Influence of Oxidative Stress on Aryl Hydrocarbon Receptor Signaling

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تقديم به معلمان بزرگ زندگیم پدر و مادر مهربانم که با ایثار ذره ذره
وجودشان بالیدن هر روزه مرا به نظره نشسته‌اند.
ABSTRACT

The aryl hydrocarbon receptor (AHR), a multifunctional protein and a key regulator of drug metabolizing enzymes, belongs to the basic-helix-loop-helix (bHLH)/PAS (Per-Arnt-Sim) super-family of transcription factors. The AHR responds to exogenous and endogenous chemicals by induction or repression of a large number of genes involved in many physiological processes and normal development.

The diverse spectrum of AHR activators from well-known planar hydrophobic halogenated aromatic hydrocarbons (HAHs) to chemical compounds whose structure and physicochemical properties are very different from classical AHR ligands suggests that the AHR has a tremendously promiscuous ligand binding pocket. Due to the absence of a 3D structure of the ligand binding domain, promiscuity of the AHR has remained elusive. However, increasing experimental evidence indicate that the non-typical AHR ligands might activate the AHR signaling pathway indirectly by inhibiting the metabolic turnover of an endogenous ligand of the AHR. Therefore, the objective of this thesis was to characterize the inhibition of degradation of 6-formylindolo[3,2-b]carbazole (FICZ), the suggested natural high affinity AHR ligand, as a mechanism that could explain the earlier described agonistic properties of structurally very diverse AHR activators. The obtained results show that FICZ is a potent AHR agonist in vitro and in vivo which can distribute to the body through systemic circulation and induce cytochrome P450 1A1 (CYP1A1) the prototypical AHR target in various organs. The studies presented in this thesis demonstrate that if the metabolic clearance of FICZ is compromised, femtomolar concentrations of FICZ are sufficient to activate AHR signaling.

The AHR signaling pathway seems to be sensitive to oxidative stress but the redox regulation of AHR has not been well characterized. Studies on dioxin and other reactive oxygen species (ROS) producing agents have demonstrated that the AHR is a mediator of oxidative stress. Indeed, AHR works in close concert with the master regulator of antioxidant responses, nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Multiple sources of ROS appear to be involved in modulating AHR signaling and probably via three major systems, microsomes, mitochondria and NADPH oxidase enzymes (NOXs). Furthermore, it has been observed that many environmental pollutants, including metals and other NOX-activators increase the levels of the diffusible molecule hydrogen peroxide (H₂O₂) and change the cellular redox status and thereby interfere with cell growth kinetics and the endogenous functions of the AHR. To increase the understanding of downstream adaptive responses to oxidative stress, including up-regulation of antioxidant genes and modulation of AHR signaling was another objective of this work. The findings demonstrate that superoxide anion (O₂⁻) or H₂O₂ produced by NOXs can negatively and positively modulate the AHR signaling pathway. The importance of cellular redox levels which can influence endogenously activated AHR signaling broadens our earlier knowledge and explains why many oxidants behave both as AHR antagonists and agonists.

In summary, this thesis extends the mechanistic understanding of the promiscuity of AHR and provides important information with regard to the redox regulation of AHR endogenous signaling.
LIST OF PUBLICATIONS


III. Mohammadi-Bardbori A, Vikström Bergander L, Rannug U, and Rannug A. NADPH oxidase has a central role in aryl hydrocarbon receptor signaling and regulation of cell growth by arsenic, cadmium, mercury and nickel. (Manuscript)
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<tr>
<td>AHR</td>
<td>aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AHRR</td>
<td>aryl hydrocarbon receptor repressor</td>
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<tr>
<td>AP-1</td>
<td>activator protein 1</td>
</tr>
<tr>
<td>ARE</td>
<td>antioxidant response element</td>
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<tr>
<td>ARNT</td>
<td>aryl hydrocarbon receptor nuclear translocator</td>
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<tr>
<td>As</td>
<td>arsenic</td>
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<tr>
<td>bHLH</td>
<td>basic helix-loop-helix</td>
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<tr>
<td>Cd</td>
<td>cadmium</td>
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<tr>
<td>CGD</td>
<td>chronic granulomatous disease</td>
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<tr>
<td>CUR</td>
<td>curcumin</td>
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<tr>
<td>CYP1A1</td>
<td>cytochrome P450 1A1</td>
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<tr>
<td>DPI</td>
<td>diphenyleneiodonium</td>
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<tr>
<td>DOUX</td>
<td>dual oxidases</td>
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<tr>
<td>EGF</td>
<td>epidermal growth factor</td>
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<tr>
<td>FICZ</td>
<td>6-formylindolo[3,2-b]carbazole</td>
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<tr>
<td>GSH</td>
<td>reduced glutathione</td>
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<tr>
<td>GSSG</td>
<td>oxidized glutathione</td>
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<tr>
<td>HaCaT</td>
<td>immortalized human keratinocyte cell line</td>
</tr>
<tr>
<td>HepG2-XRE-Luc</td>
<td>human hepatoma HepG2-derived cell line</td>
</tr>
<tr>
<td>Hg</td>
<td>mercury</td>
</tr>
<tr>
<td>HO-1</td>
<td>heme oxygenase 1</td>
</tr>
<tr>
<td>H2O2</td>
<td>hydrogen peroxide</td>
</tr>
<tr>
<td>HSP90</td>
<td>heat shock protein 90 kDa</td>
</tr>
<tr>
<td>IL-1α and β</td>
<td>interleukin-1α and β</td>
</tr>
<tr>
<td>MNF</td>
<td>3'-methoxy-4'-nitroflavone</td>
</tr>
<tr>
<td>NF-kB</td>
<td>nuclear factor kappa-light-chain-enhancer of activated B cells</td>
</tr>
<tr>
<td>Ni</td>
<td>nickel</td>
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<tr>
<td>NOXs</td>
<td>NADPH oxidase enzymes</td>
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<td>Nrf2</td>
<td>nuclear factor (erythroid-derived 2)-like 2</td>
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<tr>
<td>PAS</td>
<td>Per-ARNT-sim</td>
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<tr>
<td>QUE</td>
<td>quercetin</td>
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<tr>
<td>RES</td>
<td>resveratrol</td>
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<td>ROS</td>
<td>reactive oxygen species</td>
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<tr>
<td>TAD</td>
<td>transactivation domain</td>
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<tr>
<td>TCDD</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TGFα and β</td>
<td>growth factors α and β</td>
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<td>TNFα</td>
<td>tumor necrosis factor α</td>
</tr>
<tr>
<td>Trp</td>
<td>tryptophan</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet radiation (200-400nm)</td>
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<tr>
<td>XAP2</td>
<td>hepatitis B virus X-associated protein 2; AIP, aryl hydrocarbon receptor interacting protein</td>
</tr>
<tr>
<td>X-CGD</td>
<td>X chromosome- linked CGD human promyelocytic leukemia cell line</td>
</tr>
<tr>
<td>XRE/DRE</td>
<td>xenobiotic or dioxin response element</td>
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1 INTRODUCTION

The presence of aryl hydrocarbon receptor (AHR) homologues in early animals (about 570 million years ago) suggests that this protein family has ancient evolutionary origins (Peterson and Butterfield, 2005). The AHR was first identified in 1976 by Allan Poland, a physician who was interested in the etiology of chloracne among workers in a factory producing 2,4,5-trichlorophenol (Poland et al., 1976). The most toxic man-made anthropogenic compound, dioxin, was documented by German chemists as early as 1827 (reviewed in White and Birnbaum, 2009). The focus of research was aimed at identifying the biochemistry of AHR in 1980s. The ligand binding and DNA binding domains of the receptor were the most important findings during this period. In the early 1990s, the aryl hydrocarbon receptor nuclear translocator (ARNT), the nuclear partner of AHR, was identified. The crucial roles of AHR in physiology and biology were discovered by using knockout animals in the beginning of the 21th century (Gasiewicz and Henry, 2012). From current findings in the field of the AHR, it has become clear that AHR is more than a xenobiotic-interacting protein. The AHR turns out to be an important player in many physiological processes including cell cycle regulation, tumor suppression and immunity (reviewed by Barouki et al., 2007; Fujii-Kuriyama and Kawajiri, 2010).

The AHR can be activated by multitude of chemicals (Denison et al., 2011) and most of them do not fit into the ligand-binding pocket of the AHR. This thesis aimed at studying this claimed promiscuity of the AHR and to explain the earlier described agonistic and antagonistic properties of structurally very diverse molecules.

There is a paucity of data regarding how stress, in particular oxidative stress, influences the endogenously activated AHR signaling. It has been suggested that the AHR is a mediator of cellular stress responses (Matsumura, 2003; Matsumura and Vogel, 2006) and works in close concert with the Kelch-like ECH-associated protein 1 (Keap1)-nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response elements (ARE) pathway. Another aim of this study was therefore to fill this knowledge gap by studying the mechanisms of interaction between oxidants and ligand-mediated AHR signaling. The most interesting directions of future studies on the function of the AHR protein are expected to be:

- To learn the biology and physiology of the AHR in more detail
- To understand thoroughly the AHR endogenous signaling
- To know which human diseases may have AHR-dependent etiology and how to develop therapeutic treatments based on AHR modes of action
- To fully understand the molecular mechanism(s) of toxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and other xenobiotics
2 BACKGROUND

2.1 STRUCTURE OF THE AHR PROTEIN

AHR along with its nuclear partner, ARNT, the aryl hydrocarbon receptor repressor (AHRR), the single-minded protein (Sim), the hypoxia inducible factors and many other proteins with roles for example in sensing oxygen, light and nitric oxide belong to the basic-helix-loop-helix (bHLH)/PAS (Per-Arnt-Sim) super-family of transcription factors (Gu et al., 2000; McIntosh et al., 2010). Among PAS proteins, only the AHR is a ligand-dependent transcription factor (Furness et al., 2007) and the AHR is not able to bind to a xenobiotic or dioxin response element (XRE/DRE) in the promoter region of target genes without activation by a ligand. The AHRR has been described as an inducible protein that competes with the AHR for dimerization with ARNT for binding to DNA (Mimura et al., 1999). The AHRR binding to XRE/DRE has also been reported to be independent from ARNT heterodimerization (Hahn et al., 2009).

As shown in figure 1, the N-terminal region of AHR comprises a bHLH domain followed by PAS A and B motifs. The C-terminal region consists of a transactivation domain (TAD) (Jain et al., 1994). AHR/ARNT heterodimerization, DNA binding and recognition are mediated by the TAD domain (Reyes et al., 1992; Dolwick et al., 1993; Swanson, 2002). A nuclear localization signal (NLS) and nuclear export signal (NES) overlap with the DNA binding domain (Ikuta et al., 1998). Another NES overlaps with the PAS domain (Berg and Pongratz, 2001). Unmasking of NLS by the release of heat shock protein (HSP90) is required for localization of AHR into the nucleus (Ikuta et al., 1998) and translocation of AHR is important for AHR mediated responses. Nuclear export of AHR is also important for AHR proteasomal degradation (Pollenz et al., 1999). Interaction with chaperones, ligand recognition and AHR/ARNT heterodimer formation are mediated by the PAS domain. It consists of two regions of PAS A and B with high sequence homology (Dolwick et al., 1993; Fukunaga and Hankinson, 1996; Taylor and Zhulin, 1999; Gu et al., 2000). The TAD domain consists of three acidic, glutamine-rich and prolin-serin-threonin sub-domains (Ma et al., 1995). Each sub-domain has its own transactivation activity and all sub-domains show synergic activities in combination (Whitelaw et al., 1994). An inhibitory domain (ID) between the PAS-B domain and the TAD domain consisting of 81 residues was identified with inhibitory effects on TAD domain in the absence of an agonist (Ma et al., 1995).

![Figure 1. Functional domains of the AHR. Figure representing basic regions for DNA and ligand binding, dimerization and transactivation.](image_url)
2.2 FUNCTIONS OF THE AHR PROTEIN

2.2.1 Involvement in metabolism of endogenous and exogenous chemicals

The AHR is involved in up-regulating a large number of xenobiotic metabolizing enzymes including phase I and II metabolizing enzymes and phase III transporters in response to noxious chemicals and numerous endogenous molecules. The cytochrome P450 monooxygenase 1 proteins (CYP1s), glutathione S-transferases (GSTs) and UDP-glucuronosyltransferases (UGTs) are examples of AHR-dependent metabolizing enzymes. One role of bio-transformation is to terminate the biological or toxic effects of lipophilic chemicals by incorporating a functional groups such as -OH, -NH, -SH or -COOH in the molecule to make them more water soluble (Parkinson, 1996). Another role is to form biologically active products such as arachidonic acid metabolites (prostaglandins). However, in some cases oxidative metabolism of xenobiotics by CYP enzymes may lead to the formation of reactive intermediates and increase the toxicity (reviewed by Shimada, 2006). Involvement of CYPs in both detoxification of xenobiotics and formation of reactive intermediates that is associated with toxicity, mutagenesis and carcinogenesis has been investigated in knockout animal models. CYP1A1 (−/−) knockout mice exposed daily to benzo[a]pyrene (BaP) were dying within 30 days, whereas CYP1A1 (+/+) wild type mice didn’t exhibit any signs of toxicity. BaP- DNA adducts were higher in knockout animals compare to wild type mice (Uno et al., 2004). The toxicity of oral BaP in double-knockout mice for both CYP1A1 and 1B1 was also higher than in the other genotypes investigated and the authors concluded that CYP1A1 mediates detoxification of BaP by speeding up the metabolism and excretion (Uno et al., 2006).

The most common reactions catalyzed by CYPs are insertion of one oxygen molecule into the lipophilic substrate (reviewed by Hollenberg, 2002). The reaction can be summarized as follows: 1) binding of substrate to the active site of enzyme 2) reduction of prosthetic heme iron from ferric (Fe³⁺) to ferrous (Fe²⁺) 3) binding of oxygen to ferrous molecule 4) transferring electron from NADPH via cytochrome P450 reductase to ferrous-oxygen-substrate complex 5) formation of activated oxygen intermediate and water 6) insertion of activated oxygen intermediate into the substrate to make an oxygenated product. In this reaction, several steps including steps number one, three and six are more susceptible for inhibition by CYPs inhibitors. In general, chemicals that can inhibit the reactions before the formation of ferrous-oxygen-substrate complex are reversible inhibitors and are simply divided into competitive, noncompetitive and uncompetitive inhibitors. Those inhibitors which act after the formation of ferrous-oxygen-substrate complex are either quasi-irreversible or irreversible inhibitors. The type of inhibition of quasi-irreversible or irreversible inhibitors is dose-time-dependent whilst reversible inhibitors only show dose-dependent inhibition manner (reviewed in Hollenberg, 2002).

In phase II of bio-transformation conjugation with glucuronic acid, sulfate, or acetic acid is catalyzed by UDP-glucuronosyl transferases (UGTs), sulfotransferases (SULTs) and N-acetyltransferases (NATs) respectively (Negishi et al., 2001; Wells et al., 2004; Sim et al., 2008; Omiecinski et al., 2010). The role of phase III is to transport drugs and xenobiotics via membrane transporters such as ATP-binding cassette family (ABC) of proteins across the cellular membranes (Omiecinski et al., 2010). Expression of ATP-dependent transporters were shown to be induced by AHR ligands (Maher et al., 2006).
2.2.2 Physiological functions of the AHR

The abnormal phenotypes of AHR deficient animals clearly have shown that AHR is an important protein for proper development of liver (Fernandez-Salgueiro et al., 1995; Schmidt et al., 1996), ovaries (Benedict et al., 2000), heart (Fernandez-Salgueiro et al., 1996; Mimura et al., 1997; Lahvis and Bradfield, 1998), as well as cardiovascular (Lahvis et al., 2000; Vasquez et al., 2003; Lahvis et al., 2005) and reproductive systems (Baba et al., 2005). The role of AHR in cell proliferation and apoptosis is not well-known. In AHR-defective cells, the rate of cell growth seems to be slower than those with normal phenotypes (Ma and Whitlock, 1996; Weiss et al., 1996). The same observation has been made in mouse embryonic fibroblasts derived from AHR null mice (Elizondo et al., 2000). The AHR may be involved in apoptosis and cell death and it has been suggested that the AHR may bind to the transcription factor E2F1 and inhibit apoptosis (Marlowe et al., 2008). In addition to the suggested physiological role of AHR in cell cycle progression and normal development, AHR plays important roles in the normal function of immune and endocrine systems, circadian rhythm, skin physiology, hematopoiesis, and stem cell expansion (Schmidt et al., 1996; Baba et al., 2005; Fritsche et al., 2007; Mukai and Tischkau, 2007; Esser et al., 2009; Boitano et al., 2010; Casado et al., 2010).

2.3 ACTIVATION OF AHR SIGNALING

The unliganded AHR resides in the cytoplasm in complex with two molecules of chaperons, HSP90, a low molecular weight AHR interacting protein (AIP) also called XAP2 or ARA9 and p23. Interaction with HSP90 seems to be essential for ligand and DNA binding of the receptor (Perdew, 1988; Wilhelmsson et al., 1990; Pongratz et al., 1992; Carver et al., 1994; Ma and Whitlock, 1997). The AHR binds to the middle part of HSP90 (amino acids 272-617) (Meyer and Perdew, 1999) and its interaction with HSP90 and co-chaperones seems to be central to the stability of the AHR complex in the cytoplasm (Song and Pollenz, 2002). On the other hand, protein-protein interaction is a key component of the folding machinery. The chaperons make the receptor more sensitive to its ligands (Carver and Bradfield, 1997; Carver et al., 1998; Kazlauskas et al., 1999; Meyer and Perdew, 1999; Bell and Poland, 2000; Meyer et al., 2000). p23 enhances the ligand binding activity and transcription activity of AHR and increases the ability of AHR to bind to XRE/DRE (Young et al., 2001; Cox and Miller, 2002; Shetty et al., 2003). Upon ligand binding and activation, AHR translocates into the nucleus, detaches from chaperons, and dimerizes with its nuclear partner ARNT (Carver et al., 1994). Nuclear translocation is followed by a reduction in the size of the receptor, which can be the result of detachment from chaperons and dimerization with ARNT (McGuire et al., 1994). Studies on ARNT deficient mouse hepatoma Hepa1c4 cells showed that ARNT is required for AHR binding to specific response elements in DNA (XRE/DRE) (Probst et al., 1993; Whitelaw et al., 1993). The AHR/ARNT heterodimer complex binds to XRE/DRE and up-regulates a battery of genes involved in bio-transformation of xenobiotic and endobiotic compounds and hundreds of other genes (reviewed by Nebert and Dalton, 2006) (Figure 2).
Figure 2. Schematic structure of the AHR and molecular mechanism of AHR activation by an AHR ligand (based on Fujii-Kuriyama & Mimura, 2005)

2.3.1 AHR ligands

The physiochemical properties of AHR ligands have been tested for more than 30 years. Structure-activity relationship studies suggest that hydrophobic and planar compounds are well fitted to the ligand-binding pocket of AHR. According to molecular modeling studies, the ligand-binding pocket of AHR doesn’t accept a ligand bigger than 14x12x5 Å (Waller and McKinney, 1995). However, a large number of natural, endogenous and synthetic compounds that do not fit into the described pocket can activate AHR signaling (reviewed in Denison et al., 2002). This suggests that the AHR has a more promiscuous ligand binding pocket. Due to the absence of 3D crystal/NMR/ X ray structure of the ligand binding domain (LBD) the promiscuity of the ligand-binding pocket of AHR remains elusive.

2.3.1.1 Xenobiotic compounds

Environmental pollutants such as halogenated aromatic hydrocarbons (HAHs) and polycyclic aromatic hydrocarbons (PAHs) are known as synthetic ligands of the AHR (reviewed in Denison et al., 2002; Denison and Nagy, 2003; Fujii-Kuriyama and Mimura, 2005). Among xenobiotic compounds, TCDD (Figure 3) has the highest affinity for binding and activation of the AHR. Among the described xenobiotic ligands, TCDD has the highest toxicity and bio-accumulation with the half-life in human of about 7.5 years (Whitlock, 1999; Van den Berg et al., 2006). Induction of CYP1A1 by TCDD in the body is highly persistent due to the long halftime of TCDD (Ma and Baldwin, 2000).
2.3.1.2 Naturally-occurring compounds

In addition to synthetic ligands, numerous naturally occurring and endogenous compounds are able to bind and activate the AHR (Denison and Nagy, 2003; Nguyen and Bradfield, 2008). Indole-3-carbinol derivatives such as indolo[3,2-b]carbazole (ICZ) (Bjeldanes et al., 1991; Chen et al., 1995), phytochemicals including alkaloids and flavonoids and heme metabolites have been introduced as agonists of AHR (Gillner et al., 1989; Gasiewicz et al., 1996; Adachi et al., 2001). Arachidonic acid products (prostaglandins and lipoxins A4) (Schaldach et al., 1999; Seidel et al., 2001), and tryptophan (Trp) derivatives (tryptamine, indole acetic acid, indigo and indirubin) (Heath-Pagliuso et al., 1998; Adachi et al., 2001) are natural activators of AHR signaling.

A large body of evidence has pointed to the role of an endogenous ligand of the receptor for activation of AHR in the absence of exogenously added AHR ligands (Paine, 1976; Hankinson et al., 1985; Sadek and Allen-Hoffmann, 1994; Singh et al., 1996; Crawford et al., 1997; Chang and Puga, 1998; Chiaro et al., 2007). Most of the suggested endogenous ligands for the AHR so far have exhibited low affinity for binding and activation of the receptor. Among all tested compounds a photoproduct of the amino acid Trp, 6-formylindolo[3,2-b]carbazole (FICZ), is receiving increasing attention (Ma, 2011; Bock, 2012) (Figure 4). FICZ exerts several unique properties, which make it a possible endogenous ligand of the receptor. It exhibits the highest affinity for binding and activation of the receptor (Rannug et al., 1987; Nguyen and Bradfield, 2008). FICZ can be formed in aqueous solutions containing Trp when exposed to visible and UV light (Oberg et al., 2005; Diani-Moore et al., 2006; Wincent et al., 2009) and FICZ has been found in human skin (Magiatis et al., 2013) and its sulfate conjugates have been detected in human urine (Wincent et al., 2009). Furthermore, FICZ is an excellent substrate for CYP1A1 (Wincent et al., 2009) and was suggested to contribute to the auto-regulatory feedback control of AHR signaling.
2.4 REGULATION OF THE AHR

Because the AHR is a multifunctional protein that plays a fundamental role in normal development and cell physiology, there are multiple mechanisms designed to suppress sustained AHR activation. These include an AHR repressor, proteasomal degradation of the AHR, inducible CYP1A1 mediated degradation of ligands and epigenetic mechanisms. This shows that tight regulation of AHR signaling pathway is very important (Figure 5).

Figure 4. 6-formylindolo[3,2-b]carbazole (FICZ)

Figure 5. AHR activation can be regulated by 1) AHR/CYP1A1/FICZ-dependent auto-regulation 2) AHRR/ARNT complex formation 3) proteasomal degradation of AHR (based on Fujii-Kuriyama & Mimura, 2005)
2.4.1 AHR/CYP1A1-dependent auto-regulation

The AHR induces the expression of metabolizing enzymes in particular CYP1A1, after exposure to AHR ligands. CYP1A1 regulates AHR activity by elimination of the ligands and thereby decreases the duration of exposure. In this way the AHR/CYP1A1 auto-regulation feedback causes transient activation of AHR (Nebert and Dalton, 2006; Chiaro et al., 2007).

2.4.2 Repression by the AHR repressor

The AHRR was first identified to repress the AHR activity by competing with the AHR/ARNT heterodimer formation (Mimura et al., 1999; Baba et al., 2001). It is becoming more apparent that the AHRR/ARNT heterodimer doesn’t competitively displace the AHR protein from AHR/ARNT heterodimer and AHRR DNA binding seems to be independent to ARNT (Hahn et al., 2009). AHRR plays important roles in repression of AHR mediated responses in some cell lines such as human dermal fibroblasts (Haarmann-Stemmann et al., 2007). However, expression of AHRR is cell-, tissue-, and species specific. In addition, repression of AHR by AHRR in in vivo studies remains elusive (Haarmann-Stemmann and Abel, 2006). Furthermore, AHRR also can repress the activity of the other transcription factors such as estrogen receptor alpha (Ohtake et al., 2003).

2.4.3 Regulation of AHR activity by modulation of receptor degradation

Levels of AHR can be regulated by the rate of expression and the rate of degradation of the protein. Expression of AHR metabolizing enzymes and AHR protein is under the control of the AHR signaling pathway and the keap1-Nrf2-antioxidant response element (Shin et al., 2007). The half-life of liganded-AHR is much shorter than unliganded-AHR, indicating that the ligand-activated AHR undergoes degradation after activation and binding to DNA (Ma and Baldwin, 2000). The down regulation of AHR through the 26S proteasomal pathway starts by labeling of the protein with a polypeptide, ubiquitin, then, the protein is recognized by the 26S proteasome and is degraded into short peptides (Davarinos and Pollenz, 1999). There seems to be a regulatory feedback control between AHR transcriptional activity and degradation (Davarinos and Pollenz, 1999; Roberts and Whitelaw, 1999; Pollenz, 2002; Song and Pollenz, 2003).

2.4.4 Crosstalk between the AHR and other pathways

It has been observed that TCDD has anti-estrogenic effects (Kociba et al., 1978). Crosstalk between the AHR signaling pathway and retinoic acid receptor (Wanner et al., 1995; Lorick et al., 1998), estrogen receptor (Spink et al., 1990; Heimler et al., 1998; Tian et al., 1998), growth factors and cytokine signaling (Haarmann-Stemmann et al., 2009), transcription factor SP1 (Fisher et al., 1990) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) (Tian et al., 1999; Kim et al., 2000) has been reported. Several protein kinases such as protein kinase C (PKC) and protein tyrosine kinase (PTK) have been implicated in the regulation of AHR by phosphorylation of its C-terminal region (Gradin et al., 1994; Mahon and Gasiewicz, 1995). NF-kB activators such as bacterial endotoxins and pro-inflammatory cytokines are reported repressors of CYP1A1. Other authors suggested competition for common
co-activators (p300) to be a mechanism for suppression of CYP1A1 by NF-kB (Tian et al., 1999; Ke et al., 2001). The presence of multiple copies of XRE/DREs within the promoters of human Nrf2 genes and induction of Nrf2 and Nrf2 target genes by TCDD indicate that the AHR interacts with Nrf2 via two possible mechanisms. First, the AHR ligands such as TCDD directly up-regulate expression of Nrf2 and phase II metabolizing enzymes and second, AHR/ARNT/Nrf2 engage in the mutual binding to XRE/DRE (Ma et al., 2004; Yeager et al., 2009). Moreover, it has been shown that the level of AHR mRNA in Keap-1 knockout mice is higher than in normal animals suggesting a direct crosstalk between AHR and Nrf2 signaling pathways (Shin et al., 2007).

2.4.5 Epigenetic effects on AHR target genes regulations

DNA methylation and chromatin remodeling by histone acetylation and de-acetylation are epigenetic mechanisms shown to take part in regulation of human AHR target genes after exposure to exogenous and endogenous chemicals. Histone acetylation and de-acetylation modify the structure of chromatin to be accessible or inaccessible for the transcription factors and co-activators/co-repressors. Recruitment of histone acetylase (HAT), co-activators such as p300, and steroid receptor co-activator 2 (SRC-2) to the enhancer and promoter of both CYP1A1 and CYP1B1 genes are required for proper transcription activity (Taylor et al., 2009). Also different patterns of DNA methylation of AHR target genes provide valuable information with regard to the epigenetic modification of AHR signaling. It has for example been observed that CpG dinucleotides in the promoter and enhancer of the CYP1B1 gene exhibit DNA methylation in some cell lines while CYP1A1 is not fully methylated and is inducible (Han et al., 2006; Habano et al., 2009; Beedanagari et al., 2010).

2.5 MODULATION OF AHR SIGNALING BY OXIDATIVE STRESS

The dual function of reactive oxygen species (ROS) in biological systems is well established for several years. Regulatory events for ROS production mainly by the NADPH oxidase enzyme (NOX) family and maintenance of redox homeostasis by the activation of redox sensitive transcription factors such as Nrf2, the activator protein 1 (AP-1) and NF-kB are key events in the normal physiology of living cells. Diffusible H$_2$O$_2$ can act as a fundamental signaling molecule in activation/inhibition of several transduction signaling pathways. ROS sometimes play opposing roles in cellular signaling. For instance, activation of extracellular signal-regulated kinase (ERK1/2) by ROS can promote cell survival whereas activation of c-Jun N-terminal (JNK)/stress-activated protein kinase can induce apoptosis (reviewed in Groeger et al., 2009).

The redox regulation of AHR signaling is not well characterized but it has been shown previously that the sulfhydryl groups in the AHR complex and the DNA binding domain of the AHR are sensitive to oxidative stress (Denison et al., 1987; Pongratz et al., 1992; Ireland et al., 1995; Xu et al., 1998; Cumming et al., 2004).

2.5.1 Different sources of ROS

Superoxide anion (O$_2$•-) is a product of the one-electron reduction of an O$_2$ molecule. In mammalian cells, ROS can be generated from different sources such as mitochondria (mainly from complex I and III), endoplasmic reticulum (microsomes fractions),
peroxisomes, NO synthases and lipoxygenases in cytosol and NOX enzymes in the plasma membrane (Halliwell and Cross, 1994). ROS also can be produced by xanthine oxidase in extracellular spaces (Brown and Borutaite, 2012).

2.5.1.1 **NADPH oxidase**

Activation of NOX enzymes in macrophages and phagocytes produces millimolar concentrations of H$_2$O$_2$ to kill bacteria and other pathogens (Rhee, 2006). In contrast, activation and expression of NOXs in non-phagocytes is a highly regulated process that is involved in many cellular signaling pathways (reviewed in Droge, 2002). The acute activation of NOX family members (NOX1-4, NOX5 and the dual oxidases DUOX1 and DUOX2) are regulated either by intracellular levels of calcium or phosphorylation of regulatory subunits. Non-phagocyte NOXs can be activated by different stimuli such as transforming growth factors α and β (TGFα and β), interleukin-1α and β (IL-1α and β), epidermal growth factor (EGF), tumor necrosis factor α (TNFα), mechanical forces stimulation (in endothelial cells), changes in oxygen tension, hormones or local metabolic changes (reviewed by Jiang et al., 2011). The glycosylated catalytic moiety of NOX enzymes, gp91$^{\text{phox}}$ consists of a flavin-adenine dinucleotide (FAD) sequestered in the cell membrane in association with its trans-membrane partner p22$^{\text{phox}}$ (Figure 6). Complex formation with the p22$^{\text{phox}}$ subunit is needed for NOX activation (Sumimoto et al., 1996; Ambasta et al., 2004; Martyn et al., 2006). This complex is referred to as cytochrome b558. Phosphorylation of the P47$^{\text{phox}}$ subunit and recruitment of a small Rho GTPase protein (Rac) result in release from auto-inhibition, translocation to the membrane and binding to p22$^{\text{phox}}$ (reviewed in Groeger et al., 2009). The N-terminal of NOXs comprises six predicted α-helic regions for electron transferring (reviewed by Lambeth et al., 2007). The maximal activity of NOXs can be determined by the activation state of regulatory subunits and expression of NOX proteins. The highest level of NOX1 protein is found in colon and to some extent in other tissues and cell lines such as vascular smooth muscles and pulmonary epithelial cell line. NOX2 and NOX3 are expressed in mature myeloid cells and in the inner ear respectively (reviewed in Lambeth et al., 2007). Among NOX families, NOX4 is constitutively active (reviewed by Droge, 2002) and can be activated by various types of activators. Acute NOX4-dependent generation of ROS by stimulation with insulin (after five min) and lipopolysaccharide (after 30 min) has been reported (Mahadev et al., 2004; Park et al., 2004; Lambeth et al., 2007). Expression of NOX4 is predominantly observed in the kidney and liver and moderate levels of NOX4 are expressed in other organs (Cheng et al., 2001; Mahadev et al., 2004). High levels of NOX5 are expressed in spleen, testis and vascular smooth muscle (reviewed in Lambeth et al., 2007). DOUX1 and DOUX2 are found in the thyroid (De Deken et al., 2002). A compensatory expression mechanism between NOX4 and NOX2 has been observed in some cell lines (Petry et al., 2006; Pendyala et al., 2009).
2.5.2 Cellular defenses against ROS

\( \text{O}_2^- \) is an unstable radical species with a very short half-life. It can be converted to non-radical species such as \( \text{H}_2\text{O}_2 \) enzymatically by superoxide dismutase (SOD) or spontaneously in cells. \( \text{H}_2\text{O}_2 \) in the presence of reduced transition metals can be converted into the very reactive and harmful hydroxyl radical (HO). To neutralize and use the advantage of ROS, antioxidants play important roles in living cells (Halliwell, 2007). Some cellular antioxidants are present at low levels but work with high efficiency (catalase and glutathione peroxidase) and some are present in high levels but work with low efficiency (free amino acids and proteins). Living organisms also use nonenzymatic antioxidants such as vitamins and glutathione to scavenge ROS (reviewed by Droge, 2002). In mammalian cells SOD, catalase and glutathione peroxidase convert \( \text{H}_2\text{O}_2 \) to water and oxygen.

2.5.2.1 Glutathione

The tripeptide \( \gamma \)-L-glutamyl-L-cysteinylglycine or glutathione is an abundant non-protein antioxidant present in all mammalian cells at 1-10 mM concentration. Glutathione exists in both reduced (GSH) and oxidized (GSSG) form. More than 98% of glutathione at normal physiological conditions is present in the reduced form (Akerboom et al., 1982). Unusual linking between glutamate and cysteine (\( \gamma \)-carboxyl instead of \( \alpha \)-carboxyl) makes it resistant to degradation by intracellular enzymes. Glutathione is degraded by an extracellular enzyme, \( \gamma \)-glutamyltranspeptidase (GGT), on the surface of certain cell types (Meister and Anderson, 1983; Lu, 2012). Glutathione is synthesized in two ATP-dependent reactions. First, formation of \( \gamma \)-glutamylcysteine by a glutamate cysteine ligase (GCL) and second, the addition a
glycine amino acid to the \( \gamma \)-glutamylcysteine by glutathione synthetase (GS) forms the tripeptide, \( \gamma \)-L-glutamyl-L-cysteinylglycine. GCL is composed of catalytic and modifier subunits (GCLC and GCLM) and is a crucial enzyme in GSH homeostasis. Glutathione synthesis can be limited by the availability of cysteine amino acid. It has been shown that the reducing agent dithiothreitol is able to reversibly inactivate GCL whilst oxidative stress facilitates the holoenzyme formation (Seelig et al., 1984; Franklin et al., 2009). GSH plays several important functions in living cells including 1) detoxification of electrophiles enzymatically in a reaction catalyzed by glutathione-S-transferases 2) scavenging of ROS in a reaction catalyzed by glutathione peroxidases 3) regulation of redox-dependent cell signaling pathways by redox-sensitive transcription factors and 4) serving as a continuous source of cysteine (Meister and Anderson, 1983; DeLeve and Kaplowitz, 1991; Lu, 2012). In the reaction catalyzed by glutathione peroxidase, a sulfhydryl moiety of the cysteine residue of GSH donates a reducing equivalent to neutralize \( \text{H}_2\text{O}_2 \) to water. In this reaction two molecules of GSH are oxidized to GSSG. GSSG can be reduced back to GSH by glutathione reductase using NADPH as an electron donor.

GSH plays an important role in maintenance of the cellular redox state that is crucial for regulation of signal transduction pathways and gene expression through activation of transcription factors. The intracellular redox state of the cells is maintained by two systems, the GSH/GSSG ratio that is present at millimolar and thioredoxin (Trx) at micromolar concentrations. The steady-state balance of GSH and GSSG during oxidative stress conditions is different. The redox state of cells is determined in a reversible reaction catalyzed by thiol-transferase as follows (reviewed in Lu, 1999):

\[
\text{Protein-disulfide} + 2 \text{(GSH)} \rightleftharpoons \text{GSSG} + \text{Protein-(GSH)}_2
\]

A convenient expression has also been introduced for the calculation of GSH redox potential according to the Nernst equation as follows:

\[
E_h = E^\circ + (RT/nF)\ln([\text{acceptor}]/[\text{donor}])
\]

In the above equation, \( E_h \) (mV) at defined pH is calculated relative to a standard hydrogen electrode. In this expression \( R, T, n \) and \( F \) are gas constant, absolute temperature, the number of electrons transferred and Faraday’s constant respectively (see a review by Jones, 2002). Extracellular and intracellular redox potentials provide useful indicators in association with redox-dependent cellular signaling. For instance, the intracellular GSH/GSSG potential redox is reduced during proliferation and becomes oxidized in differentiation and apoptosis (from -260mV to -150mV). The extracellular Cys/CySS redox follows the same pattern (Moriarty-Craige and Jones, 2004). While more than 98% of cellular glutathione under normal physiological conditions is present in the reduced form (Akerboom et al., 1982), any changes in the intracellular GSH level can be useful for predicting cellular signaling.

2.5.2.2 *The Nrf2-ARE signaling pathway*

Nrf2 is a redox sensitive transcription factor that controls the expression of a group of enzymes protecting against reactive oxygen intermediates. Nrf2-deficient mice are more susceptible to developing different types of cancer and chronic diseases (Ramos-Gomez et al., 2001; Rangasamy et al., 2004; Khor et al., 2006). Under non-stress physiological conditions, Nrf2 is sequestered by its cysteine-rich, Kelch domain-
containing partner (Keap1) in the cytoplasm. The Keap1-Nrf2 complex is rapidly degraded by the ubiquitin proteasome pathway after oxidation (Itoh et al., 2003). Both the N-terminal Broad complex, Tramtrack and Bric-à-Brac (BTB) and intervening-region (IVR) domains of Keap1 are required for Nrf2 degradation and a subunit of E3 ubiquitin ligase (Cul3) interacts with the IVR domain of Keap1 (Kobayashi et al., 2004). Overexpression of Cul3 leads to rapid degradation of Nrf2 and this has been shown to enhance the risk of breast cancer (Loignon et al., 2009).

**The Nrf2 pathway can be activated not only by chemical compounds with the capacity to undergo redox cycling or by reactive oxygen intermediates but also by compounds that have the ability to react with sulfhydryl groups.** Thus, alterations in the glutathione cellular levels and the redox state of cells can trigger the Nrf2 signaling pathway (Talalay et al., 2003). Modification of two cysteine (C273 and C288) residues in the IVR domain by ROS and formation of intermolecular disulfide bridges leads to the release of Nrf2 and its translocation to the nucleus (Dinkova-Kostova et al., 2002; Kobayashi et al., 2004; Wakabayashi et al., 2004) where it dimerizes with Maf, one of the small bZIP proteins. Activated Nrf2 binds to the antioxidant response element (ARE) and causes ARE-dependent transcription of antioxidant genes such as GST, NAD(P)H:quinone oxidoreductase 1 (NQO1), heme oxygenase 1 (HO-1), ferritin, GCL, glutathione reductase and aldehyde dehydrogenase (Hayes and McLellan, 1999; Talalay et al., 2003; Kensler et al., 2007) (Figure 7). It has been suggested that Keap1 regulates Nrf2 activity but the mechanism of stabilization of Nrf2 activity is not well known. Nrf2 has been found to be an unstable protein with a short half-life (t1/2 around 15 min). After activation with ROS, Cul3 targets Nrf2 for ubiquitin-dependent degradation by the 26S proteasome (Nguyen et al., 2003; Nguyen et al., 2009).

![Figure 7](image)

Figure 7. General scheme for the activation of the Keap1-Nrf2-ARE signaling pathway. ROS disrupts the Keap1-Nrf2 association leading to formation of intermolecular disulfide bridges and accumulation of Nrf2 in nucleuses where it dimerizes with small protein Maf, binds to ARE and stimulates expression of ARE responsive genes.
3 COMMENTS ON METHODOLOGY

3.1 ANIMALS

In order to determine whether FICZ can be distributed to the body through the systemic circulation, experiments were carried out on female C57BL/6J mice (Paper I). The experiment was conducted in accordance with the regulations for animal experimentation at Karolinska Institutet and was preapproved by the Ethical Committee on Animal Experimentation in Stockholm.

3.2 CELL LINES

The immortalized human keratinocyte cell line (HaCaT) was kindly provided by N. E. Fusenig (DKFZ, Heidelberg, Germany). HaCaT cells were grown at a high density to elucidate the AHR activation by different AHR activators and CYP1A1 inhibitors (Paper I, II and III). Higher expression of differentiation markers (involutrin and transglutaminase) and AHR protein was documented when HaCaT cells were grown at high density (Paper I).

The human hepatoma HepG2-derived cell line HepG2-XRE-Luc, containing a pTX.DIR- luciferase reporter under the control of two XRE/DRE sequences of the rat CYP1A1 gene, was kindly provided by K. Gradin, Karolinska Institutet. HepG2-XRE-Luc was used to investigate CYP1A1 reporter gene activation in response to different types of AHR activators and CYP1A1 inhibitors (Paper II and III).

The human X chromosome-linked chronic granulomatous disease (X-CGD) derived-PBL-985 cell line (X-CGD cells) carrying a mutated gp91phox gene (Zhen et al., 1993) and the X-CGD cell line re-transfected with gp91phox (X-CGD-gp 91phox cells), originally constructed by M.C. Dinauer (Ding et al., 1996), were kindly provided by B. Fadeel, Karolinska Institutet. The X-CGD and X-CGD-gp 91phox cells were used to study cell growth and NADPH oxidase activation after treatment with several metal and metalloid compounds (Paper III).

3.3 PRIMARY CELLS

Supplementary studies were performed on the primary human epidermal keratinocytes (HEKa cells) (Paper I).

3.4 MATERIALS

3.4.1 AHR agonists

An indolocarbazole compound, FICZ (Figure 4) instead of the highly toxic compound TCDD (Figure 3) was chosen to assess the biological functions of the AHR in order to understand the intrinsic AHR signaling. TCDD is the prototype for a family of persistent and structurally similar compounds with toxicological and biological effects on human and experimental animals through activation of the AHR signaling (Poland and Knutson, 1982; White and Birnbaum, 2009). The AHR high affinity ligand FICZ was first described in 1987 by Rannug et al. as a photoproduct formed after ultraviolet irradiation of Trp. Among AHR ligands, FICZ exhibits the lowest $K_d$ (70 pM) for AHR binding of all compounds tested so far and it has been suggested as
an endogenous signaling molecule (Rannug et al., 1987). FICZ is an excellent substrate for CYP1A1 ($k_{cat}/K_m$ of 8.1 x 10$^7$ M$^{-1}$s$^{-1}$). The catalytic efficiency for FICZ seems to be close to the limit of diffusion and it is a 50 times better substrate compared to the model CYP1A1 substrate 7-ethoxyresorufin (Wincent et al., 2009). However, the pharmacokinetics and pharmacodynamics of FICZ has to be investigated in more details in future studies. AHR activation by TCDD and FICZ was examined in Paper I.

### 3.4.2 CYP1A1 inhibitors

Different types of chemicals including oxidants, clinical drugs, endogenous and natural substances, biochemical inhibitors, phytochemicals and metals have been shown to inhibit the activity of CYP1A1 (Moorthy et al., 2000; Ueng et al., 2002; Bozcaarmutlu and Arinc, 2004; Oliveira et al., 2004; Baliharova et al., 2005; Chaudhary and Willett, 2006; Mikstacka et al., 2007; Zhang et al., 2008; Sergent et al., 2009a). In most studies, inhibition of 7-ethoxyresorufin-O-deethylase activity (EROD) was chosen to test the inhibitory effects of chemicals on CYP1A1 enzyme activity. Depending on the structure different chemicals can act as a substrate, inhibitor or both substrate/inhibitor of CYP1A1. A study with different types of flavonoids containing methoxy- and hydroxyl-group substitutions suggests that compounds demonstrating high substrate affinity for CYP1A1 might be stronger inhibitors of EROD activity (Androutsopoulos et al., 2011). Shimida et al. suggested that the number and position of hydroxyl and methoxy groups in the structure of flavones are important factors for inhibitory effects of flavones on CYP1A1 (Shimada et al., 2010).

#### 3.4.2.1 Oxidants

H$_2$O$_2$ and UVB were chosen to evaluate the inhibitory effects of oxidants on CYP1A1 gene expression and enzyme activity (Paper I). For UVB exposure, HaCaT cells were exposed to UVB light in dishes containing PBS. UVB lamps consisting of six (Philips TL20W/12RS lamp) or two (Philips PL, 36W, UV240 DT, IP20) UV tubes were used to obtain 5-20 mJ/cm$^2$ doses of UVB (280-320nm). After irradiation, the PBS was removed and fresh medium was added to the dishes.

#### 3.4.2.2 Polyphenols

AHR activation by the polyphenols, 3′-methoxy-4′-nitroflavone MNF (Paper I), quercetin (QUE), resveratrol (RES) and curcumin (CUR) (Paper II) was investigated (for chemical structure see Figure 8-10). It has been reported that polyphenols are potent inhibitors of CYP1A1 (Chaudhary and Willett, 2006; Mikstacka et al., 2007). Polyphenols have been claimed to exhibit both agonistic and antagonistic effects on AHR signaling (Ciolo\no et al., 1998a; Ciolo\no et al., 1998b; Casper et al., 1999; Ciolo\no et al., 1999; Zhang et al., 2003; Pohl et al., 2006; Sergent et al., 2009b).
Figure 8. 2-(3,4-dihydroxyphenyl)-3,5,7-trihydroxychromen-4-one (quercetin)

Figure 9. 5-\{(E)-2-(4-hydroxyphenyl)ethenyl\}benzene-1,3-diol (resveratrol)

Figure 10. (1E,6E)-1,7-bis(4-hydroxy-3-methoxyphenyl)hepta-1,6-diene-3,5-dione (curcumin)
3.4.2.3 Metals and metalloids

AHR activation by metals and metalloids was investigated in Paper III. Arsenic (As), cadmium (Cd), mercury (Hg) and nickel (Ni) were chosen to investigate the mechanism of AHR activation by compounds that are not typical AHR ligands. To investigate the oxidative interaction of metals with AHR signaling was another purpose of this study.

3.5 METHODS

3.5.1 Experimental designs

Dose-response and time course studies were carried out in cells exposed to CYP1A1 inhibitors alone or in combination with FICZ. In all experiments excluding studies with UVB, cells were exposed to fresh medium containing compounds of interest without adding FBS. The treatments were terminated by removing the medium and rinsing the cells with PBS (Paper I, II and III). In UVB experiments, cells were exposed in dishes containing PBS (Paper I). Data were collected and normalized according to the protein content.

In the preconditioning studies, cells were pre-treated with the compounds of interest in the FBS free medium for 5 hours. The pre-treatments were terminated by removing the medium, rinsing the cells with PBS and adding new medium with 10% FBS for 24 hours. After recovery time, second treatments were performed by changing the medium to new medium containing DMSO or FICZ for up to 48 hours (Paper III).

3.5.2 CYP1A1 inhibition assays

Human recombinant CYP1A1 + NADPH-450 reductase suprameresomes were used to investigate inhibitory effects of chemicals on pure human CYP1A1 by using 7-ethoxyresorufin as a standard substrate (Paper I, II, and III).

3.5.3 Analyses of cellular FICZ content

A HPLC method was used to quantify the levels of FICZ in HaCaT cells exposed to FICZ alone or in combination with CYP1A1 inhibitors. A solid-phase extraction column coupled to a reverse-phase C18 column (Alltech Alltima; 250 mm ×4.6 mm) and a mobile phase consisting of acetonitrile and water were used. FICZ was detected by fluorescence at excitation and emission wavelengths of 390 and 525 nm (Paper I, II and III).

3.5.4 AHR activation assays

Several types of assays have been established to investigate AHR activation by different AHR ligands and CYP1A1 inhibitors. In order to quantify AHR activation at the transcriptional levels either qRT-PCR or luciferase reporter assays were used. CYP1A1 enzyme activity was quantified by the EROD assay (Paper I, II and III).

3.5.4.1 AHR activation in commercial and purified medium

In order to find out whether AHR activation by CYP1A1 inhibitors is due to the presence of FICZ in the cell culture media, experiments were performed in a
commercial medium or in a Trp-free medium which was supplemented with fresh and re-crystallized Trp before each use (Paper I, II and III).

3.5.5 Detection of oxidative stress

3.5.5.1 NADPH oxidase activity
Superoxide anion or H$_2$O$_2$ produced by NOXs activation was determined by increased lucigenin activity as described previously (Smith et al., 2001). Chemiluminescence was measured on a Genios Pro plate reader after addition of NADPH and lucigenin (Paper III).

3.5.5.2 GSH assay
The level of GSH was determined spectrophotometrically after reaction with 5-5'-dithiobis[2-nitrobenzoic acid] (DTNB) at 405 nm utilizing a Genios Pro plate reader (Paper III).

3.5.5.3 Up-regulated antioxidant genes
Expression of HO-1, CYP1A1 and AHR were determined by RT-qPCR after exposure of HaCaT cells to As. In parallel, adaptive responses to oxidative stress induced by As were determined by predesigned multiple RT-PCR array plates (Stress & Toxicity PathwayFinder PCR Array, PAHS-003Z, SABioscience) consisting of 84 genes involved in 6 biological pathways including oxidative stress, osmotic stress, DNA damage, inflammation, hypoxia and heat shock proteins/unfolded proteins (Paper III).

3.5.6 NOX4 and Nrf2 silencing
A SMART pool siGENOME NOX4 siRNA consisting of four small interfering NOX4 RNA sequences and siRNA against Nrf2 were used to silence NOX4 and Nrf2 pathways. SiRNA complexes were formed in antibiotic- and serum-free DMEM medium, using DharmacoFECT transfection reagents (Paper III).

3.5.7 Cell proliferation assay
The CyQUANT NF cell proliferation assay kit was used to assess cell proliferation in PLB-985 and HaCaT cells treated with different concentrations of metals alone or together with diphenyleneiodonium (DPI). Dye binding to DNA (fluorescence) was measured at excitation/emission wavelengths 492/535 nm (Paper III).
4 AIMS OF PRESENT STUDY

The overall aim of the work included in this thesis was to improve current knowledge regarding the redox regulation of AHR signaling. A particular objective was to study AHR activation in the absence of exogenously added AHR ligands. The project includes characterization of enzymatic breakdown of a metabolizable natural AHR ligand, FICZ.

The specific aims were:

- To explore the effect of some compounds, known to activate AHR without being good ligands for the receptor
- To investigate if the effect of these compounds is caused by the presence of FICZ in the cell culture medium
- To evaluate inhibition of CYP1A1 mediated degradation of the natural AHR ligand FICZ
- To clarify the influence of ROS-generating compounds on AHR signaling pathways
5 RESULTS AND DISCUSSION

5.1 FICZ IS A POTENT CYP1A1 INDUCER IN VITRO AND IN VIVO

Among AHR agonists known to date, FICZ exhibits the highest affinity to bind and activate AHR signaling (Rannug et al., 1987; Fritsche et al., 2007; Jonsson et al., 2009; Wincent et al., 2009). In contrast to TCDD the effects of FICZ on AHR signaling are transient (Wei et al., 1998; Wincent et al., 2009). The ability of FICZ to distribute in the body through systemic circulation and induce CYP1A1 gene expression in peripheral tissues was determined by administration of 10 ng FICZ on one ear of female C57BL/6J mice (Paper I). The results of this experiment showed that FICZ, in spite of its rapid metabolism (Wincent et al., 2009), can be distributed in the body through the systemic circulation and induce CYP1A1 gene expression in several tissues. In our study, the expression of CYP1A1 gene in liver and adipose tissues was transient and a prolonged induction was observed at the site of application. Sustained induction of CYP1A1 at the site of application can be explained by pharmacokinetic parameters of FICZ and presence of FICZ at high concentrations at the site of application. The inhibitory effects of FICZ on its own metabolism by CYP1A1 has been previously reported (Wincent et al., 2009). Therefore, studying pharmacokinetics parameters including absorption, distribution, metabolism and excretion from the body might provide valuable information in order to understand the intrinsic physiology of AHR.

Next, we examined the potential of FICZ as an inducer of CYP1A1 gene expression in vitro. The EC50 value (half maximal effective concentration) of FICZ was found to be at picomolar concentration which is lower than the EC50 value earlier described for TCDD (Denison et al., 2002).

Taken together, this study showed that FICZ is a potent inducer of CYP1A1 in vivo and in vitro. FICZ can distribute in the body through the systemic circulation and induce CYP1A1 in various organs.

5.2 CYP1A1 INHIBITORS TURN INTO AHR ACTIVATORS

5.2.1 Metabolic clearance of FICZ is inhibited by CYP1A1 inhibitors

The potential ability of different groups of chemicals such as H2O2, MNF (Paper I), polyphenols (Paper II) and metals (Paper III) to inhibit human recombinant CYP1A1 was evaluated in this thesis. H2O2 at 200 µM and MNF at 2.5 µM concentrations gave approximately 90% and 80% inhibition of microsomal CYP1A1 activity, respectively. The half maximal inhibitory concentration (IC50 value) of the other chemicals tested in the experiments was determined by constructing dose-response curves. According to the IC50 values, their potencies to inhibit human recombinant CYP1A1 can be ranked as follows:

QUE (1.2 µM)> CUR (7.3 µM)> RES (11.8 µM)
Hg (0.16 µM) > Cd (1.70 µM) > Ni (14.70 µM) > As (16.02 µM)

Our results are consistent with earlier studies, which have shown that the polyphenols QUE, RES, and CUR are able to inhibit the catalytic activity of CYP1A1 enzyme (Oetari et al., 1996; Ciolino et al., 1998a; Chun et al., 1999; Chaudhary and Willett, 2006). In studies performed with human recombinant CYP1A1 both K_m and V_max changed with increasing doses of QUE and RES suggesting a mixed type of inhibition by QUE and RES (Chaudhary and Willett, 2006; Mikstacka et al., 2007). Metals and
metalloids have also earlier been shown to be able to inhibit the activity of CYP1A1 enzyme (Anwar-Mohamed et al., 2009). Metals inhibit the function of CYP1A1 enzymes by interfering with the heme moiety at the catalytic site (Gonzalez, 1988; Vernhet et al., 2003; Kaminsky, 2006). Metals also are able to generate ROS and ROS may oxidize thiol groups in the cysteine residues of proteins (Bogdan, 2001). The inhibition of CYP1A1 by metals seems to be through reversible inhibition. These inhibitors exhibit only dose-dependent inhibition pattern (Hollenberg, 2002). CYP1A1 inhibition by metals can be reversed by addition of GSH or antioxidant (Bozcaarmutlu and Arinc, 2004; Oliveira et al., 2004) suggesting that in addition to interfering with the heme moiety of CYP1A1, generation of ROS is also involved in the inhibition of CYP1A1 by metals.

The effects of CYP1A1 inhibitors on metabolic degradation of FICZ were evaluated by HPLC in HaCaT cells. The cells were treated with FICZ alone or in combination with different doses of H$_2$O$_2$ and MNF (Paper I), QUE, RES and CUR (Paper II), As, Cd, Hg and Ni (Paper III) for different lengths of time. All tested compounds were able to inhibit the metabolic degradation of FICZ. Among the three polyphenol compounds tested in this thesis, QUE was the most potent inhibitor of human recombinant CYP1A1 and QUE was also a strong inhibitor of FICZ metabolism in HaCaT cells. Among the metals tested, Hg was the most potent inhibitor of CYP1A1 while no major differences in efficacy of metals to inhibit FICZ degradation were observed. It can be speculated that the pharmacokinetics of FICZ can be influenced by co-treatments with metals. Heavy metals not only interfere with the normal function of metalloproteins such as CYP1A1 but also they are able to disrupt the normal functions of cellular carriers and transporters.

5.2.2 CYP1A1 inhibitors activate AHR indirectly

It has been shown that commercial media contain FICZ and can activate AHR signaling especially if the media had been exposed to light (Oberg et al., 2005).

The ability of AHR agonists such as FICZ and TCDD and several CYP1A1 inhibitors including oxidants, clinical drugs, endogenous and natural substances, and compounds used as biochemical inhibitors (Paper I), polyphenols (Paper II) and metals (Paper III) to activate AHR signaling in the absence of the high affinity ligand FICZ was tested by exposing the cells in a commercial DMEM and a Trp-free DMEM which was supplemented with freshly re-crystallized Trp before each use. The results obtained in the medium lacking FICZ were significantly different from the experiments performed in commercial DMEM.

Next, the background levels of FICZ were quantified in commercial batches of media to find out how much of FICZ is sufficient to activate AHR signaling. The level of FICZ was determined by HPLC and a series of experiments were performed in the purified medium by adding different concentrations of FICZ. The concentration of FICZ in commercial DMEM was found to be in the order of 0.1 pM (Paper I) and this level of FICZ was sufficient to activate AHR signaling as determined in the EROD assay (Paper I).
Based on our studies with different groups of CYP1A1 inhibitors (paper I, II and III), previously established AHR activation by αNF (Wincent et al., 2009), and a wide range of CYP1A1 inhibitors listed in the supplementary table of Paper I (Figure 11) we suggest that an indirect mechanism for AHR activation explains the findings of AHR-induction by compounds that do not fit into the ligand binding pocket of the AHR.

Figure 11. Examples of CYP1A1 inhibitors that are also AHR activators

5.3 OXIDATIVE STRESS MODULATES AHR SIGNALING

5.3.1 Oxidative stress antagonizes AHR activation

Temporal inhibition of CYP1A1 transcription activity at the early time of incubation was seen in HaCaT and HepG2 cells treated with FICZ together with oxidants (Paper I), polyphenols (Paper I and II) and metals followed by an up-regulation of both CYP1A1 transcription and enzyme activity (Paper III). An obvious correlation between AHR activation and intracellular levels of reduced glutathione was suggested by the results presented in Paper III.

Up-regulation of CYP1A1 required relatively reducing conditions as shown by measurement of cellular GSH in parallel with CYP1A1 induction in HepG2 cells. We further confirmed this hypothesis by using a precursor of glutathione, N-Acetyl-L-cysteine (NAC), and a depletory agent of glutathione, buthionine-(S,R)-sulfoximine (BSO) in CYP1A1 induction experiments. In HepG2-XRE-Luc cells co-treated with BSO the attenuation of CYP1A1 transcription activity caused by the metals was potentiated, while NAC treatments efficiently reduced the inhibitory effects.
The effects of polyphenols on CYP1A1 transcription activity were biphasic (Figure 12). FICZ-stimulated AHR transcription was potentiated by low concentrations of polyphenols and inhibited in a dose-dependent manner by high concentrations. It can be speculated that polyphenols at low concentrations act as antioxidants and increase the antioxidant capacity of cells. Phenolic compounds have been suggested to interfere with the oxidation of macromolecules in the cells by donating a hydrogen atom to free radicals. However, under certain conditions polyphenols may act as pro-oxidants (Shahidi and Wanasundara, 1992; Bravo, 1998). It seems plausible that the inhibition of AHR reporter activity that was only observed with µM concentrations was explained by the pro-oxidative effects of the polyphenols.

![Figure 12. Biphasic effects of polyphenols on the transcription activity of AHR](image)

It is well known that metals induce oxidative stress and cause depletion of GSH by binding to sulphydryl groups of proteins (Kaminsky, 2006; Jomova and Valko, 2011). Down-regulation of CYP1A1 by oxidants and depletion of GSH has been reported in several studies (Morel and Barouki, 1998; Xu et al., 1998). The AHR protein and the DNA-binding of many transcription factors were earlier shown to be sensitive to oxidative stress (Denison et al., 1987; Pongratz et al., 1992; Droge et al., 1994; Cumming et al., 2004). We hypothesize that the sulphydryl groups in the chaperone protein HSP90 in the AHR complex and DNA-binding of AHR may be the sensitive targets to oxidative stress.

FICZ, TCDD and some other high affinity AHR ligands function as potent agonists and activators of AHR in vitro and in vivo. Conversely, α-naphthoflavone, MNF and the most potent AHR inhibitor described so far i.e. CH223191 are compounds used as specific antagonists of AHR (Lu et al., 1995; Gasiewicz et al., 1996; Kim et al., 2006;
Zhao et al., 2010). However, most of the AHR antagonists exert partial agonistic activity at high concentrations as shown by their capacity to compete with a high affinity AHR agonist, in most cases [3H]TCDD, for binding to the receptor and inhibit agonist activated AHR-ARNT complex to bind and activate XRE/DRE-dependent transcription. We have shown that the agonistic properties of some AHR activators to some extent can also be attributed to the presence of the AHR high affinity ligand FICZ in the cell culture media.

CH223191 (Figure 13) has not been reported to activate AHR and is therefore suggested to be a pure and potent AHR antagonist. CH223191 also inhibited the AHR-activation in HepG2-XRE-Luc cells in our studies (Figure 14). However, the antagonistic effects of CH223191 on FICZ stimulated AHR activation could, to some extent, be reversed by addition of the potent antioxidant NAC (Figure 14), which suggest that CH223191 is an oxidant which like other oxidants can lower the levels of GSH and thereby cause a temporal inhibition of AHR regulated responses. This result suggests that like the commonly used AHR antagonists α-naphthoflavone and MNF, also CH223191 can work as an oxidant and inhibit AHR signaling by this mechanism. The sensitivity of AHR complex to oxidants has been further confirmed by results showing the ability of the reducing agent dithiothreitol to restore the displacement of [3H]TCDD from AHR complex by the sulfhydryl-modifying reagent, Hg (Denison et al., 1987).

Figure 13. Structure of the potent AHR antagonist 2-methyl-N-[2-methyl-4-[(2-methylphenyl)diazenyl]phenyl]pyrazole-3-carboxamide (CH223191)
5.3.2 Oxidative stress up-regulates antioxidant genes

In response to As treatment we observed a substantial (1000-fold) up-regulation of HO-1 and more than 2 fold induction of 16 genes related to oxidative stress, osmotic stress, DNA damage, inflammation, hypoxia and heat shock proteins/unfolded proteins (Paper III). In parallel with up-regulation of the modifier subunit and the catalytic subunit of GCL we observed that the level of GSH increased during the time of incubation. The common mechanisms for up-regulation of antioxidant genes are mainly mediated by activation of redox-sensitive transcription factors. Low intensity of ROS activates the Keap1/Nrf2 pathway system to up-regulate genes encoding antioxidant enzymes such as the GCL. Intermediate intensity of ROS activates NF-kB and MAP-kinases pathways to induce antioxidant genes and inflammation responses and high levels of ROS lead to apoptosis and necrosis which is mainly mediated by mitochondria-dependent death cascades (Gloire et al., 2006; Lushchak, 2011).

5.3.3 Preconditioning with oxidants leads to super-induction of CYP1A1

Preconditioning with H$_2$O$_2$ or metals significantly elevated the level of GSH in HepG2-XRE-Luc cells. In accordance with these results the AHR transcription machinery induction by endogenous ligand FICZ was very effective, when cells were in a highly reduced form, (Paper III).

The steady-state level of CYPs mRNA is a result of mRNA synthesis and mRNA degradation. Among CYPs mRNAs, CYP1A1 possesses a short half-life (2.4-4.4 h) (Lekas et al., 2000; Suzuki and Nohara, 2007). After translation of CYPs mRNA to protein, the level of enzymes can be regulated by post-translational modifications (Aguiar et al., 2005; Oesch-Bartlomowicz and Oesch, 2005). This suggests that ROS-mediated increases of antioxidant enzymes change the redox levels of the cells so that
the AHR transcriptional machinery or the half-life AHR-regulated RNAs or proteins are prolonged. Accordingly, overexpression of antioxidant enzymes such as superoxide dismutase or catalse in mouse aortic endothelial cells (MAECs) has been shown to increase the induction of CYP1A1 mRNA and protein after benzo[a]pyrene treatment (Wang et al., 2009; Tang et al., 2010).

Since the AHR protein has a considerably short half-life, stabilization of the protein could be another explanation for super-induction of CYP1A1.

5.4 NADPH OXIDASE HAS A CENTRAL ROLE IN REGULATING CELL GROWTH AND ARYL HYDROCARBON RECEPTOR SIGNALING

5.4.1 NOXs stimulate cell growth

NOXs can be activated by metals as documented in our studies (Paper III) and with other stimuli (Chou et al., 2004; Rockwell et al., 2004; Cuypers et al., 2010; Furieri et al., 2011). Superoxide anion or \( \text{H}_2\text{O}_2 \) produced by NOX enzymes are known to play a crucial role in stimulation of cell proliferation (Sturrock et al., 2006; Chan et al., 2009). We observed that administration of a NOX inhibitor or using a mutant X-CGD cell line lacking a functional gp91\(^{phox}\) inhibited the stimulation of cell growth that was caused by exposure to low levels of metals. This clearly showed that NOX-derived superoxide anion or \( \text{H}_2\text{O}_2 \) was responsible for the growth stimulation by As and the other metals. Stimulation of cell growth at low concentrations and inhibition at high concentrations of metals as observed in this study has also been reported in other studies with inorganic chemicals (Calabrese and Baldwin, 2003).

5.4.2 NOXs have biphasic effects on AHR signaling pathway

The mechanism of down-regulation of CYP1A1 by oxidants is not well known. Another approach of this study was therefore to evaluate the interference of NOX activation on the AHR signaling pathway. It has been reported that several NOX activators such as \( \text{H}_2\text{O}_2 \), oxidized low density lipoproteins (oxLDL), TGF\( \alpha \), and \( \beta \), IL-1\( \alpha \) and \( \beta \), EGF, TNF\( \alpha \) and INF\( \gamma \) are repressors of CYP1A1 (Hohne et al., 1990; Ballou et al., 1992; Abdel-Razzak et al., 1993; Barker et al., 1994; Muntane-Relat et al., 1995; Muller et al., 2000; Thum and Borlak, 2004; Hollingshead et al., 2008). All metals tested in this study were able to activate NOXs and inhibit the activity of CYP1A1 (Paper III). The inhibitory effects of metals on FICZ-stimulated AHR activation were transient. We found that the temporal inhibition of CYP1A1 was attenuated by siRNA against NOX4 or administration of DPI a pharmacological inhibitor of NOXs, suggesting an important role for NOX-derived ROS in down-regulation of CYP1A1.

Temporal inhibition of CYP1A1 was followed by a prolonged and sustained induction of CYP1A1 at later incubation times. This late induction of CYP1A1 was abolished by silencing of the Nrf2 pathway (Paper III) indicating that Nrf2 gives up-regulation of antioxidant genes and increasing the level of GSH and thereby increasing the efficacy of the AHR as was seen in the cases of pre-conditioning with metals and \( \text{H}_2\text{O}_2 \). NOX-derived ROS or \( \text{H}_2\text{O}_2 \) seems to be important signals for activation of the keap1/Nrf2 complex and up-regulation of antioxidants and phase II genes through the antioxidant response element (Chan et al., 2009; Brewer et al., 2011; Jiang et al., 2011; Schroder et
al., 2012). Taken together, oxidative stress can negatively and positively modulate the AHR signaling pathway. Therefore, FICZ/AHR/CYP1 auto-regulation is highly influenced by oxidative stress and this might have potentially harmful consequences.
6 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The inhibition of metabolic turnover of the endogenous ligand FICZ was found to be a mechanism for activation of AHR signaling by several oxidants, metals and phytochemicals. This finding explains how compounds can activate AHR indirectly. In many studies the AHR-activating capacity of natural and synthetic molecules has been established in the presence of the high affinity AHR ligand FICZ (commercial media which contain FICZ) and it can be assumed that the background levels of FICZ have contributed to the results of such experiments.

We found that redox-active compounds regulate the catalytic turnover of the endogenous AHR activator FICZ by inhibiting and inducing AHR-mediated transcription of CYP1A1 and the catalytic efficiency of the CYP1A1 protein. We can conclude that the FICZ/AHR/CYP1 auto-regulation is highly influenced by changes in the redox status of cells. The current in vitro studies thus can explain why many oxidants behave both as AHR antagonists and agonists. To further confirm these findings, additional in vivo experiments and mechanistic studies are needed.

We also found that the induction of AHR target genes such as CYP1A1 by the AHR endogenous ligand FICZ is transient and suggest that prolonged induction of AHR activity by CYP1A1 inhibitors might have potentially harmful consequences and lead to developmental and physiological disorders.

Most often, humans are exposed to a mixture of toxic chemicals. Co-exposure to synthetic chemicals and heavy metals may enhance the risk of cancer as well as non-cancerous diseases in human. Due to the persistence of heavy metals, PAHs and other AHR activators in the environment, drinking water and food supplies interaction of these compounds with the endogenously activated AHR signaling pathway can be of considerable importance.
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