability to inhibit 1A1, 1B1 and 1A1/1A2/1B1, respectively (Shimada, et al., 1998). Ellipticine, primaquine and cyclohexene oxide (CHO) were utilized with regard to their capacity to inhibit CYP1A1, CYP1A2 and mEH, respectively.
RESULTS AND DISCUSSION

TRANSIENT INDUCTION OF CYP1A1 BY FICZ (Paper I).

An autoregulatory feedback loop has been suggested to be present in the regulation of CYP1A1. The hypothesis was primarily based on the observation that cell lines defective in CYP1A1 activity were found to have higher constitutive levels of CYP1A1 mRNA than the wild type cells (Hankinson, et al., 1985; Kimura, et al., 1987) and that insertion of a functional CYP1A1 gene, restored the repression (RayChaudhuri, et al., 1990; Chang and Puga, 1998). This may reflect the presence of an endogenous ligand that accumulates in the CYP1A1 deficient cells.

In Paper I, the regulation by FICZ was studied in mouse Hepa-1 cell lines. The induction of CYP1A1 gene expression was found to be different in wild-type and c37 cells, a cell line lacking functional CYP1A1. The wild-type cells displayed a transient induction that declined after 3 hours of exposure to FICZ, while the c37 cells demonstrated a sustained induction. The transient induction of CYP1A1 mRNA by FICZ, in addition to the transient inhibition of cDNA-expressed human CYP1A1 enzyme (Wei, et al., 1998), indicated that FICZ is a competing substrate for the CYP1A1 enzyme. This hypothesis was verified by the differences in metabolism of FICZ observed when using S9 preparations of the two cell lines. Metabolism of FICZ and detection of metabolites were only detected in the wild-type cells and not in the c37 cells, pointing to a CYP1A1 dependent metabolism of FICZ. Further support for the CYP1A1 dependent metabolism of FICZ was obtained in experiments where ellipticine was added. Ellipticine, an inhibitor of CYP1A1 (Lesca, et al., 1979) and an antagonist of the AhR (Gasiewicz, et al., 1996), totally blocked the metabolism of FICZ at the lowest concentration used (5 µM). In addition, treatment of the Hepa-1 wild-type cells with ellipticine significantly increased the basal CYP1A1 mRNA level. Taken together, these results showed that the CYP1A1 enzyme may regulate both the constitutive and the FICZ-induced CYP1A1 mRNA levels. It can therefore be suggested that CYP1A1 substrates, either endogenous or present in the cell culture media, affect the transcription of the CYP1A1 gene.
CHARACTERIZATION OF PRIMARY METABOLITES OF FICZ (Papers I, III and IV)

The metabolic pathways of FICZ were examined in different metabolic systems, namely induced and non-induced rat liver fractions and human liver fractions. At first, when utilizing Aroclor induced rat liver S9, three main metabolite peaks were detected by HPLC and their formation was time dependent. The metabolite peaks were designated metabolites 1 (M1), 2 (M2) and 3 (M3) according to their HPLC retention time and hydrophilicity. Consequently M1 is the most polar metabolite and M3 the most non-polar metabolite. The UV absorption spectra of these metabolites were highly similar to the spectrum of FICZ and therefore their identification in complex mixtures was facilitated. However, the metabolites exhibited changed fluorescence properties compared to FICZ. The formation of M1-M3 was NADPH dependent, pointing to a cytochrome P450 dependent metabolism. Furthermore, a product precursor relationship between M3 and M1 and M2 was observed since the most non-polar metabolite (M3) was formed ahead of the more polar metabolites M2 and M1.

The human liver S9 and the non-induced rat liver S9 caused a more intricate metabolic profile of FICZ compared to the Aroclor induced rat liver S9. The major HPLC peaks M2 and M3 were detected in all three S9 systems. M1 was not detected with human S9 but was a distinct peak when a microsomal preparation from the human S9 was used. The HPLC peak termed MA was one of the predominant peaks in the chromatogram from the human liver incubations. A number of other metabolites (h1-h5) were also produced from FICZ by the human liver S9 and observed in small amounts in non-induced rat liver and in separate incubations of metabolites. Another human metabolite, h6, was formed when M2 was used as a substrate. In contrast to M1-M3, h1 exhibits strong fluorescence at the specific wavelengths utilized for FICZ analysis and the UV spectra of h1-h5 are shifted to shorter wavelengths compared to FICZ.

The rate of FICZ metabolism differed between the metabolic systems. Induced rat liver causes a rapid increase of the metabolites M1-M3, followed by a decrease of all metabolites. On the other hand, the metabolites were all present or still increasing at the last sampling point (80 min) when utilizing the non-induced rat liver or human liver. This observation indicated a further
metabolism of M1-M3 in the induced system to more polar metabolites that could not be detected by the HPLC method used.

STRUCTURE IDENTIFICATION OF THE PRIMARY METABOLITES OF FICZ (Papers II and IV)

The studies presented in Papers II and IV were performed with the intention to structurally identify the three major metabolite fractions of FICZ (M1-M3) formed by rat liver enzymes and the additional metabolites (MA and h1-h6) formed mainly by human liver enzymes.

In the work described in paper II, FICZ was metabolized in the presence of Aroclor-induced rat liver S9. Extracts were fractionated by HPLC and pooled metabolite fractions were subsequently evaporated and analyzed by LC-MS/MS and NMR. The LC-MS analysis of the crude extracts and pooled metabolite fractions assigned the molecular weights of M1, M2 and M3 to 316, 316 and 300, respectively, corresponding to the insertion of one or two hydroxy groups to the parent compound FICZ (Mw 284). The NMR analysis of combined fractions revealed that fraction M3 and M2 in fact each contained two compounds, respectively. A strong attraction between the hydrogen on the nitrogen in position 5 (Fig 8) and the oxygen of the formyl group resulted in a nuclear Overhauser effect (NOE) between the hydrogen of the formyl group and the hydrogen in position 7. This cross peak in the NOSEY spectrum enabled the identification of the position of the hydroxy
group in ring E. However, it was not possible to decisively confirm the position of the hydroxy group in ring A due to a missing NOE effect, but the high electron density of positions 2 and 4, in ring A, most likely would direct the hydroxylations to these positions. The M3 fraction contained two coeluting substances in a 5:4 ratio and the hydroxy group of the major compound was assigned to position 8 (8-hydroxyindolo[3,2-b]carbazole-6-carboxaldehyde; 8-OH-FICZ). The minor substance of M3 was designated to be hydroxylated in position 2 (2-hydroxyindolo[3,2-b]carbazole-6-carboxaldehyde; 2-OH-FICZ). Moreover the M2 fraction also contained two coeluting substances in a 5:2 ratio. The major substance was hydroxylated in position 2 and 10 (2,10-dihydroxyindolo[3,2-b]carbazole-6-carboxaldehyde; 2,10-dOH-FICZ) and the minor substance was hydroxylated in position 4 and 8 (4,8-dihydroxyindolo[3,2-b]carbazole-6-carboxaldehyde; 4,8-dOH-FICZ). The M1 fraction contained one compound identified as 2,8-dihydroxyindolo[3,2-b]carbazole-6-carboxaldehyde (2,8-dOH-FICZ). The structures of all metabolites were verified by Wahlström and coworkers in a subsequent synthesis of the above mentioned compounds (Wahlström, et al., 2004).

In Paper IV, the structures of the metabolites h1, h3 and h5, which were observed with human S9, were resolved by LC-MS/MS. The structure of h2 and h4 were postulated from product-precursor studies using microsomal as well as cytosolic fractions of human liver. In addition, LC-MS analyses indicated a molecular weight of 300 for MA. This pointed to a cytochrome P450 catalyzed formation of a mono-hydroxylated metabolite since its NADPH dependent formation was observed in microsomes. The structures of h1, h3, h5 and h6 were identified by comparing the molecular weights, retention times and fragmentation patterns and/or UV and fluorescence spectra to standards synthesized by Wahlström and coworkers (Wahlström, et al., 2004). An oxidation of the aldehyde moiety resulted in h1, i.e. indolo[3,2-b]carbazole-6-carboxylic acid (CICZ). As mentioned above the metabolite fractions M1–M3 are ring-hydroxylated derivatives of FICZ with an intact formyl group and thus, their UV spectra are comparable to the UV spectrum of FICZ. The metabolites h2–h6 are more polar than h1 and have analogous UV spectra and may therefore be hydroxylated derivatives of h1.

A product-precursor investigation of the human metabolites performed prior to structure analysis revealed that h1-h6 are formed independently of
NADPH in the cytosolic fraction after metabolism of FICZ (to h1), 8-OH-FICZ (to h2), 2-OH-FICZ (to h3), 2,10-dOH-FICZ (to h4), 2,8-dOH-FICZ (to h5) and 4,8-dOH-FICZ (to h6). However, h3 was also formed in a NADPH dependent manner from h1. The structure identification confirmed the formation of 2-hydroxyindolo[3,2-b]carbazole-6-carboxylic acid (h3) and 2,8-dihydroxyindolo[3,2-b]carbazole-6-carboxylic acid (h5) and 4,8-dihydroxyindolo[3,2-b]carbazole-6-carboxylic acid (h6). Since h4 is a metabolic product of 2,10-dOH-FICZ and h2 of 8-OH-FICZ and LC-MS/MS analysis indicated a carboxylic acid group in both h2 and h4 together with one and two hydroxy groups respectively, these metabolites most likely are 8-hydroxyindolo[3,2-b]carbazole-6-carboxylic acid and 2,10-dihydroxyindolo[3,2-b]carbazole-6-carboxylic acid. A summarized scheme of the identified and proposed metabolites is given in Fig 9.

CHARACTERIZATION OF ENZYMES CRITICAL FOR METABOLIC TRANSFORMATION OF FICZ (Papers I, III and IV).

In the study presented in Paper II, in vitro metabolites of FICZ were identified to be preferentially mono- and di-hydroxylated derivatives (see Fig 9). Papers III and IV describe work that was undertaken in order to identify the enzymes involved in the hydroxylations and potential conjugation reactions.

It was previously reported that FICZ transiently inhibits the cDNA-expressed human CYP1A1 enzyme (Wei, et al., 1998), indicating that FICZ is a competing substrate for the enzyme. In Papers I, III and IV the importance of CYP1A1 in the metabolism of FICZ was shown by i) the lack of metabolite formation using a CYP1A1 deficient cell line, ii) the total block of metabolite formation in the presence of the CYP1A1 inhibitors, ellipticine and 21PP and iii) the metabolism of FICZ by heterologously expressed human CYP1A1.

Dalton and coworkers (2000) have argued that CYP1A2, due to the overlapping substrate specificity between CYP1A1 and CYP1A2, could compensate for the lack of CYP1A1 in liver. In the study presented in Paper IV, we used heterologously expressed human CYPs, and found that both
Figure 9. Summarized scheme of metabolite formation of FICZ as well as responsible human cytochrome P450 enzymes involved in the reactions using human liver fractions and heterologously expressed enzymes.
CYP1A1 and CYP1A2 participate in the formation of the mono-hydroxylated metabolites 2-OH-FICZ and 8-OH-FICZ. Preliminary data showed that CYP1A2 has a somewhat higher specificity for FICZ although the efficiency of the CYP1A1 enzyme is higher than the corresponding efficiency of the CYP1A2 enzyme.

Similar to the hydroxylations of the estrogens, estrone and estradiol, the CYP enzymes show some regioselective hydroxylations in the metabolism of FICZ. CYP1A2 was concluded to be essential for the subsequent hydroxylation at positions 4 and 10, forming M2 (4,8-dOH- and 2,10-dOH-FICZ) since (i) the CYP1A2 inhibitor primaquine caused a substantial decrease in the amount of M2 in the induced rat liver and (ii) the heterologously expressed human CYP1A2 favored a formation of M2 in extended incubations of FICZ. On the other hand, 2,8-dOH-FICZ (M1) was the major di-hydroxylated metabolite from FICZ using heterologously expressed human CYP1A1. The formation of 2,8-dOH-FICZ from the mono-hydroxylated metabolites was catalyzed by CYP1A1 when using 2-OH-FICZ as a substrate and by CYP1A2 when using 8-OH-FICZ. In addition, CYP1A2 was solely responsible for the second hydroxylation of 8-OH-FICZ to 4,8-dOH-FICZ as well as the formation of MA but CYP1A2 and, to a lesser degree, CYP1A1 catalyzed the formation of 2,10-OH-FICZ from 2-OH-FICZ. We speculate that the mono-hydroxylated metabolite MA is N-hydroxylated since CYP1A2 is known to cause N-hydroxylations (Butler, et al., 1989).

There are several reports on CYP1A1 induction that appears to be AhR-independent and one example is the CYP1A1 induction by primaquine in human hepatocytes in the absence of AhR binding (Fontaine, et al., 1999). As mentioned, CYP1A2 is highly expressed in liver and primaquine causes an inhibition of the enzyme. Since FICZ is substantially metabolized by CYP1A2 in liver, the reported CYP1A1 induction by primaquine could instead be explained by un-metabolized FICZ due to the CYP1A2 inhibition.

Aldehyde oxidase is probably responsible for the oxidation of the aldehyde group of FICZ since the formation of the carboxylic acid CICZ takes place in the cytosol and is NADH/NADPH independent. The lack of CICZ formation using induced Sprague-Dawley rat liver fraction in the present study could be explained by the fact that Sprague-Dawley rats display three different phenotypes, high, intermediate and deficient with
regard to aldehyde oxidase activity (Stanulovic and Chaykin, 1971). Thus, the liver fraction used could have been derived from aldehyde oxidase deficient rats. The hydroxylations of CICZ are mainly performed by CYP1A1 and result in the formation of 2-OH-CICZ, 8-OH-CICZ, 2,8-dOH-CICZ and to a much lower extent 4,8-dOH-CICZ and 2,10-dOH-CICZ. In addition, CYP1A2 takes part in the formation of 2-OH-CICZ from CICZ.

The CYP1B1 enzyme does not seem to be involved in the primary hydroxylations of FICZ but rather in the further metabolism of the di-hydroxylated metabolites. This conclusion is drawn because the effect of the CYP1B1 inhibitor 3EP caused an accumulation of the most polar metabolites 2,8 and 2,10-dOH-FICZ when using 2-OH-FICZ as the substrate but caused no reduction in metabolism of FICZ.

By adding the cofactors (UDPGA and PAPS) necessary for the conjugating enzymes UGT and SULT, the S9 metabolic system more closely resembles the in vivo situation. These additions resulted in a substantial reduction of the di-hydroxylated metabolites. The FICZ metabolites formed by the human metabolizing system were more susceptible to sulfation than to glucuronidation. Cleavage of the conjugated metabolites by β-glucuronidase confirmed the formation of glucuronides.

The substrate specificity of different human SULT enzymes towards the hydroxylated metabolites of FICZ were analyzed. No sulfate conjugation of any of the tested metabolites was observed with SULT1A3 and SULT2A1. The enzymes SULT1A1 and SULT1A2 displayed the highest detected activities towards all tested metabolites and SULT1A1 was approximately twice as active as SULT1A2. By comparing the mono-hydroxylated metabolites 2-OH-FICZ and 8-OH-FICZ, both SULT1A1 and SULT1A2 demonstrated a 6 times higher activity towards 2-OH-FICZ than towards 8-OH-FICZ. In contrast to SULT1A1 and SULT1A2, SULT1B1 only showed activity towards 8-OH-FICZ, while SULT1E1 displayed a higher activity towards 8-OH-FICZ than towards 2-OH-FICZ. Regarding the di-hydroxylated metabolites, SULT1A1 was most efficient towards 2,8-dOH-FICZ although SULT1A2 was more capable in sulfonating 4,8-dOH-FICZ and 2,10-dOH-FICZ. SULT1A1 is reported to have broad substrate specificity and exhibits the highest hepatic expression of all SULT1 enzymes. It is also
expressed at a lower level, in nearly every extrahepatic tissue including platelets (Glatt and Meinl, 2004).

mEH was also found to take part in the metabolic transformation of FICZ. This can probably explain the low proportion of metabolized FICZ that was accounted by the identified metabolites. Other more polar metabolites such as dihydrodiols are most likely also formed by the cytochrome P450 enzymes in combination with EH but were not detected in the HPLC system used.

It seems plausible that the enzyme aldehyde oxidase is the enzyme responsible for the oxidation of the aldehyde moiety of FICZ. This enzyme is highly expressed in liver, but is not detected in human skin (Rooseboom, et al., 2004). For that reason the carboxylic acid derivatives of FICZ are not likely to be formed in human skin. However, CYP1A1 is constitutively expressed in skin which implies that the mono-hydroxylated metabolites of FICZ in combination with the 2,8-dihydroxylated metabolite are the most likely formed skin metabolites of FICZ.

IMPACT OF TRYPTOPHAN IN CELL CULTURE MEDIUM (Paper I and V).

As discussed above, the TRP photoproduct FICZ has been suggested to play a role as an endogenous AhR ligand that regulates the expression of CYP1A1 in an autoregulatory manner. A transient and weak induction of CYP1A1 gene expression and activity has repeatedly been observed to occur in cell culture following medium change (Paine, 1976a; Nemoto and Sakurai, 1991; Kocarek, et al., 1993; Harvey, et al., 1998; Segner, et al., 2000; Sadar and Andersson, 2001; Feng, et al., 2002). Furthermore, it has been speculated that the cell culture medium may contain a TRP derived AhR ligand responsible for the observed effect (Paine, 1976a; Kocarek, et al., 1993; Harvey, et al., 1998; Segner, et al., 2000). The essential amino acid TRP is present in all media used for cell culturing. Our previous results indicated that TRP photoproducts are formed in UV exposed water solutions of TRP and in UV irradiated cells in the presence of TRP (Rannug, et al., 1987; Wei, et al., 1999). Hankinson and co-workers reported high constitutive CYP1A1 mRNA expression in Hepa-1c1c7 cells lacking AHH activity. They argued that this effect was controlled by an inducer accumulated in the cells and was independent of any
component in the cell medium (Hankinson, et al., 1985). Interestingly, in the study presented in Paper I, using mouse hepatoma Hepa-1 cells, we found that a higher amount of TRP in cell culture medium (20 µg/ml) significantly increased the basal level of CYP1A1 mRNA expression in the same CYP1A1 defective c37 cells as used by Hankinson and co-workers. Such an effect was not found in wild-type cells. The study presented in Paper V aimed at identifying the TRP-derived substance(s) contributing to the effect seen by cell medium on CYP1A1 mRNA expression.

One new and important observation was that ordinary cell culture medium exposed to visible light caused a significantly increased CYP1A1 activity after only three hours of light exposure. The induction of CYP1A1 activity caused by illuminated medium was transient and comparable to the induction seen by FICZ when using a short incubation time. The results suggested that an easily metabolized, TRP-derived, AhR ligand in the medium regulates the basal CYP1A1 expression. To identify the formed CYP1A1 inducers, cell culture media, with or without TRP, were exposed to visible light for 24 hours and subsequently fractionated by HPLC. Seven fractions collected according to polarity were tested for CYP1A1 inducing activity and significant TRP dependent effects were observed in the non-polar fractions (4-7), even though the large majority of components detected by UV and fluorescence eluted at retention times less then 20 minutes (fractions 1-4). Four distinct TRP-related fluorescent peaks were seen in fractions 5 to 7 in the TRP-containing medium. One of the two peaks in fraction 7 was identified by LC-MS/MS as FICZ and the concentration in the cell medium was estimated to be 8 pM. The unfractionated cell medium showed a similar inducing efficiency as that by FICZ at a concentration of about 10 pM which means that the amount of FICZ in the cell medium could explain the bulk of the CYP1A1 inducing effect of the unfractionated cell medium, analyzed in parallel with the fractions.

Thus, this study showed that normal laboratory light can significantly induce the constitutive CYP1A1 activity through formation of TRP-derived photoproducts. The formation of FICZ in cell media is a plausible explanation for the many reports in literature on CYP1A1 induction caused by change in cell medium and light exposure. However, more importantly, the results point to the fact that the presence of FICZ, which at short term
incubations is a more potent inducer than TCDD, may have a major direct or indirect effect on results from in vitro experiments.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The evolution of cytochrome P450 enzymes has been extensively discussed and in 1990, Gonzalez and Nebert speculated that drug metabolizing enzymes of the cytochrome P450 family originally evolved as animal-plant ‘warfare’ enzymes i.e. a means for animals to protect themselves from toxic chemicals produced by plants (Gonzalez and Nebert, 1990). Later, Nebert (1991) and Denison and Whitlock (1995) argued that the regulatory pathways of the cytochrome P450 enzymes have possibly been developed in the cells as a mechanism to maintain homeostasis with respect to endogenous lipophilic substances. The results presented in the present thesis supports the latter hypothesis.

In biological systems TRP is an important amino acid, and there are numerous indole-containing substances that have the ability to influence the AhR signaling pathway. One could speculate that the CYP1 family has developed to regulate steady state levels of lipophilic indoles with important endogenous functions. The amount of TRP in whole blood, sweat and several tissues is about 1 mg/100 ml or 100 mg tissue. Our results indicate a 0.0006‰ formation of FICZ after irradiation of TRP solutions with normal laboratory light, but the conversion by UV light is orders of magnitude higher. The formation of oxidized TRP derivatives could be the explanation for several reports on CYP1A1 activation in the absence of exogenous AhR ligand. UV-B exposure of human skin results in an enhanced expression of mRNA and protein of both CYP1A1 and CYP1B1 in the epidermis (Katiyar, et al., 2000) and the novel CYP2S1 (Rylander, et al., 2001) has been identified to be expressed and induced by UV-A in mammalian skin (Smith, et al., 2003). A recent report identified CYP2S1 as an AhR-inducible CYP (Rivera, et al., 2002) which indicates that FICZ might be a substrate also for this enzyme.

Interestingly, a seasonal variation of AHH activity in human lymphocytes has been reported (Paigen, et al., 1981) where the maximum activity occurred during late summer and early fall. Additionally, a daily up-regulation of AhR, Arnt and CYP1A1 mRNA in liver has been described (Plewka, et al.,
The CYP1A1 mRNA up-regulation occurred after the light hours and the AhR and Arnt mRNAs were in antiphase to CYP1A1 mRNA (Huang, et al., 2002). It is attractive to speculate that the daily cycles of AhR, Arnt and CYP1A1 as well as the seasonal variation in AHH activity are secondary to changes in the levels of tryptophan photoproducts.

One should not rule out the possibility that FICZ might be formed in TRP rich nutrients and thereby ingested as a food constituent. For example, milk contains 50 mg tryptophan/100 ml and daily light might convert TRP in milk to FICZ to an approximate concentration of 1 nM. The preparation of dairy products implies conditions of normal light as well as high temperature surroundings.

In the studies presented in this thesis, it has been demonstrated that the TRP photoproduct, FICZ not only transiently up-regulates CYP1A1 but is metabolized by the CYP1 family (1A1, 1A2 and 1B1) of enzymes as well. All members of the CYP1 family are regulated by the AhR pathway and the TRP photoproduct FICZ fits into a model in which the ligand-activated AhR signaling is autoregulated by the induced metabolizing enzymes. However the regulation could be different in different organs. According to unpublished data, 8-OH-FICZ one of the three possible metabolites of FICZ (2-OH-FICZ, 8-OH-FICZ and 2,8-OH-FICZ) in human skin, possesses approximately half of the AhR affinity of FICZ and is not further metabolized by CYP1A1. In that way the AhR signaling in skin could be different from the regulation in liver.

Several criteria ought to be fulfilled in order to classify a substance as an endogenous AhR ligand. These are i) AhR binding affinity, ii) formation in vivo and iii) metabolism by its induced enzymes. There is a range of reported CYP1A1 inducers of natural origin. However for most of these, the CYP1A1 induction as well as AhR affinity is very low and only a few have been reported to be substrates of the CYP1 family (FICZ, ICZ, bilirubin, TA and indirubin) (Heath-Pagliuso, et al., 1998; Wei, et al., 1998; Zaccaro, et al., 2001; Spink, et al., 2003). For endogenous AhR ligands exhibiting high affinity, low in vivo concentrations should be sufficient for receptor activation. Low AhR affinity ligands could also act as endogenous ligands if they are present in high in vivo concentrations. FICZ, ICZ and indirubin are promising...
candidates but indirubin is the only natural AhR ligand so far that seems to fulfill these requirements. Indirubin has been detected in human urine (Adachi, et al., 2001) although the endogenous function as well as origin of indirubin has not yet been clarified.

The studies on the TRP-derived FICZ compound as well as its metabolites need to be extended. For the further identification of FICZ \textit{in vivo}, it is crucial to identify metabolites of FICZ in urine or blood. If the fate of FICZ \textit{in vivo} is comparable to steroid hormones, FICZ metabolites could like many steroid hormones be circulating as a conjugate, acting as a precursor reservoir (Adessi, et al., 1982; Barnes, 2001). The resemblance between the estrogen receptors and the AhR is striking. Both are: i) high affinity ligand binding transcription factors ii) binding to transactivation domains, XRE resp. ERE, iii) activated by in general planar compounds iv) promiscuous receptors, with regard to ligand binding and v) susceptible to down-regulation by rapid metabolism of the ligands by phase I and phase II enzymes.

It has been suggested that some of the estrogen metabolites may have specific biological functions that are different from the functions of their parental hormones, estradiol and estrone (Zhu and Conney, 1998). The regioselective hydroxylations seen with estrogens are also seen with FICZ, which makes it relevant to speculate about specific biological functions of the metabolites of FICZ. The di-hydroxylated metabolites of FICZ do not possess any AhR binding affinity (unpublished). However the structural requirement for ligand binding to the estrogen receptors \(\alpha\) and \(\beta\) are generally planar compounds with two oxygens atoms (of which one is phenolic) spaced 11-12 Å apart which means that it is of interest to examine whether the di-hydroxylated metabolites, of FICZ have the ability to activate the estrogen receptors. In addition, Ohtake and coworkers have shown that the AhR can associate with the estrogen receptors when TCDD is bound to AhR but when estradiol is bound to the estrogen receptor, TCDD represses the estradiol induced response (Wormke, et al., 2003). In an analogous way, FICZ and its metabolites might give similar estrogenic and anti-estrogenic effects.
ACKNOWLEDGEMENTS

Det är många jag vill tacka för min tid som doktorand. Jag är bättre på att prata än att formulera mig i skrift men jag gör i alla fall ett försök.

Först och främst vill jag tacka mina handledare:
Ulf Rannug, för att du initierat mig i projektet med FICZ och för din osvikliga optimism som hjälpt mig framåt när det känts motigt. Jag har uppskattat möjligheten till att arbeta själstablet och alla våra livliga diskussioner med många inslag av skratt.

Agneta Rannug, för att du är en driva person som delat med dig av din kunskap och särskilt tack för dina värdefulla kommentarer som varit till stor hjälp i arbetet.

Jag vill även tacka:

Emma Wincent för att du skapat trivsel på jobbet och för att du dragit ett så tungt lass till min avhandling. Ny i gruppen så organiserade du dessutom genast en preussisk ordning på labb, det behövdes!

Niklas Wahlström, för ett värdefullt samarbete. Utan din insats med NMR analyser och metabolit synteser så hade detta ej gått vägen.

Mattias Öberg, för ett trevligt samarbete. Du gav mig möjlighet att bredda mina laborativa kunskaper.

Tomas Alsberg, för ditt tålamod med LC-MS analyser.

Fredrik, du har varit en lysande vän både på och utanför jobbet! Tack även för ALL hjälp med "fix och trix"! Hoppas att vi håller kontakten.

Hasse, du var som en mentor för mig när jag började på Walle, och en väldigt snäll sådan. En stor kram och tack för den tiden!
Ett stort tack vill jag rikta till ALLA övriga arbetskamrater på institutionen, nya som gamla, men framför allt till Ingrid (du var en toppen rumskamrat som skämde bort mig med presenter) Cissi (gladare än gladast för jämnan!), Björn (för all datorsupport), Dag (alltid lika hjälpvän), Klaus (glad och omtänksam), Niklas (du är en underbar person, min idoll!), Håkan (för givande och roliga diskussioner), Anna RL (tack för bra kommentarer...), för en trevlig atmosfär och intressanta lunch och fika samtal.

Karin, en bättre vän kan man inte ha, du står mig mycket nära. Det har varit en förmån att ha dig som glädjespridande kollega under tiden som doktorand. Våra många lååångata telefoner har verkligen lyst upp min vardag, tack för att du finns!

Jag vill även tacka alla övriga på miljökemi som var delaktiga i den trevliga stämningen och de minnesvärda festerna under tiden på Wallenberg lab. Ett särskilt tack till, Britta (du är en varm och osjälvisk vän), Charlotta (med magen i väder klarade du dig galant, så jag vågade chansa!) och Per (för alla glada skratt).

Gary, tack för språklig granskning av kappan till min avhandling

_Sist men inte minst vill jag tacka:_


Övrig släkt och familj speciellt Christina för allt stöd och intresse.

Malin, en kär vän som till fullo förstådd hur det kan känna att slutföra en avhandling med barn hemma och en på väg!

Alla övriga vänner, ingen nämnd ingen glömdu, för att ni finns i mitt liv. Jag har inte varit närvarande på sistone men tack för all förståelse och hoppas att det blir mer frekventa träffar framöver.
REFERENCES


Enan, E. and Matsumura, F. (1996) Identification of c-Src as the integral component of the cytosolic Ah receptor complex, transducing the signal of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) through the protein phosphorylation pathway. *Biochem Pharmacol* **52:**1599-1612.


Sinal, C.J. and Bend, J.R. (1997) Aryl hydrocarbon receptor-dependent induction of cyp1a1 by bilirubin in mouse hepatoma hepa 1c1c7 cells. Mol Pharmacol 52:590-599.


by inducible cytochrome P450 is more important than metabolic activation. *Mol Pharmacol* **65**:1225-1237.


