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Dynamic Regulation of Aryl Hydrocarbon Receptor Function and Activity by Different Stimuli

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“What we know is a drop, what we don´t know an ocean.”
Isaac Newton

ABSTRACT

Cells have to sense and adapt to changes in their surrounding environment. Most of the cellular responses to environmental and developmental stimuli are mediated by PAS (Per-ARNT-Sim) proteins. A member of this family of proteins is the aryl hydrocarbon receptor (AhR), a ligand-activated bHLH transcription factor and E3 ubiquitin ligase. Many xenobiotics, in particular the highly toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), bind to and activate the AhR giving this receptor a critical role in mediating xenobiotic-induced toxicity and carcinogenesis. Developmental defects in AhR-/- mice, the strong evolutionary conservation of the AhR and its ubiquitous expression imply a role of this receptor beyond xenobiotic-induced toxicity and xenobiotic metabolism. The physiological function of the AhR has long remained nebulous, but more and more experimental evidence emerges for a role of the AhR in proliferation and differentiation processes and in the maintenance of cellular homeostasis.

AhR signaling has mainly been studied upon activation by TCDD, an experimental condition that certainly does not mimic the physiological situation. The photoproduct 6-formylindolo[3,2-*b*]carbazole which is formed upon irradiation of tryptophan with UVB or visible light, has been suggested as a physiological AhR ligand. As shown in paper I, FICZ is both a high affinity ligand for the AhR and a good substrate for the cytochromes P450 (CYP) family of enzymes and induces its own metabolism suggesting a regulatory circuit between the AhR and CYP enzymes. The identification of FICZ metabolites in human urine indicates the presence of this ligand or its metabolites in biological systems and underlines its physiological relevance.

The regulation of AhR function and activity in a normal, xenobiotic-free context was analyzed in paper II using indolocarbazoles as physiological ligands. Due to their rapid metabolism, these ligands evoke a very transient activation of the transcription factor and ubiquitin ligase function of the AhR. On the contrary, AhR stimulation by TCDD results in a sustained transcriptional activation and a persistent down-regulation of AhR and estrogen receptor levels, thereby impairing the receptors responsiveness to new stimulation. This mode of action of TCDD and presumably other non-metabolizable xenobiotics may contribute to their toxicity, whereas dynamic AhR regulation seems to be a prerequisite for normal physiological cell signaling.

The regulation of the AhR when activated by UVB was studied in paper III. Upon UVB irradiation, AhR activity and CYP1A1 gene expression were found to be modulated by nuclear factor κB, probably as part of the cellular stress response to UV.

Exposure to UVB and xenobiotics stimulate the formation of protective melanin polymers in melanocytes. As shown in paper IV, these cells enhance both their activity of tyrosinase, the key enzyme for melanin biosynthesis, and their intracellular melanin content upon AhR activation. Melanogenesis was shown to be induced by the AhR through the transcriptional up-regulation of melanogenic genes, without effecting melanocyte proliferation.

The results of this study contribute to our knowledge of dioxin toxicity, but first and foremost extend our mechanistic understanding of the regulation and functioning of AhR signaling under physiological conditions. Moreover, a novel function of the AhR in the skin was identified.

LIST OF PUBLICATIONS

I. Wincent E, Amini N, **Luecke S**, Glatt H, Bergman J, Crescenzi C, Rannug A and Rannug U (2009). The Suggested Physiologic Aryl Hydrocarbon Receptor Activator and Cytochrome P4501 Substrate 6-Formylindolo[3,2-*b*]carbazole Is Present in Humans.
J Biol Chem, 284(5), 2690-2696

II. **Luecke S**, Gradin K, Zheng X, Backlund M, Lindebro M, Poellinger L and Rannug A. Dynamic Regulation of the Transcription Factor and Ubiquitin Ligase Functions of the Aryl Hydrocarbon Receptor by Physiological Ligands.
Manuscript

III. **Luecke S,** Wincent E, Backlund M, Rannug U and Rannug A (2010). Cytochrome P450 1A1 Gene Regulation by UVB Involves Crosstalk between the Aryl Hydrocarbon Receptor and Nuclear Factor κB.
Chemico-Biologcial Interactions, in press

IV. **Luecke S,** Backlund M, Jux B, Esser C, Krutmann J, Rannug A. The Aryl Hydrocarbon Receptor (AhR), a Novel Regulator of Human Melanogenesis.
Mansucript

CONTENTS

LIST OF ABBREVIATIONS

AhR	aryl hydrocarbon receptor; DR, dioxin receptor
AhR-CA	constitutive active AhR
AhR -/-	AhR-deficient
AhRR	aryl hydrocarbon receptor repressor
ARNT	aryl hydrocarbon receptor nuclear translocator
α-NF	α-naphthoflavone
bHLH	basic helix-loop-helix
CYP1A1	cytochrome P450 1A1
CYP1B1	cytochrome P450 1B1
E_2	17-β estradiol
ER	estrogen receptors α and β
ERE	estrogen receptor response element
FICZ	6-formylindolo[3,2-*b*]carbazole
hAhR	human AhR
HAHs	halogenated aromatic hydrocarbons
HSP90	heat shock protein 90kDa
ICZ	indolo[3,2-*b*]carbazole
LBD	ligand binding domain
mAhR	murine AhR
MCR-1	melanocortin receptor 1
MITF	microphthalmia-associated transcription factor
MNF	3´-methoxy-4´-nitroflavone
α-MSH	α-melanocyte stimulating hormone
NES	nuclear export signal
NFκB	nuclear factor kappa light polypeptide gene enhancer in B cells
NLS	nuclear localization signal
PAHs	polycyclic aromatic hydrocarbons
PAS	Per-ARNT-Sim
PCBs	polychlorinated biphenyls
PKC	protein kinase C
Pmel17	mouse silver locus protein homolog; gp 100
Rb	retinoblastoma protein
ROS	reactive oxygen species
TAD	transactivation domain
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TYR	tyrosinase; monophenol monooxygenase; EC 1.14.18.1
TYRP1	tyrosinase-related protein 1; gp75; EC 1.14.18.
TYRP2	tyrosinase-related protein 2; DCT, dopachrome tautomerase; EC 5.3.3.12
UV	ultraviolet radiation (200-400nm)
XAP	hepatitis B virus X-associated protein 2; ARA 9, AhR associated protein 9; AIP, Ah receptor-interacting protein
XRE	xenobiotic response element; DRE, dioxin response element; AhRE, AhR response element

1 INTRODUCTION

1.1 GENERAL INTRODUCTION

Cells have to cope and adapt themselves to a variety of environmental conditions. In order to respond to stimuli of the changing environment, cells possess extracellular and intracellular receptors which sense and transduce the received signals into changes in gene expression.

One of these receptors is the aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor and ubiquitin ligase of the Per-ARNT-Sim family of proteins that is ubiquitously expressed in all cells. The AhR was originally discovered as a binding site for planar, non-halogenated and halogenated xenobiotics, in particular the highly toxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Nearly all toxic and carcinogenic effects of these anthropogenically produced compounds are mediated by the AhR. With the identification of naturally-occurring compounds as AhR ligands, the sole role of this receptor as a sensor for xenobiotics and a mediator of toxicity and carcinogenesis were questioned. AhR function and activity have mainly been studied upon activation by xenobiotics, an experimental condition that certainly does not mimic the physiological situation. Studies on AhR-deficient mice, however, revealed that AhR signaling is essential for normal physiological cell signaling. In a number of different tissues, including the immune system, the gastrointestinal tract, the reproductive organs, the liver and the skin, the AhR regulates proliferation and differentiation processes thereby maintaining homeostasis. As a transcription factor and ubiquitin ligase, the AhR is able to modulate the expression levels of proteins involved in mediating important normal cellular functions. Being such an important modulator of intracellular protein levels, the activity of the AhR itself has to be tightly regulated. Numerous molecular mechanisms exist to induce and restrict AhR signaling in response to changing environmental conditions. Now, it becomes more and more evident that the disturbance and dys-regulation of normal AhR activity and responsiveness account for the pleiotropic toxic effects observed after exposure to xenobiotics. It therefore seems that the AhR can rather be considered as a "sensor" of developmental and environmental stimuli than exogenous signals in the form of xenobiotics.

1.2 PER-ARNT-SIM (PAS) PROTEINS

Many transcription factors that are involved in the modulation of gene expression in response to developmental and environmental stimuli belong to the superfamily of Per-ARNT-Sim (PAS) proteins. The name PAS originates from the first letters of the proteins Per, ARNT and Sim. Per, the Period protein, regulates circadian rhythmicity in *Drosophila.* The aryl hydrocarbon receptor nuclear translocator, abbreviated ARNT, was identified as a dimerization partner for both the AhR and the hypoxia-inducible factor (HIF-1α), a PAS protein regulating cellular responses to low oxygen. The single-minded protein (Sim) fulfills important functions in the control of midline cell development in *Drosophila*. In general, PAS proteins can be divided into (i) sensor proteins such as AhR and HIF and (ii) partner proteins such as ARNT and Bmal (reviewed in Crews and Fan, 1999; Gu et al., 2000).

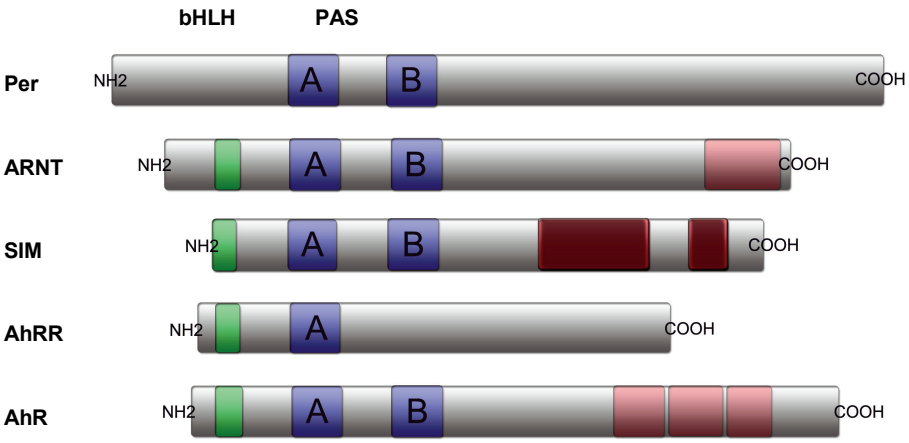

Figure 1. Members of the PAS superfamily of proteins. The basic helix-loop-helix (bHLH) domain is depicted in green, the PAS domain with its two repeats, PAS A and B, in blue. The C-terminal region of these proteins is variable containing often domains for transactivation (light red) or transrepression (dark red).

PAS proteins share a common structural organization (Figure 1) (for review see Kewley et al., 2004). The PAS domain, the hallmark of these proteins, consists of a region of 230-300 amino acids with two adjacent, highly degenerated repeats, called PAS A and PAS B. The PAS domain allows interactions with PAS partner proteins and molecular chaperones. In spite of its relative low amino acid sequence homology, this region is structurally highly conserved. PAS-like proteins, involved in light sensing and oxygen regulation, have also been identified in prokaryotes (Taylor and Zhulin, 1999). At their N-terminal end most PAS proteins possess a basic helix-loop-helix (bHLH) motif which mediates binding to cognate response elements in the major groove of the DNA and interactions with other bHLH proteins. The C-terminal region of these proteins often contains a transactivation or transrepression domain and is rather variable in its amino acid sequence composition.

1.3 THE ARYL HYDROCARBON RECEPTOR (AhR)

1.3.1 Structure of the AhR

Based on its structure, the AhR is classified as a bHLH-PAS protein (Figure 2). Among all bHLH-PAS proteins that have been characterized with regard to their ability to bind ligands, so far only the vertebrate AhR has been shown to bind ligands (reviewed in Furness et al., 2007).

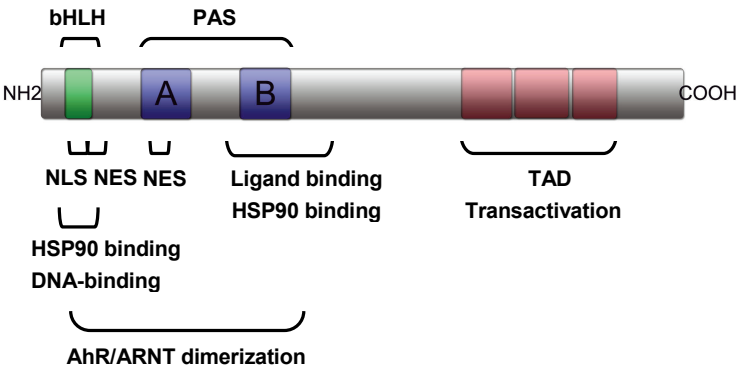

Figure 2. Schematic representation of the structural and functional organization of the AhR. bHLH, basic helix-loop-helix motif; NLS, nuclear localization signal; NES, nuclear export signal; PAS, Per-ARNT-Sim domain; TAD: transactivation domain with subdomains.

1.3.1.1 The bHLH motif and the nuclear localization and nuclear export signals of the N-terminus

The basic region of the N-terminal bHLH motif of the AhR mediates sequence-specific binding to gene regulatory elements, called xenobiotic response elements (XREs), whereas the two α-helices of this region, together with the PAS domain, promote dimerization with the partner bHLH-PAS-protein ARNT (Fukunaga and Hankinson, 1996). Heterodimer formation with ARNT is a prerequisite for DNA-binding. Activated AhR fails to bind to DNA in the absence of ARNT, indicating that the AhR is not able to interact as a homodimer with its response elements (Probst et al., 1993; Whitelaw et al., 1993a). Even though the bHLH motif clearly functions as the DNA-binding domain of the AhR (Dong et al., 1996; Swanson and Jun-hua, 1996), other amino acids outsides this region may as well contribute to interactions with the DNA (Sun et al., 1997).

In addition to the bHLH motif, the N-terminus also harbors a nuclear localization signal (NLS) and nuclear export signal (NES), two sequences commonly found in proteins shuttling between the cytoplasm and the nucleus. Both sequences, the minimum bipartite NLS and the leucine-rich NES, lie in the region of the bHLH motif partly overlapping with the basic region respectively the second helix of this motif (Ikuta et al., 1998). A second NES, which seems to promote mainly nuclear export of unliganded receptor, has been identified in the PAS A repeat (Berg and Pongratz, 2001). The AhR undergoes dynamic nucleocytoplasmic shuttling, but is mostly cytoplasmic in the absence of ligand (Ikuta et al., 2000; Richter et al., 2001). The inactive receptor seems to be retained in the cytoplasm due to masking of its NLS by chaperone binding and rapid active nuclear export (Antonsson et al., 1995; Ikuta et al., 1998; Whitelaw et al., 1993b). Translocation of the AhR into the nucleus is essential for functional AhR signaling, because mice bearing a receptor variant with a mutated NLS show no signs of toxicity after treatment with TCDD (Bunger et al., 2003). AhR translocation is mediated by the nuclear pore complex proteins importin α and β, which

recognize the dephosphorylated NLS and allow nuclear import (Ikuta et al., 2004b). Nuclear export of the receptor occurs through interactions between the chromosome region maintenance 1 (CRM1) export receptors and the leucine residues of the NES (Ikuta et al., 2000). Nuclear export is required for the restriction of AhR activity by degradation via the 26S proteasomal pathway (Davarinos and Pollenz, 1999; Pollenz and Barbour, 2000).

Thus, it seems that the balance between nuclear import and export determines the intracellular localization of inactive and activated AhR. As the molecular mechanisms underlying this regulation, masking and unmasking as well as phosphorylation and dephosphorylation of the two signal sequences have been identified (Ikuta et al., 1998; Ikuta et al., 2004a, b; Ikuta et al., 2000).

1.3.1.2 The PAS domain – heterodimer formation, chaperone and ligand binding

Deletion analyses revealed that the PAS domain is involved in (i) AhR/ARNT heterodimer formation, (ii) chaperone binding and (iii) ligand recognition (Dolwick et al., 1993b; Fukunaga and Hankinson, 1996). By providing a secondary dimerization surface in addition to the bHLH region, the PAS domain mediates dimerization with ARNT. Within this domain, the PAS A and PAS B repeats fulfill different functions. In particular, the PAS A repeat seems to stabilize the dimer formation (Chapman-Smith et al., 2004; Lindebro et al., 1995), whereas the PAS B repeat mediates binding of the molecular chaperone heat shock protein 90 (HSP90) to the AhR. An additional HSP90 binding site in the region of the bHLH motif suggests that HSP90 spans over the N-terminal part of the AhR from the PAS B repeat to the bHLH motif (Fukunaga and Hankinson, 1996; Perdew and Bradfield, 1996). Both PAS repeats play an important role in mediating the transformation of activated AhR into its DNA-binding form by promoting heterodimerization and replacement of HSP90 (Soshilov and Denison, 2008).

In addition to the HSP90 binding site, the PAS B repeat covers also the ligand-binding domain (LBD) of the AhR. Even though the structural and functional domains of the AhR have been well characterized, an NMR or X-ray structure of the receptor has not yet been solved. By using crystal structures of other PAS proteins (FixL, ARNT, HIF) as templates for homology modeling, the LBD of the mouse AhR was predicted as a 5-stranded antiparallel β-sheet with a central helix coupled to three small helices (Pandini et al., 2007; Procopio et al., 2002). Based on the 3D-structure, a model proposes that ligand molecules like TCDD may enter the cavity by displacing the central helix. Detailed site-directed mutagenesis, using TCDD as a model ligand, identified numerous amino acid residues within the ligand-binding pocket that essentially facilitate the entrance and optimal binding of ligand molecules (Pandini et al., 2009). For example, alanine 375 has been defined as a key residue for ligand recognition in the LBD of the mouse AhR (mAhR) (Murray et al., 2005). Replacement of this residue by amino acids with bulky side chains e.g. valine sterically hinders ligand binding and leads to profound inter- and intraspecies-specific differences in ligand binding affinities (Ema et al., 1994; Moriguchi et al., 2003). The human AhR (hAhR), which contains a valine at position 381 (equal to position 375 in mAhR), shows a 10-fold lower affinity to TCDD than its murine counterpart. Similar to humans, DBA/2J mice possess a receptor variant with a valine residue (at position

375). These mice are nearly resistant to the toxic effects of TCDD, whereas C57BL6 mice, containing a receptor with an alanine residue, are very sensitive (Pohjanvirta and Tuomisto, 1994). Binding of molecules other than TCDD, low affinity agonists or antagonists, however, seems to be mediated by other amino acids in the ligand-binding pocket, suggesting that binding of structurally different molecules is promoted by specific subsets of residues (Backlund and Ingelman-Sundberg, 2004; Goryo et al., 2007; Henry and Gasiewicz, 2008). Partial removal of the LBD renders the receptor into a state in which it has lost its capacity to bind both ligands and HSP90 and becomes constitutively active with regard to its transcriptional activation properties and ubiquitin ligase function (AhR-CA) (Andersson et al., 2002; McGuire et al., 2001; Ohtake et al., 2008).

1.3.1.3 The C-terminal transactivation domain (TAD)

Both the AhR and its partner protein ARNT contain a transactivation domain (TAD) in their C-terminal region (Jain et al., 1994). TADs mediate transcriptional initiation by recruiting general transcription factors and co-regulators to the transcriptional site. Detailed analysis revealed that the TAD of the AhR consists of three subdomains. Each of these subdomains shows a rather low transactivation potential itself, but is able to act in synergy with the others. The TAD of the hAhR, for instance, consists of an acidic-, glutamine (Q)-, and proline/serine/threonine (PST)-rich subdomain, of which the Q-rich domain is absolutely essential for transactivation activity (Kumar et al., 2001; Rowlands et al., 1996) .

Another possible function of the C-terminal region including the TAD was revealed by degradation studies using AhR mutant variants. Receptor variants truncated at the carboxyl-end and TAD were improperly degraded upon ligand activation, suggesting that the TAD may also serve as a degron, a structural motif important for proteasomal degradation of proteins (Ma and Baldwin, 2000).

1.3.2 The dual function of the AhR

The AhR has been shown to possess two intrinsic functions (Figure 3). On the one hand, the receptor serves as a ligand-activated transcription factor regulating the expression of genes containing XREs in their 5´-flanking regions (see a review in Beischlag et al., 2008). On the other hand, the AhR functions as a ligand-dependent ubiquitin E3 ligase targeting itself and other substrate proteins for proteasomal degradation (reviewed by Ohtake et al., 2009). So far, this ubiquitin ligase function seems to be unique for the AhR among all sequence-specific transcription factors.

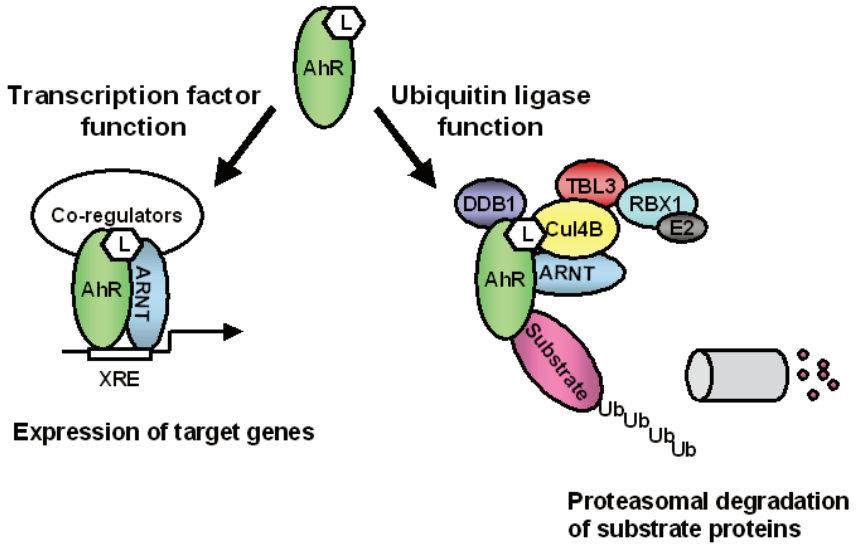

Figure 3. The dual function of the AhR. The AhR is both a ligand-activated transcription factor and a ligand-dependent ubiquitin E3 ligase targeting itself and substrate proteins such as steroid receptors and β-catenin for proteasomal degradation (modified after Ohtake et al., 2009).

1.3.2.1 The AhR as a transcriptional regulator - translocation, XRE-binding and transcriptional activation of target genes

The molecular events, following AhR activation by ligands that lead to the transcriptional up-regulation of AhR target genes, are fairly well understood (Figure 4). In its latent form, the AhR resides in the cytoplasm bound to a complex of molecular chaperones (reviewed in Petrulis and Perdew, 2002) consisting of two molecules of HSP90 (Chen and Perdew, 1994; Perdew, 1988), the immunophilin hepatitis B virus X-associated protein 2 (XAP) (Carver and Bradfield, 1997; Ma and Whitlock, 1997; Meyer et al., 1998) and p23 (Kazlauskas et al., 1999). The two molecules of HSP90 assist the proper folding of the receptor and stabilize it in the cytoplasm in a conformation capable to bind ligands. Disruption of the HSP90-AhR complex impairs the receptor to bind ligands (Pongratz et al., 1992) and targets it to proteasomal degradation (Chen et al., 1997; Pollenz and Buggy, 2006; Song and Pollenz, 2002). The removal of unliganded, improper folded receptor seems not to require DNA-binding prior to degradation and employs mechanisms different from the proteolysis of ligand-activated receptor (Pollenz et al., 2005). Overall, it appears that HSP90 plays an essential role in holding the receptor in a functional, ligand-responsive state. The immunophilin XAP, also termed ARA9 (AhR associated protein 9) or AIP (Ah receptor-interacting protein), has also been implicated in the stabilization of the cytoplasmic AhR-HSP90 complex by interacting via its tetraticopeptide repeats with HSP90 and the AhR (Bell and Poland, 2000; Carver et al., 1998). However, its exact molecular role in AhR signaling remains unclear (Pollenz and Dougherty, 2005; Pollenz et al., 2006). While in mouse cells XAP modulates the localization of mAhR partly by disrupting nuclear translocation via importin β (Petrulis et al., 2003) and

hindering degradation (Kazlauskas et al., 2000), the subcellular localization of the human receptor appears not to be significantly affected by XAP (Ramadoss et al., 2004), indicating differences in XAP-AhR interactions. XAP mediates also interactions between the AhR and EBNA-3, an Epstein-Barr virus-encoded protein implied in the regulation of lymphocyte proliferation and transformation (Kashuba et al., 2006). Nevertheless, XAP seems to be important for the cytoplasmic stability and distribution of the unliganded, inactive receptor. Bound to the ATP-binding domain of HSP90, p23 has been implicated in the stabilization of the liganded complex during translocation to the nucleus (Kazlauskas et al., 1999) and in the transformation of the complex to its AhR/ARNT-DNA-bound form (Shetty et al., 2003). However, the exact role of p23 in AhR signaling is questioned, since p23- heterozygous mice and p23-deficient embryos showed no direct signs of disrupted signaling (Flaveny et al., 2009).

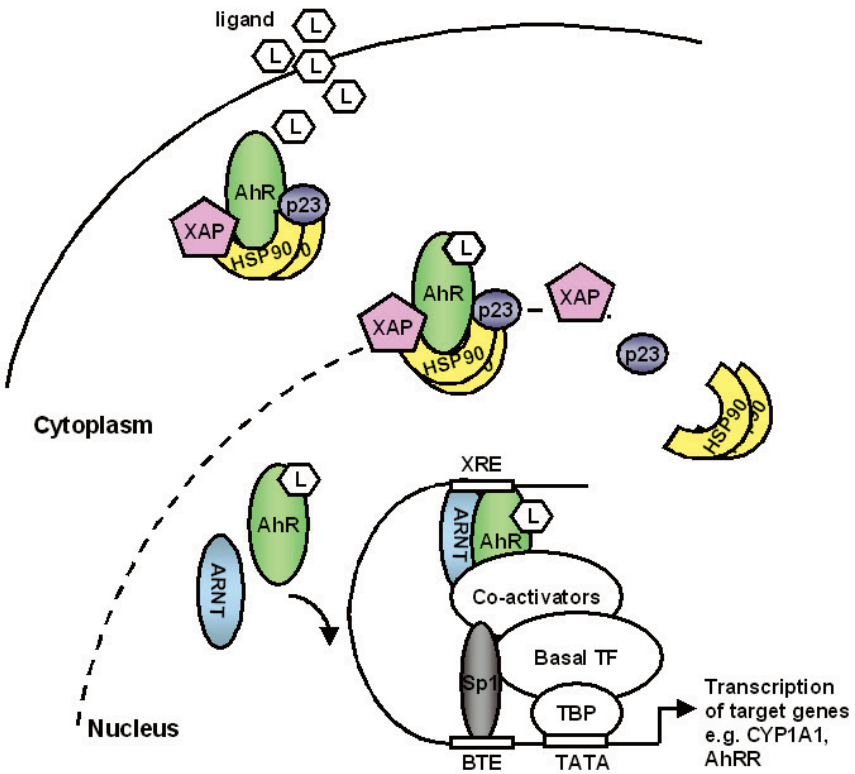

Figure 4. The molecular mechanisms underlying the activation of the transcription factor function of the AhR by ligands. In its latent form, the AhR resides in the cytoplasm bound to a complex of molecular chaperones (HSP90, XAP and p23). Upon ligand binding, the AhR translocates into the nucleus and heterodimerizes with its partner protein ARNT. The AhR-ARNT dimer binds to xenobiotic response elements (XREs) in the promoter region of target genes and regulates their transcription. BTE, basal transcription element; TF, transcription factors; TBP, TATA-binding protein.

Upon ligand binding, the complex translocates to the nucleus. Concomitantly with the release of the HSP90 molecules, the AhR can undergo dimerization with ARNT (Heid et al., 2000). Studies on ARNT-deficient cells (murine hepatoma cells Hepa-1 c4) revealed that ARNT is absolutely required for the formation of the DNA-bound, transcriptional active dimer (Probst et al., 1993; Whitelaw et al., 1993a). Once the AhR-ARNT heterodimer has been formed (Reyes et al., 1992), it binds to its cognate response elements (XREs) (core nucleotide sequence 5´-TNGCGTG-3´) in the 5´-flanking region of target genes (reviewed in Swanson, 2002). The AhR binds to the TNGC half-site of the core sequence, whereas ARNT interacts with the typical GTG site, to which many bHLH transcription factors bind (Bacsi et al., 1995). The identification of extended XREs suggests that additional nucleotides outside the core sequence contribute to dimer-XRE interactions.

Following binding of the AhR-ARNT dimer, one model proposes the bending of the DNA into a configuration that allows contact between the dimer at the enhancer and the basal transcriptional machinery at the promoter (Tian et al., 2003). Via the subdomains of its TAD, the AhR interacts with basal transcription factors (Rowlands et al., 1996) and concomitantly recruits co-factors (reviewed in Hankinson, 2005). As shown for the well-established AhR target gene CYP1A1, this gene is silenced by nucleosomes but becomes active after nucleosome repositioning following AhR activation by TCDD (Whitlock, 1999). Thus, interactions between the AhR-ARNT dimer and histone acetylases and components of the chromatin remodeling machinery may also take place at these sites and histone modifications and chromatin remodeling, including repositioning of nucleosomes, are prerequisites for the initiation and facilitation of transcription.

Among the hundreds of AhR target genes identified are e.g. genes encoding phase I metabolizing enzymes e.g. CYP1A1, CYP1A1, CYP1B1 (see a review in Fujii-Kuriyama and Mimura, 2005), phase II metabolizing enzymes e.g. UDP-glucuronosyl transferase, glutathione S-transferase Ya and NADP(H)oxidoreductase, the repressor of AhR function, AhRR, and genes involved in cell cycle regulation and cell differentiation.

1.3.2.2 The ubiquitin ligase activity of the AhR

Besides functioning as a transcriptional regulator, the AhR acts also as an ubiquitin E3 ligase. In general, substrate-specific E3 ligases associate with their substrate proteins thereby enabling E2 ubiquitin conjugating enzymes to tag the substrate with ubiquitin for degradation via the ubiquitin 26S proteasomal pathway (Schwartz and Ciechanover, 2009). Unlike typical $CUL4B^{DCFA}$ E3 ligases which contain DDB1- and CUL4-associated factors (DCFA) as substrate-specific adaptors, the $CUL4B^{AhR}$ complex uses activated AhR for substrate recognition. The $CUL4B^{AhR}$ complex consists of the scaffold protein CUL4B, the β-transducin-like protein TBL3, the ring finger protein RBX1, subunits of the proteasomal 19S particle, DDB1, ARNT and activated AhR and is only assembled and functional in the context of activated AhR (Ohtake et al., 2007). So far, the only substrate proteins identified for this atypical ligase complex are β-catenin (Kawajiri et al., 2009) and the steroid receptors, estrogen receptors (ERα, ERβ) and androgen receptor (AR) (Ohtake et al., 2007). Down-regulation of ERα protein levels was noted in cells treated with TCDD in the absence of estrogen (E_2) (Wormke et al., 2000; Wormke et al., 2003). Similar to the ERα, AhR levels are also reduced

upon TCDD treatment (reviewed by Pollenz, 2002) indicating that ligand-induced degradation of the receptor is partly mediated by its own ubiquitin ligase function (Kawajiri et al., 2009; Ohtake et al., 2007). Self-ubiquitination has also been observed for other substrate adaptors of E3 ligase complexes.

Taken together, the AhR functions both as a ligand-activated transcription factor and ubiquitin ligase. Equipped with these two functions, the AhR is able to modulate the expression, function and activity of other proteins in response to exogenous and endogenous stimuli.

1.4 REGULATION OF AhR SIGNALING

Due to its essential role in the modulation of intracellular protein levels, the AhR itself has to be tightly regulated. Several, complex mechanisms have been identified in the regulation of this signaling pathway (Figure 5) (reviewed in Fujii-Kuriyama and Kawajiri, 2010; Harper et al., 2006).

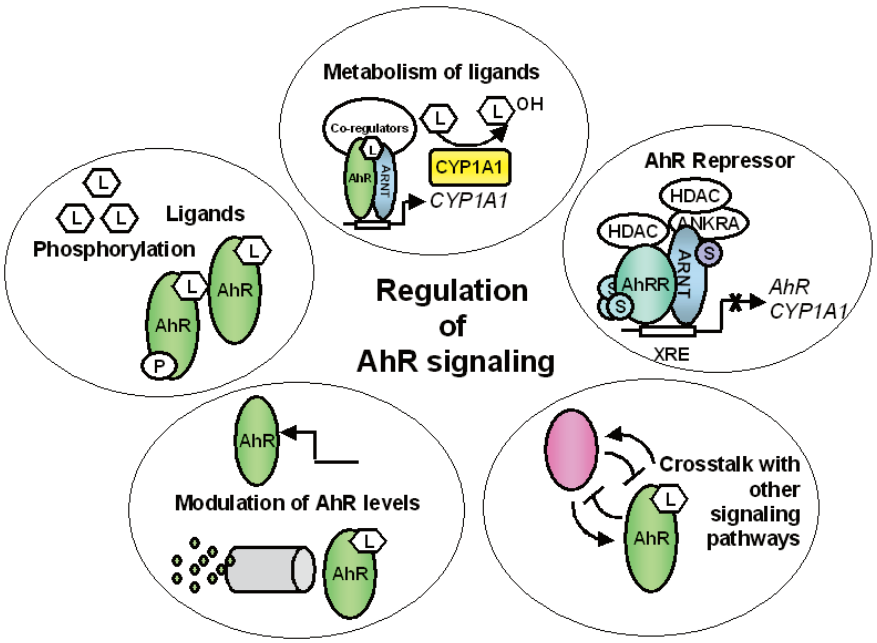

Figure 5. Schematic overview over the mechanisms regulating AhR signaling. (I) AhR activation by ligands and modulation of the activity of ligand-activated AhR by phosphorylation. (II) Ligand-induced expression of the CYP1 enzymes results in the metabolic clearance of activating ligands. (III) Activated AhR induces the expression of the AhR repressor (AhRR). The AhRR suppresses, among others, the receptors own expression by interactions with histone deacetylases (HDACs). (IV) The AhR can be positively or negatively regulated by crosstalk with other signaling pathways. (V) Regulation of AhR activity by modulation of receptor expression levels. AhR activity is restricted by ligand-induced degradation.

1.4.1 Binding of ligands

The AhR can be activated by various, structurally diverse compounds. A typical AhR ligand is hydrophobic and planar in its structure and enters the cell by diffusion across the cell membrane. The dioxin molecule complies with all these features, making this compound a prototypical high affinity AhR ligand. Molecules of the size of TCDD easily fit in the ligand-binding pocket of the AhR, which was estimated to be approximately 14Å*12Å*5Å, whereas ligands with larger size may not be accommodated in the pocket. With the continuously identification of novel ligands, however, there is more and more evidence that the ligand-binding pocket of the AhR is more promiscuous with regard to ligand size and structure than previously predicted. A promiscuous ligand-binding pocket would allow the sensing of a broader spectrum of compounds making it thereby possible for the receptor to respond to a greater variety of different environmental stimuli (reviewed in Denison et al., 2002).

Box 1. AhR activators

Xenobiotic compounds	**Naturally-occurring compounds**
Halogenated aromatic hydrocarbons 2,3,7,8-tetrachlorodibenzo-*p*-dioxin 2,3,7,8-tetrachlorodibenzofuran 2,3,6,7-tetrachloronapthalene 3,3´,4,4´-tetrachloroazoxybenzene 3, 3´,4,4´,5-pentachlorobiphenyl Polycyclic aromatic hydrocarbons benzo[a]pyrene β-napthoflavone (β-NF) 3-methylcholantrene	Arachidonic acid metabolites lipoxin 4A, prostaglandins B_3,D_3,F_{3a},G_2, H_1,H_2 Flavonoids: curcumin Carotenoids: canthaxanthin, astaxanthin, quercetin Indoles indigo, indirubin 6-formylindolo[3,2-*b*]carbazole (FICZ) 6,12-diformylindolo[3,2-*b*]carbazole (dFICZ) indolo[3,2-*b*]carbazole (ICZ) 3,3´-diindolylmethane (DIM) tryptamine (TA), indole acetic acid (IAA), kynurenic acid 2-(1´H-indole-3´-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) Heme metabolites bilirubin, biliverdin, hemin Malassezin Equilenin
Other activators cAMP, oxLDL, omeprazole, primaquine, nicotine, pyridines, disruption of cell-cell contacts	

Among the ligands that have been identified to bind to the AhR are both xenobiotic and naturally-occurring compounds (see Box 1) (reviewed by Denison and Nagy, 2003; Nguyen and Bradfield, 2007). Developmental defects in AhR-/- mice (Fernandez-Salguero et al., 1995; Schmidt et al., 1996), the strong evolutionary conservation of the

receptor (reviewed in Hahn, 2002), the inability of ancestral AhR to bind xenobiotics and its activation in cells in the absence of ligand treatment (Chiaro et al., 2007; Ikuta et al., 2004a; Sadek and Allen-Hoffmann, 1994a) suggest the existence of endogenous ligands. Several of the naturally-occurring compounds have been proposed as endogenous ligands. However, for many of these compounds their formation and physiological role *in vivo* have to be confirmed. It is tempting to speculate that the promiscuity of the ligand-binding pocket of the AhR may be due to the existence of several endogenous ligands.

Xenobiotic compounds. Among potent AhR activators are the *halogenated aromatic hydrocarbons (HAHs)* and *polycyclic aromatic hydrocarbons (PAHs)*. These synthetic compounds are mainly anthropogenically produced during incomplete combustion, waste incineration, as flame retardants and insulators and as by-products in the manufacturing process of chemicals and pesticides. Many of these environmental contaminants are fairly resistant to microbiological and chemical degradation and have the ability to bio-accumulate in the food chain. They can activate the AhR in pM (HAHs) to µM (PAHs) concentrations and evoke multiple toxic responses in exposed organisms (reviewed in Poland and Knutson, 1982).

Naturally-occurring compounds. This group of ligands contains structurally diverse compounds from all different kinds of sources. Numerous compounds are derived from plant products e.g. flavonoids, indigoids and derivates of the indole-3-carbinole (I3C). *Flavonoids* and *carotenoids* and related compounds, which are taken up through consumption of tea, fruits, and vegetables, show low AhR-activating capacity. On the other hand, indirubin and indigo, two indigoids produced by cytochromes P450s from indoles, are rather good AhR activators (Adachi et al., 2001; Guengerich et al., 2004). Even though they have been identified in serum and human urine (Adachi et al., 2001), their formation *in vivo* has not yet been shown. I3C is derived from glucobrassicin, a tryptophan-based second plant metabolite, and is found in vegetables from the *Brassica* genus. I3C can be converted to the potent AhR ligand *indolo[3,2-b]carbazole (ICZ)* by the intestinal flora and the acidic environment of the stomach (Bjeldanes et al., 1991). A second indolocarbazole and high affinity ligand, activating the AhR at pM-nM concentrations, is *6-formylindolo[3,2-b]carbazole* (FICZ). FICZ is derived by photolysis of the amino acid tryptophan (Rannug et al., 1987; Rannug et al., 1995) and can be formed intracellular in UVB-irradiated cells (Fritsche et al., 2007). Other compounds derived from tryptophan are *indole acetic acid* (IAA) and *tryptamine*, both weak AhR activators (Heath-Pagliuso et al., 1998), and the more potent ligand *kynurenic aci*d (DiNatale et al., 2010). Furthermore, *bilirubin*, *biliverdin* and *hemin*, degradation products of the heme pathway, have been identified to activate the AhR at µM concentrations. Of these three compounds, bilirubin, which is derived from hemin and biliverdin, is the most potent one. However, it remains unclear whether these compounds are present in relevant concentrations under non-pathological conditions (Phelan et al., 1998; Sinal and Bend, 1997). Also, arachidonic acid metabolites, including *lipoxin A4* (Schaldach et al., 1999) and various *prostaglandins* (PGB_3, PGD_3, $PGF_{3\alpha}$, PGG_2, PGH_1, PGH_2) (Seidel et al., 2001) have been described as AhR activators. Whereas lipoxin A4 is quite potent at higher nM concentrations, µM concentration of prostaglandins are required for AhR activation. Prostaglandins are produced with the help of cyclooxygenase (Cox) and degraded by cytochrome P450, two enzymes under

the transcriptional control of the AhR. Another potent AhR ligand, *2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE)*, has been isolated from the lung tissue of the pig (Henry et al., 2006; Song et al., 2002).

Other AhR activators. In addition to the above described ligands, AhR activation has also been observed in cells in the absence of exogenous ligands in response to cyclic adenosine monophosphate (cAMP) treatment (Oesch-Bartlomowicz et al., 2005), oxidized LDL modified by fluid shear stress (McMillan and Bradfield, 2007) and disruption of cell-cell contacts (Cho et al., 2004; Sadek and Allen-Hoffmann, 1994a, b).

1.4.2 Depletion of ligands through ligand-induced expression of metabolizing enzymes

Ligand-activated AhR induces the expression of genes coding for the first family of cytochromes. High basal, non-inducible CYP1A1 mRNA levels have been observed in cells deficient in functional CYP1A1 (e.g. murine hepatoma cells Hepa-1 c37) (Chang and Puga, 1998; RayChaudhuri et al., 1990). This implies a regulatory circuit between CYP1A1 activity and the AhR pathway. The CYP1A1 enzyme metabolizes many of the naturally-occurring ligands and thereby clearly restricts their AhR-activating potential. The metabolic clearance of ligands through ligand-induced expression of metabolizing enzymes may therefore be an important autoregulatory feedback mechanism in the control of AhR signaling (Chiaro et al., 2007).

1.4.3 Post-translational modification – phosphorylation

The activity of many proteins is modulated by phosphorylation. Even the AhR has been identified as a phosphoprotein (Berghard et al., 1993; Carrier et al., 1992), yet the exact number and positions of its phosphoresidues remain controversial (Backlund and Ingelman-Sundberg, 2005; Mahon and Gasiewicz, 1995; Minsavage et al., 2004; Park et al., 2000). Mitogen-activated protein kinases (MAPK) (reviewed in Henklova et al., 2008), tyrosine kinases (Backlund and Ingelman-Sundberg, 2005; Park et al., 2000) and protein kinase C (PKC) (Berghard et al., 1993; Carrier et al., 1992; Chen and Tukey, 1996; Long et al., 1998; Machemer and Tukey, 2005) have been implicated in the phosphorylation of the AhR. Impaired AhR/ARNT dimer formation and reduced DNA binding has been observed in cell extracts from TCDD-stimulated cells when treated with phosphatase (Berghard et al., 1993; Pongratz et al., 1991). From these studies is seems that phosphorylation of the AhR promotes DNA-binding, whereas phosphorylation of ARNT is essential for AhR/ARNT interaction upon dimerization (Berghard et al., 1993). Phosphorylation and dephosphorylation have also partly been identified as the underlying mechanism controlling nucleocytoplasmic shuttling and the subcellular distribution of the AhR (Ikuta et al., 2004a, b). Moreover, it seems that phosphorylation at the C-terminus may contribute to proteasomal degradation of the receptor, because overexpression of MAPK reduces AhR protein levels (Chen et al., 2005).

As part of the cytoplasmic AhR-chaperone complex, the c-src kinase has been implicated in the phosphorylation of the AhR (Enan and Matsumura, 1996). Its release and the activation of the epidermal growth factor signaling pathway can been observed after receptor activation by UVB irradiation (Fritsche et al., 2007). Both c-src -/- mice

(Enan et al., 1998) and rat H4IIE cells treated with c-src inhibitors or transfected with dominant-negative c-src (Backlund and Ingelman-Sundberg, 2005) showed a reduced response to receptor activation by TCDD suggesting that c-src may also be involved in the regulation of AhR signaling.

1.4.4 Crosstalk with other signal transduction pathways

The AhR regulates and is regulated by many other signaling pathways (reviewed in Carlson and Perdew, 2002). Examples of crosstalk between the AhR and some signaling pathways are listed below.

Growth factor and cytokine signaling. Several growth factors and cytokines interact with the AhR pathway (see a review in Haarmann-Stemmann et al., 2009). AhR and transforming growth factor (TGF)-β, for example, mutually modulate their expression (reviewed in Gomez-Duran et al., 2009).

Steroid receptor signaling. Crosstalk between the AhR and steroid receptors has been shown. E_2-activated ERα clearly modulates transcription of AhR target genes and can be associated with the AhR on XREs (Beischlag and Perdew, 2005). On the contrary, ER signaling can be modified by the AhR through (i) activation of ER in absence of E_2 on ER target genes (Ohtake et al., 2003), (ii) competition for ARNT and other coactivators, (iii) inhibitory XREs in the promoter region of ER target genes, (iv) modulation of the expression of estrogen synthesizing enzymes (CYP19, aromatase) and (v) targeting ER to proteasomal degradation (reviewed in Swedenborg and Pongratz, 2010).

Nuclear factor κB signaling. TCDD-induced CYP1A1 expression can be suppressed by treatment with bacterial endotoxins (lipopolysaccharide) and proinflammatory cytokines (interleukin-1β; tumor necrosis factor (TNF)-α), well known NFκB activators (Ke et al., 2001; Tian et al., 1999). The NFκB subunit p65 (RelA) and AhR have been shown to directly interact with each other (Tian et al., 1999). In the case of CYP1A1, p65 suppresses AhR transcriptional activity through chromatin remodeling and competition for common coactivators (Ke et al., 2001). AhR and p65 interactions on an NFκB-site, however, can promote the expression of the c-myc gene (Kim et al., 2000) suggesting that the AhR and p65 mutually modulate their transactivation properties. In addition, the AhR and another member of the NFκB transcription factor family, RelB, can associate and promote the induction of both RelB- and XRE-controlled genes (chemokines, reporter genes) (Vogel et al., 2007) showing that intensive crosstalk between these two signaling pathways exists.

Retinoblastoma protein. Similar to NFκB, the AhR seems also to interact with the hypophosphorylated active form of the retinoblastoma protein (Rb), which regulates cell cycle progression from G_1 to S. The direct interaction between these two proteins, but not ARNT, is mediated by the Q-rich subdomain of the TAD and a LXCXE motif at the N-terminus of the AhR (Elferink et al., 2001; Ge and Elferink, 1998). Reduced CYP1A1 induction by LXCXE-mutated AhR after ligand activation (Elferink et al., 2001) suggests Rb as a coactivator for the AhR.

1.4.5 Repression of AhR signaling by the AhR repressor (AhRR)

Another bHLH-PAS protein, referred to as the AhR repressor (AhRR), is involved in the regulation of AhR signaling (for a review see Hahn et al., 2009). The AhRR resembles in its structural and functional organization the AhR, with the exception that it lacks the TAD and PAS B repeat (Kanno et al., 2007; Mimura et al., 1999). Thus, the AhRR is unable to bind ligands (Karchner et al., 2002). AhRR expression is transcriptionally controlled by the AhR through several XREs and an additional NFκB site (Baba et al., 2001) and AhR-/- mice have clearly reduced AhRR expression levels (Bernshausen et al., 2006).

The AhRR itself is not transcriptionally active but represses the transcriptional activity of the AhR. Human dermal fibroblasts, which are non-responsive to TCDD treatment (Gradin et al., 1999), show constantly bound AhRR at their XREs in the CYP1A1 promoter (Haarmann-Stemmann et al., 2007). The exact molecular mechanisms underlying the repression of AhR signaling by the AhRR are not fully elucidated. Initially it was proposed that the AhRR competes for ARNT heterodimer formation (Mimura et al., 1999). Due to the nearly unlimited access of ARNT (Pollenz et al., 1999; Tomita et al., 2000) and temporally similar CYP1A1 and AhRR expression patterns (Tsuchiya et al., 2003), this mode of action is questionable. Studies using mutant AhR variants with impaired XRE-binding capacities as well as unlimited amounts of ARNT clearly showed that the observed inhibition is not due to competition for XRE-binding or for ARNT (Evans et al., 2008). The repressive action of the AhRR seems rather to be mediated by interactions between the SUMOylated repressor and histone deacetylases (Haarmann-Stemmann et al., 2007; Oshima et al., 2009; Oshima et al., 2007). This autoregulatory loop between AhR and AhRR signaling is evolutionary well conserved (Karchner et al., 2002) suggesting it as an important negative regulatory mechanism.

1.4.6 Modulation of AhR levels

AhR expression levels may also be reflected in the levels of receptor activity. AhR expression can be induced by growth factors (Vaziri et al., 1996) and is in parts under the transcriptional control of the transcription factor NF-E2 p45-related factor 2 (NRF2), which acts via an antioxidant response element in the 5´flanking region of this gene (Shin et al., 2007).

An important mechanism to control AhR function and activity is the down-regulation of ligand-activated receptor. The inactive AhR is constantly distributed in the cytoplasm with a half-life of ~28 hours. However, upon activation, e.g. by ligands such as TCDD, the half-life of the receptor is shortened to ~ 3 hours (Ma and Baldwin, 2000) and receptor protein levels are rapidly reduced both *in vitro* and *in vivo* in a dose and time-dependent manner, whereas ARNT levels remain unaffected (Pollenz, 1996; Pollenz et al., 1998; Roman et al., 1998). Because receptor levels decrease in both subcellular compartments (Pollenz, 1996; Reick M et al., 1994) without changes in AhR mRNA levels (Giannone et al., 1998) upon ligand treatment, repression of mRNA synthesis and shuttling of the receptor can be excluded as the underlying mechanisms for the observed decline in receptor levels. These results rather suggest the proteolytic degradation of the liganded AhR. Activated AhR undergoes partly self-ubiquitination through its own E3 ligase function (Kawajiri et al., 2009; Ohtake et al., 2007) and is then degraded in the cytoplasm via the 26S proteasomal pathway following DNA-

binding and nuclear export (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000; Roberts and Whitelaw, 1999). As the degron, the structural motif at which ubiquitination occurs, the C-terminal region enclosing the TAD has been proposed (Ma and Baldwin, 2000). Down-regulation and cleavage of the receptor via the action of the Ca^{2+}-dependent cysteine protease calpain, as suggested by Dale and colleagues (Dale and Eltom, 2006), could not be confirmed by other studies (Pollenz, 2007), excluding this pathway as the underlying proteolytic mechanism. Inhibition of TCDD-induced AhR degradation by protein synthesis inhibitors, e.g. cycloheximide, or proteasomal inhibitors, e.g. MG132, results in a so called superinduction of CYP1A1 gene expression, an induction higher than the one achieved by agonist treatment alone (Joiakim et al., 2004; Ma and Baldwin, 2000; Ma et al., 2000). It therefore seems that the control of AhR levels through degradation is an extremely important and universal mechanism for the restriction of receptor activity and the regulation of AhR signaling, occurring in all cells irrespectively of tissue and species origin.

1.5 THE ROLE OF THE AhR IN PHYSIOLOGICAL PROCESSES

The AhR has long been viewed as a receptor that mainly binds xenobiotics and mediates both their metabolism and toxicity. However, more and more evidence arises that the AhR plays pivotal roles in maintaining cellular homeostasis (Figure 6).

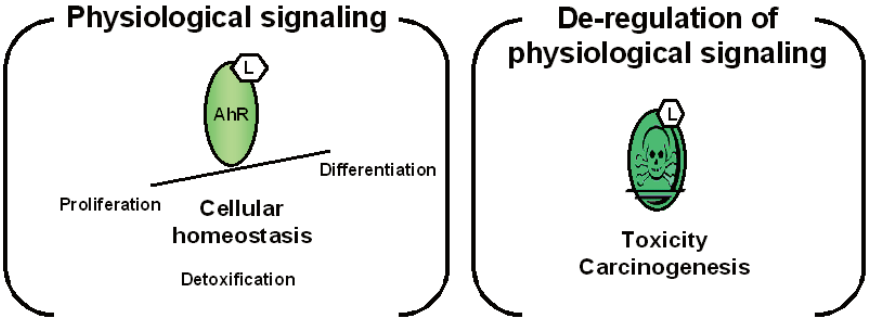

Figure 6. Schematic overview over the role of the AhR in physiological processes.

1.5.1 The role of the AhR in detoxification

The AhR is part of the detection and detoxification system of organisms. By binding a broad range of structurally diverse compounds, the AhR serves, like other PAS proteins, as a sensor protein providing important information about the environment. At the same time, AhR activation induces the transcriptional up-regulation of phase I and II biotransformation enzymes of the detoxification system. In contrast to the invertebrate AhR orthologs (Butler et al., 2001; Powell-Coffman et al., 1998), only the vertebrate AhR seems to be able to bind ligands. It has been considered that this pathway may have originally evolved in higher organisms for the detoxification of plant material and was later further adapted to sense and eliminated harmful man-made contaminants. However, it remains strongly questionable whether the existence of these

artificial, synthetic compounds alone would apply enough evolutionary selection pressure to conserve this pathway.

1.5.2 The AhR as a mediator of toxicity and carcinogenesis

Despite its important function in detoxification, the AhR plays a critical role in mediating xenobiotic-induced toxicity and carcinogenesis (reviewed in Nebert et al., 2004). Exposure to TCDD, for example, results in multiple toxic effects including thymus atrophy, liver enlargement, wasting, cardiovascular diseases, chloracne, tumorgenesis and immune suppression (reviewed in Pohjanvirta and Tuomisto, 1994; Schecter et al., 2006; White and Birnbaum, 2009). Studies on AhR-/- mice clearly showed that TCDD-induced toxicity (Fernandez-Salguero et al., 1996; Mimura et al., 1997) and PAHs-induced carcinogenesis (Shimizu et al., 2000) are mainly mediated by the AhR. Metabolic activation of these compounds by AhR-induced CYP450 enzymes leads to the formation of damaging intermediates e.g. epoxides and reactive oxygen species that subsequently contribute to tumor development. Many of these adverse effects seems to be the consequence of prolonged AhR activation and dysregulation of physiological cell signaling, since similar signs of toxicity can be observed in AhR-CA mice (Andersson et al., 2002; Brunnberg et al., 2006; Tauchi et al., 2005).

1.5.3 The role of the AhR in normal cellular functions

The expression of the AhR during early embryonic development (Abbott et al., 1995), its ubiquitous expression later in adult organisms (Dolwick et al., 1993a), developmental defects in AhR-/- mice (Fernandez-Salguero et al., 1995; Fernandez-Salguero et al., 1997; Schmidt et al., 1996) and its stong conservation throughout evolution (reviewed in Hahn, 2002) suggest a role of the receptor in physiological processes beyond the regulation of xenobiotic metabolism. Studies on the invertebrate AhR orthologs ahr-1 (*Caenorhabditis elegans*) and spineless (*Drosophila melanogaster*) revealed functions in the control of neuron differentiation in the nematode (Huang et al., 2004; Qin et al., 2006) as well as in dendrites, antenna, tarsus and ommatides development of flies (Duncan et al., 1998; Emmons et al., 1999; Kim et al., 2006; Wernet et al., 2006). It seems therefore that the ancestral AhR plays an important role in cell fate determination and differentiation.

Studies on AhR-/- mice showing a reduced liver size, skin and gastrointestinal lesions as well as defects in the reproductive organs and the immune system, point towards the importance of this receptor in normal cellular functions (Baba et al., 2005; Fernandez-Salguero et al., 1995; Fernandez-Salguero et al., 1997; Kawajiri et al., 2009; Schmidt et al., 1996).

Proliferation rates are decreased in AhR-deficient cells (Elizondo et al., 2000; Ma and Whitlock, 1996) suggesting a pivotal role of the AhR in the cell cycle regulation. The AhR appears to interact with various components of the cell cycle machinery to promote cell cycle progression. AhR-p65 interactions allow, for example, entry into the cycle by inducing c-myc gene expression (Kim et al., 2000). Receptor association with CDK4, CCND1 and Rb-E2F sequesters Rb to CDK4, thereby allowing its phosphorylation and the release of E2F which promotes the transcription of genes important for cell cycle progression to the S phase (Barhoover et al., 2010). Furthermore, interactions between the AhR and p300 promote also p300-mediated DNA-synthesis (Tohkin et al., 2000). Disruption of this signaling by xenobiotic

activation of the AhR clearly results in cell cycle arrest and growth inhibition (Gottlicher et al., 1990; Vaziri and Faller, 1997). Cell cycle progression is inhibited by TCDD-activated AhR through transcriptional up-regulation of the cell cycle inhibitor $p27^{Kip}$ (Kolluri et al., 1999) and repression of E2F-mediated transcription of S phase genes by interactions with hypophosphorylated Rb (Marlowe et al., 2004; Puga et al., 2000).

Besides its functional role in cell cycle regulation, the AhR seems also involved in the regulation of cell differentiation as shown for keratinocytes (Swanson, 2004), hematopoetic cells (Singh et al., 2009), adipocytes and immune cells (Esser et al., 2009). Especially the role of the AhR in the differentiation of immune cells has been well characterized. By modulating Stat-1-mediated cytokine expression (Kimura et al., 2008), the AhR modulates the functional differentiation of naïve T-cells into regulatory T cells (T_{reg}) and T-helper 17 (T_H17) cells (Quintana et al., 2008; Veldhoen et al., 2008). It seems that AhR activation switches the development towards T_H17 cells, a subset of effector T cells implicated in the development of autoimmunity and the defense against microbial infections. The AhR clearly transcriptionally controls the production and secretion of interleukin-17 and 22, two proinflammatory cytokines which mediate autoimmune and anti-microbial reactions. Furthermore, AhR signaling seems to contribute to Langerhans cell maturation, because AhR-/- mice show aberrant maturated cells (Jux et al., 2009).

Even in other organs than the immune system the AhR seems to be involved in modulating differentiation, proliferation and cell fate decisions and thereby contribute to tissue homeostasis. AhR-/- mice show increased hepatocyte proliferation (Fernandez-Salguero et al., 1995; Schmidt et al., 1996) and are not able to close the *ductus venosus* in the liver after birth (Schmidt et al., 1996). In female AhR-/- mice folliculogenesis is impaired due to de-regulated CYP19 expression and E_2 production (Baba et al., 2005). Recently, also a crucial role of the AhR in gastrointestinal homeostasis was revealed. AhR-/- mice show high incidences of intestinal cancer due to the accumulation of β-catenin (Kawajiri et al., 2009). β-catenin is a substrate for the E3 ligase function of the receptor and under normal physiological conditions its levels are downregulated following AhR activation by e.g. indolocarbazoles formed by the microbial intestinal flora.

Thus, by controlling important cellular functions such as cell proliferation and differentiation the AhR seems to be essential for the maintenance of tissue homeostasis under physiological conditions.

1.6 AhR SIGNALING IN THE SKIN

1.6.1 The skin

The skin, which is the outer protective barrier of the body, is composed of three layers, the hypodermis, dermis and epidermis. The epidermis, a 50-100μm thick terminally differentiated epithelium, defines the uppermost layer. Based on its morphological appearance, it can be further divided into (i) the *stratum basale/stratum germanium*, a single cell layer of keratinocytes with stem-cell like properties and melanocytes, (ii) the *stratum spinosum*, the prickle cell layer formed of irregular polyhedral-shaped keratinocytes, (iii) the *stratum granulosum*, a layer of flattened, non-dividing keratinocytes filled with granules of keratinohyalin and lipids important for the barrier

function and (iv) the *stratum corneum*, the uppermost layer consisting of terminally differentiated, metabolically inactive keratinocytes (corneocytes) building a network of cells and keratin (Figure 7).

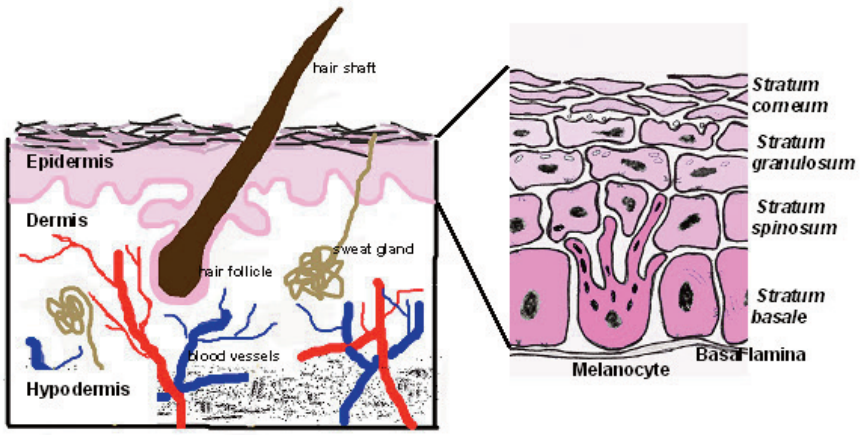

Figure 7. Cross section of the skin and the epidermal layers.

Keratinocytes. Keratinocytes are the most abundant cells of the epidermis. Keratinocyte stem cells are located in the bulge region of hair follicles and the *stratum basale* supplying the renewing epidermis continuously with cells. When passing through the epidermis (epidermal turnover time: 50-75 days), these cells undergo a complex differentiation program under the influence of growth factors and controlled by surrounding calcium levels (for review see Eckert et al., 1997).

Melanocytes. The highly dendritic melanocytes are the melanin-producing cells of the skin. They originate as non-pigmented precursors (melanoblasts) from Schwann cell precursors and the neural crest (Adameyko et al., 2009) and migrate after the closure of the neural tube to their final destinations where they undergo maturation. Melanocytes are located in the *stria vascularis* of the cochlea, the uval tract (cilliary, choroids, iris), the meninges, the epidermis and hair follicles (reviewed in Goding, 2007; Tolleson, 2005). The bulge region of hair follicles serves as a niche for the skin melanocyte stem cell population and their progenitors that provide pigment to the growing hair (Nishimura et al., 2002). During aging, the loss of these cells accounts for hair graying (Nishimura et al., 2005). The highest number of melanocytes is found intrafollicular at the dermal-epidermal junction where they form together with approx. 36 keratinocytes the so called epidermal melanin units (Quevedo, 1972).

1.6.2 The role of the AhR in normal skin functions

In the skin, the expression and inducibility of the AhR and CYP1A1 greatly increase from the lower towards the upper layers. Even though the AhR pathway is present in all skin cells (dermal fibroblasts (Gradin et al., 1999), Langerhans cells (Jux et al., 2009), melanocytes (Villano et al., 2006), keratinocytes (reviewed in Swanson, 2004), its activation seems limited in fibroblasts and Langerhans cells. In keratinocytes, AhR

expression correlates very well with the differentiation status of the cells (Wanner et al., 1995; Wanner et al., 1996). Proliferating keratinocytes show low nuclear AhR levels and are greatly unresponsive to ligand activation, whereas differentiated cells have high cytoplasmic receptor levels (Berghard et al., 1990; Ikuta et al., 2004a). In keratinocytes, the AhR is activated in response to ultraviolet radiation B (UVB) (280-320nm), resulting in the up-regulation of CYP1A1 expression and the activation of c-scr and epidermal growth factor receptor (EGFR) signaling (Fritsche et al., 2007). By sensing UVB, the AhR seems to contribute to the UV stress response system which orchestrates adaptive changes. Apart from functioning as a cytoplasmic sensor for UVB, the AhR has also been implicated to detect and mediate cellular changes in response to ozone (Afaq et al., 2009). Moreover, in epidermal cells the AhR appears to modulate the expression of genes such as slug and metalloproteinases fundamental for cell motility during skin development and renewal (Ikuta and Kawajiri, 2006; Villano et al., 2006). These observations point towards a role of the AhR in maintaining epidermal homeostasis. De-regulation of AhR-controlled processes in the skin by xenobiotics may therefore result in a variety of skin lesions.

1.7 XENOBIOTIC-INDUCED SKIN LESIONS

Environmental and occupational exposure to xenobiotics may cause the development of skin cancer, contact dermatitis, hyperpigmentation and chloracne (reviewed in Yamamoto and Tokura, 2003). Especially chloracne, the formation of acne-like eruptions, hyperkeratosis and epidermal thickening, is a hallmark of systemic exposure to dioxin in humans (Geusau et al., 2000; Schecter et al., 2006). The observed symptoms point towards a disruption of AhR-regulated keratinocyte proliferation and differentiation. Indeed, keratinocytes treated with TCDD showed increased terminal differentiation. As a consequence of increased corneocyte formation, the *stratum corneum* is abnormally thick in TCDD-exposed organisms (Greenlee et al., 1985; Loertscher et al., 2002; Loertscher et al., 2001a; Loertscher et al., 2001b; Sutter et al., 2009).

1.8 MELANOGENESIS AND XENOBIOTIC-INDUCED HYPERPIGMENTATION

Often the development of chloracne is accompanied by an increase in pigmentation. The production of pigment (melanin), a process called melanogenesis, is essential for the protection of the skin against UV. Melanin is exclusively produced in melanocytes in specialized organelles, called melanosomes, which are passed to surrounding keratinocytes.

1.8.1 The process of melanin formation

The formation and distribution of melanin is a highly regulated process that requires (i) the biogenesis of melanosomes, (ii) the expression of melanogenic proteins and their correct trafficking into melanosomes and (iii) the transport and transfer of melanosomes to keratinocytes.

1.8.1.1 Biogenesis of melanosomes

Melanin production is restricted to melanosomes, which are membrane-bound, lysosomal-like organelles that derive from the endosomal pathway (for review see Raposo and Marks, 2007). Melanosomes undergo a maturation process and based on their morphological appearance and the expression of melanogenic proteins they can be classified into four stages (for review see Marks and Seabra, 2001; Wasmeier et al., 2008). Stage I premelanosomes are filled with irregular arranged filaments, but do not contain melanin. Even though all melanogenic proteins are present in premelanosomes, they are catalytically inactive due to the protolytic and acidic milieu (Kushimoto et al., 2001). The proteolytic cleavage of the structural melanosomal membrane protein Pmel17 releases filaments that form the characteristically striated appearance of stage II melanosomes. In stage III melanosomes, melanin starts to accumulate on these filaments. Finally, fully maturated stage IV melanosomes are completely filled with melanin. The proper biogenesis of melanosomes is a prerequisite for the successful production of melanin

1.8.1.2 Biosynthesis of melanin

Melanocytes produce two types of melanin in their melanosomes, the black-brownish eumelanin and the yellow-reddish pheomelanin. Based on the type of melanin produced and its photoprotective properties, different skin types can be assigned (Cripps, 1981). In general, the constitutive skin color depends on (i) the number, size and distribution of melanosomes (Alaluf et al., 2002; Szabo et al., 1969; Thong et al., 2003), (ii) the total melanin content (Alaluf et al., 2002) and (iii) the ratio of eu- to pheomelanin (Wakamatsu et al., 2006). Even though melanosome numbers differ between different skin types, the number of melanocytes is rather equal at the same donor site (Tadokoro et al., 2005). In light-pigmented skin melanosomes appear grouped, whereas melanosomes in dark skin are more dispersed. The lower skin cancer incidence rates in dark-pigmented individuals are probably due to better photoprotection by eumelanin-filled, dispersed melanosomes and higher DNA repair rates (Landi et al., 2002).

Melanin polymers are formed by a complex biochemical pathway, called the Raper-Mason pathway (Land et al., 2004). Polymer formation is initiated by hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-Dopa) and its oxidation to L-dopaquinone. From this point on the chemical composition of the produced melanin is determined by the availability of melanogenic proteins and substrates. In the presence of thiol-containing compounds (del Marmol et al., 1996; Smit et al., 1997), L-dopaquinone converts spontaneously to 3 or 5-cysteinyldopa or glutahionyldopa which is further converted to pheomelanin polymers. In the absence of thiol-containing compounds, eumelanogenesis occurs and L-dopaquinone is spontaneously decarboxylated to 5,6-dihydroxyindole (DHI) or converted by tyrosinase-related protein 2 to 5,6-dihydroxyindole-2-carboxylic acid (DHICA). DHI and DHICA are then further polymerized to the black eumelanin polymers (Figure 8).

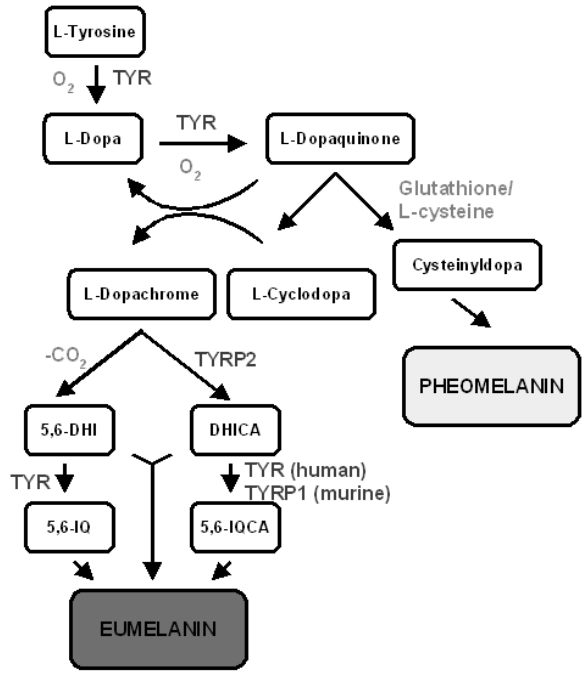

Figure 8. Representation of the Raper-Mason pathway, the biochemical pathway of melanin synthesis. TYR, tyrosinase; TYRP1, tyrosinase-related protein 1; TYRP2, tyrosinase-related protein 2; L-Dopa, 3,4-dihydroxyphenylalanine; 5,6-DHI, 5,6-dihydroxyindole; DHICA, 5,6-dihydroxyindole-2-carboxylic acid; 5,6-IQ, 5,6-indolequinone; 5,6-IQCA, 5,6-indolequinone-2-carboxylic acid.

Eu- and pheomelanin differ not only in their chemical composition, but also in their photoprotective properties. Whereas eumelanin is highly photoprotective and effectively in scattering and absorbing photons and UV-produced ROS (Ortonne, 2002), pheomelanin is photolabile and shows reduced absorptive capacities (Chedekel et al., 1978). Besides its role in photoprotection, melanin is also involved in the chelation of xenobiotics.

1.8.1.3 Melanogenic proteins

Melanin formation is catalyzed by a family of melanosomal transmembrane type I glycoproteins called tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1) and 2 (TYRP2). The expression of these proteins and protein kinase C-β (PKC-β), a kinase involved in the posttranslational regulation of tyrosinase, is under the control of the microphthalmia-associated transcription factor (MITF) (Park et al., 2006; Steingrimsson et al., 2004). All three proteins share a common structural organization and a high amino acid sequence homology and contain (i) a C-terminal transmembrane

motif, (ii) two putative metal binding sites in the catalytic center, (iii) an EGF motif allowing interactions with the other melanogenic proteins and (iv) an N-terminal signal sequence for correct sorting and trafficking into melanosomes. Proper processing through the endoplasmic reticulum and Golgi and correct targeting of these proteins by specific adaptor proteins towards the melanosomal pathway is fundamental for melanin production (Setaluri, 2000; Wang and Hebert, 2006). Dislocation of tyrosinase by mutated adaptor protein 3, for example, accounts for the partial albinism observed in patients with the Hermansky-Pudlak syndrome.

Tyrosinase (TYR). Tyrosinase (monophenol monooxygenase; EC 1.14.18.1) is the key and rate-limiting enzyme of the melanogenic pathway. This enzyme catalyzes (i) the first step in the melanin biosynthesis, the hydroxylation of L-tyrosine to L-dopa, (ii) the oxidation of L-dopa to L-dopaquinone and (iii) the oxidation of DHI thus removing DHI which acts inhibitory on the oxidation of L-tyrosine (Lerner et al., 1949). L-dopa acts both as a co-factor and a substrate for tyrosinase (Pomerantz and Warner, 1967) and is supplied by the phenylalanine hydroxylase (PAH, EC 1.14.16.1) and melanosomal tyrosine hydroxylase isoform I (THI, EC 1.14.16.2) (reviewed in Schallreuter, 2007).

Tyrosinase activity correlates well with the amount of melanin produced and the constitutive skin color (Iozumi et al., 1993; Iwata et al., 1990). In the skin of light-pigmented individuals, tyrosinase activity is lower due to the acidic milieu of melanosomes, whereas its activity is optimal at neutral pH in the lumen of melanosomes of dark-pigmented skin (Fuller et al., 2001).

In contrast to its activity, tyrosinase mRNA and protein levels do not always match the melanin content (Iozumi et al., 1993; Naeyaert et al., 1991). Alterations in tyrosinase activity have been observed upon modulation of the PKC-β signaling pathway indicating that posttranslational modifications seems to be involved in the control of this enzyme. Inhibition of PKC-β reduces basal melanin production *in vitro* and *in vivo* (Park et al., 2004a; Park et al., 1993) and decreases UV-induced melanogenesis *in vivo* (Park et al., 2004a). Upon stimulation, PKC-β is targeted by the receptor for activated C-kinase (RACK) to the melanosomes (Park et al., 2004b) where it phosphorylates serine 505 and 509 of the cytoplasmic domain of tyrosinase (Park et al., 1999). This phosphorylation enhances the catalytic activity of tyrosinase and concomitantly favors the formation of the melanogenic multi-enzyme complex (Wu and Park, 2003), consisting of tyrosinase together with the tyrosinase-related proteins 1 and 2.

The catalytic activity of tyrosinase is essential for the melanogenic pathway and silencing of this gene completely abolishes melanin production (An et al., 2009). In humans, mutations in the tyrosinase gene are linked to the type I oculocutaneous albinism (Tripathi et al., 1992).

Tyrosinase-related protein 1 (TYRP1). The exact catalytic activity of the tyrosinase-related protein 1 (gp75; EC 1.14.18) has not been fully elucidated. TYRP1 functions as a DHICA oxidase in murine melanocytes (Jackson, 1988; Jimenez-Cervantes et al., 1994; Kobayashi et al., 1994), but has no DHICA oxidase activity in human melanocytes (Boissy et al., 1998). In fact, TYRP1 may take part in the biogenesis of melanosomes, because melanosomes lacking TYRP1 appear deformed and are abnormally melanized (Setaluri, 2003). Furthermore, TYRP1 seems to support

tyrosinase stability by forming a complex together with tyrosinase and TYRP2 (Kobayashi et al., 1998b; Orlow et al., 1994). Mutations in the TYRP1 gene result in a light brown skin color in otherwise dark-pigmented individuals, a pathological condition called oculocutaneous albinism 3 (reviewed in Sarangarajan and Boissy, 2001). Thus, functional TYRP1 is required for proper eumelanogenesis.

Tyrosinase-related protein 2 (TYRP2). Tyrosinase-related protein 2 (dopachrome tautomerase; EC 5.3.3.12) catalyzes the conversion of dopachrome to DHICA in the later steps of eumelanogenesis (Bouchard et al., 1994). Mice homozygote for the slaty mutation, a single base pair mutation in the TYRP2 gene, show reduced TYRP2 activity and gray instead of black coat color (Jackson et al., 1992), demonstrating the importance of TYRP2 for the eumelanogenic pathway. In humans, however, no pigmentary disorders have been linked to mutations in TYRP2.

1.8.1.4 Transport and transfer of melanosomes to keratinocytes

Once melanosomes are maturated and filled with melanin, they are transported to the dendritic tip and passed onto surrounding keratinocytes by mechanisms that may include (i) the fusion of membranes of both cells, (ii) exocytosis and endocytosis, (iii) phagocytosis of melanocyte filopodia or (iv) direct inoculation (Seiberg, 2001).

In keratinocytes, melanosomes are arranged in clusters, individually or, in response to UV irradiation, as supranuclear caps to shield against UV-induced DNA damage (Kobayashi et al., 1998a).

1.8.2 Stimulation of melanogenesis by ultraviolet radiation

UV radiation (200-400nm), the major physiological stimulus for melanogenesis, evokes epidermal thickening and increased melanin production, a response known as the tanning response. The tanning response can be divided into (i) intermediate pigment darkening resulting in the photooxidation of existing melanin immediately after UV, in particular UVA, exposure and (ii) delayed tanning that occurs over days to weeks by *de novo* melanin synthesis and melanocyte proliferation.

UV stimulates melanogenesis by both directly acting on melanocytes and by affecting keratinocytes (Figure 9). Human melanocytes undergo cell cycle arrest and upregulate the formation of melanin in response to UVB radiation (Abdel-Malek et al., 1994). The main chromophore for UVB in these cells is DNA. UV-induced DNA damage i.e. the formation of cyclobutane pyrimidine and thymine dimers, and DNA repair mechanisms have been shown to initiate melanogenesis (Eller et al., 1996; Eller et al., 1994) while at the same time inducing p53. T-oligos, sequences that resemble the uncapped telomers, can also induced p53 and enhance melanin formation (Arad et al., 2006). Besides its important role in DNA repair and cell cycle arrest, p53 is able to transcriptionally upregulate tyrosinase mRNA (Khlgatian et al., 2002). Apart from DNA, UVB is also able to damage melanocyte membranes leading to the release of diacylglycerol which stimulates tyrosinase activity via the activation of PKC-β (Carsberg et al., 1995; Gordon and Gilchrest, 1989). As the result of the up-regulation of tyrosinase mRNA levels and activity, the intracellular melanin content increases and irradiated cells enhance their melanosome and dendrite numbers (Friedmann and Gilchrest, 1987; Ramirez-Bosca et al., 1992).

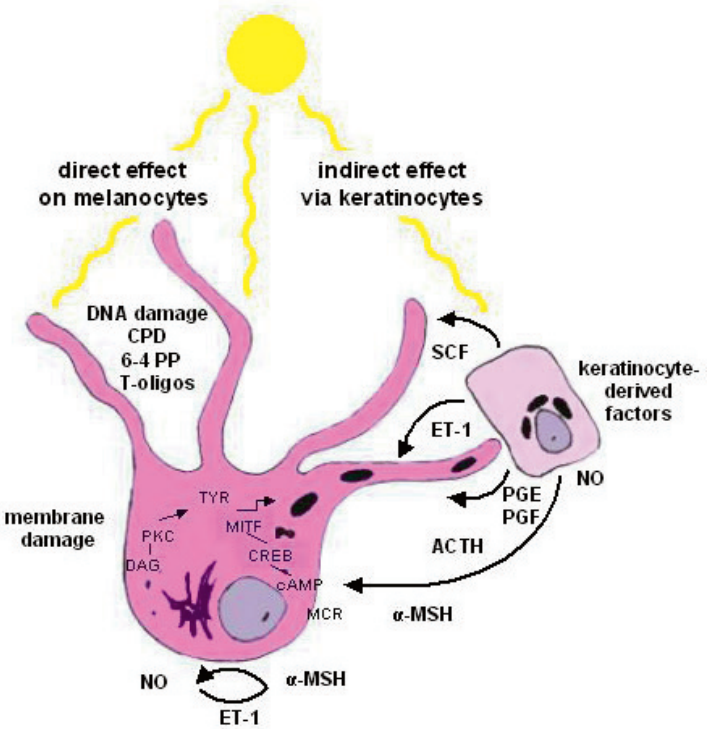

Figure 9. Schematic overview over UV-induced melanogenesis. Upon UV radiation, melanogenesis is stimulated (i) directly in melanocytes by DNA and membrane damage, (ii) autocrine signaling and (iii) paracrine signaling via factors released from stimulated keratinocytes. CPD, cyclobutane pyrimidine dimers; 6-4 PP, thymine dimers; SCF, stem cell factor; ET-1, endothelin-1; PG, prostaglandins; NO, nitric oxide; α-MSH, α-melanocyte stimulating hormone; ACTH, adenocorticotrophic hormone; MCR, melanocortin receptor; DAG, diacylglycerol; CREB, cAMP response element (CRE)-binding protein; MITF, microphthalmia-associated transcription factor; TYR, tyrosinase.

Factors secreted from keratinocytes in response to UV irradiation facilitate further the induction of the melanogenic pathway, but stimulate also melanocyte proliferation. POMC-derived α-melanocyte stimulating hormone (α-MSH), produced both in keratinocytes and melanocytes, plays an important role in the induction of melanogenic gene expression and DNA repair mechanisms (Bohm et al., 2005) as well as in the stimulation of melanocyte proliferation (Abdel-Malek et al., 1995; Im et al., 1998). α-MSH acts via the melanocortin receptor 1 (MCR-1), which is also upregulated in response to UV. MCR-1 activation upregulates cAMP production and subsequently stimulates MITF expression through cAMP response-element binding protein (CREB). Besides tyrosinase-related proteins and PKC-β, MITF controls also the transcription of various other genes of the melanogenic pathway, e.g. the melanosomal structure protein pmel17 (Du et al., 2003) and the melanosome transport protein RAB27 (Chiaverini et al., 2008). In addition to α-MSH, several other factors including endothelin-1 (ET-1), granulocyte-macrophage colony stimulating factor (GM-CSF), nitric oxide (NO), prostaglandins (PGE_2, $PGF_{2\alpha}$), stem cell factor (SCF) and adenocorticotrophic hormone (ATCH) are released from surrounding keratinocytes or, in case of NO, ET-1 and α-

MSH, by the melanocyte itself (reviewed in Hirobe, 2005). These factors act as melanogenic or mitogenic stimuli in an autocrine or paracrine manner on the melanocyte population.

1.8.3 Xenobiotic-induced hyperpigmentation

Increased skin pigmentation is not only observed in response to UV radiation, but also as a consequence of exposure to xenobiotics. In the early 1970´s, two incidences of contamination of cooking rice oil in Taiwan ("Yu-Cheng") (Hsu et al., 1985; Lu and Wu, 1985) and Japan ("Yusho") (Kikuchi, 1984; Urabe and Asahi, 1985) led to acne-like eruptions, comedome formation as well as hyperpigmentation of the nails, mucosa and skin in exposed individuals. Even babies born by exposed mothers showed hyperpigmentation, a phenomenon called the fetal PCB syndrome (Yamashita and Hayashi, 1985). Similar symptoms can also be observed in excessive smokers who usually show hyperkeratosis and increased gingival pigmentation (Axell and Hedin, 1982; Haresaku et al., 2007). Detailed histopathological analyses have revealed elevated melanin deposition in the hyperpigmented tissues (Hashiguchi et al., 1987; Pastor et al., 2002), thereby strengthening the evidence for an induction of the melanogenic pathway by these xenobiotics.

Taken together, these reports clearly indicate that environmental contaminants, particularly PCBs, PAHs and dioxins, are able to interfere with the melanogenic pathway leading to the development of hyperpigmentation in exposed individuals. Given that these compounds are potent AhR activators, these data point towards a role of the AhR in melanogenesis.

2 COMMENTS ON METHODOLOGY

2.1 CELL LINES

The human hepatoma cell line HepG2 and the HepG2-derived cell line HepG2-XRE-Luc were used to elucidate the regulation of AhR signaling by ligands and UVB (Paper I, II & III). HepG2-XRE-Luc cells are stably transfected with a pTX.DIR luciferase reporter under the control of two XREs of the rat CYP1A1 gene (Peters et al., 2006).

The human breast cancer cell line MCF7 was used to study the effects of AhR ligand treatment on estrogen receptor signaling (Paper II). Since both fetal bovine serum (FBS) and phenol red show estrogenic properties (Berthois et al., 1986; Darbre et al., 1983), these cells were treated in phenol red-free medium containing 5% charcoal-treated FBS.

For studies on the role of AhR signaling in melanogenesis, the intermediately pigmented human melanoma cell line FM55 was used (Paper IV).

Supplementary experiments (Paper III & IV) were performed on the human keratinocyte cell line HaCaT (Boukamp et al., 1988) and HaCaT AhR knockout cells carrying AhR silencing shRNA (Fritsche et al., 2007).

2.2 PRIMARY CELLS (MELANOCYTES)

Normal human melanocytes were used to investigate the role of the AhR in melanogenesis (Paper IV). Primary cells were isolated from human foreskin collected from anonymous donors under the age of 10 years undergoing circumcisions. Cells derived from tissue samples of young persons are preferred for the establishment of cultures, since these cells generally have higher proliferation rates and longer lifespans compared to melanocytes collected from adult tissue (Eisinger and Marko, 1982; Gilchrest et al., 1984). Following circumcision, foreskin tissue samples were stored at 4°C in growth medium and processed within 24 hours. Tissue samples were cleaned from excessive fat and shortly sterilized in antibiotics. For the separation of epidermis and dermis, tissue samples were incubated in dispase II solution (2mg/ml) at 4°C overnight. Dispase digestion separates dermis from epidermis along the basal membrane (Kitano and Okada, 1983), thereby favoring the isolation of a higher number of viable cells and reducing the risk for contamination with dermal fibroblasts (McLeod and Mason, 1995). After dispase treatment and removal of the dermis, epidermal sheets were trypsinized at 37°C for 40 minutes and single cell suspensions were collected by filtration through 40µm cell strainers. Cultures were purified by selective trypsinization. Short (~2 min) trypsin treatment at room temperature favors melanocyte detachment, whereas keratinocytes and fibroblasts detach after longer times (~5-7 min).

Pure melanocyte cultures were propagated in commercially available melanocyte M2 growth medium (PromoCell) and kept subconfluent to avoid growth inhibition. In contrast to other cells including murine melanocytes, human melanocytes require the presence of two mitogens for proliferation in culture stimulating the MAPK and cAMP/PKA pathway (reviewed in Halaban, 2000). In early studies, melanocytes could only be cultured in the presence of phorbol 12-myristate 13-acetate (PMA) and cholera toxin (CT) (Eisinger and Marko, 1982) or bovine pituitary extracts (BPE) (Wilkins et

al., 1985). Both PMA and CT exert also anti-proliferating effects on keratinocytes and fibroblasts (Eisinger and Marko, 1982). The replacement of these components and BPE by physiological factors such as ET-1, basic fibroblast growth factor (bFGF) and α-MSH (Swope et al., 1995) as well as the reduction of the serum content in the medium (Abdel-Naser, 2003) allow now melanocyte culture under more physiological conditions.

To provide physiological growth conditions, the antibiotic-, serum- and PMA-free M2 growth medium was selected for this study. Melanocytes grown in this medium show an increased pheomelanin/eumelanin ratio, which suggests that M2 growth medium favors pheomelanogenesis (Duval et al., 2002).

2.3 AhR AGONISTS AND ANTAGONISTS

The following AhR agonists (Figure 10) and antagonists were used in these studies.

Indolocarbazoles. The two indolocarbazoles, 6-formylindolo[3,2-*b*]carbazole (FICZ) and indolo[3,2-*b*]carbazole (ICZ), were chosen as examples of physiological ligands. FICZ has been identified as a photoproduct formed from the amino acid tryptophan when irradiated with UV (Rannug et al., 1987; Rannug et al., 1995) or visible light (Diani-Moore et al., 2006; Oberg et al., 2005) and its intracellular formation has been shown in UV-irradiated HaCaT cells (Fritsche et al., 2007). ICZ, the second indolocarbazole studied, is formed in the acidic environment of the stomach and by the intestinal flora from the secondary plant metabolite I3C (Bjeldanes et al., 1991), which is mainly present in vegetables of the *Brassica* genus e.g. Brussels sprouts, cabbage and cauliflower. Both indolocarbazoles are excellent AhR activators with binding affinities in the pM-nM range (Gillner et al., 1993; Gillner et al., 1985; Kleman et al., 1994; Rannug et al., 1987) and are rapidly metabolized (Bergander et al., 2004; Chen et al., 1995).

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The prototypical AhR ligand TCDD activates the AhR at low nM concentrations. This environmental pollutant is metabolically inert and resistant to degradation (for review see Poland and Knutson, 1982).

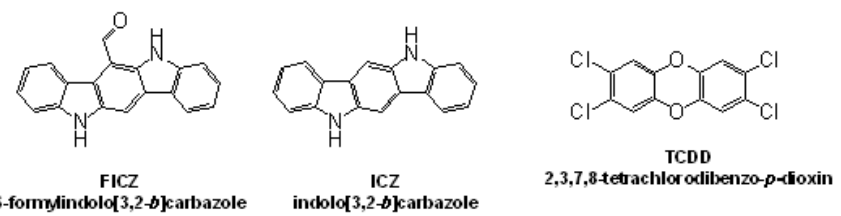

Figure 10. Structures of AhR agonists used in this study.

α-naphthoflavone (α-NF) and 3´-methoxy-4´-nitroflavone (MNF). These two flavones exhibit both antagonistic and agonistic properties. At nM-μM concentrations α-NF has been shown to inhibit TCDD-induced CYP1A1 gene expression and activity

(Blank et al., 1987; Merchant et al., 1990), whereas at higher μM concentrations and in the absence of TCDD, α-NF can stimulate AhR activation (Wilhelmsson et al., 1994).

Similar to α-NF, MNF has been shown to compete with TCDD for binding sites thereby hindering nuclear translocation, DNA-binding, CYP1A1 induction and down-regulation of activated receptor (Henry et al., 1999; Lu et al., 1995). Although MNF was first classified as a "pure AhR antagonist", it was later identified as an agonist inducing CYP1A1 expression (Zhou and Gasiewicz, 2003). Due to both their agonistic and antagonistic properties, these compounds are also termed partial antagonists (Wilhelmsson et al., 1994; Zhou and Gasiewicz, 2003).

2.4 ULTRAVIOLET LIGHT TREATMENT

In the studies on AhR activation by UVB light (Paper III), a Philips TL20W/12RS lamp was used as an ultraviolet light source. The six UV tubes of this lamp emit ultraviolet light in the range of 280-320nm with an emission peak at 310nm. To obtain an UVB dose of 10mJ/cm^2 cells were irradiated for 18sec at a distance of 40cm. Since phenol red and serum components can act as photosensitizers during irradiation, cells were irradiated in Mg^{2+}/Ca^{2+}-PBS in dishes with the lid removed. After irradiation, the PBS was immediately replaced by fresh cell culture medium. Sham-irradiated cells were handled similarly, but shielded with aluminum foil against irradiation.

2.5 TRANSFECTION AND PLASMID CONSTRUCTS

Plasmids were introduced into cells by transient transfections using Fugene-6 as transfection reagent (Paper II & III). The *pTX.DIR* construct contains a herpes simplex virus thymidine kinase (TK) promoter and luciferase reporter gene under the control of two XREs of the rat CYP1A1 gene (Berghard et al., 1993). The *2xERE-TK-Luc* plasmid contains the TK promoter and the luciferase reporter gene under the control of two estrogen response elements (ERE) (Pettersson et al., 1997). The *pCMX-GFP-AhR* expression vector codes for a fusion protein of the murine AhR (amino acids: 1-805) fused to green fluorescent protein (GFP) (Kazlauskas et al., 2000). The *pCMX-GFP-AhR-A375I* plasmid was constructed by subcloning pIRES-DRA375I into pCMX-GFP-AhR. GFP-AhR-A375I contains a mutation at position 375 of the LBD substituting alanine by isoleucine. The *pFA-AhR* encodes a Gal4-AhR fusion protein in which the first 37 amino acids of the rat AhR are replaced by the Gal4 DBD. In the Gal4 transactivation assay, the luciferase reporter gene (*pFR-Luc)* was under the control of the upstream activation sequence (UAS) to which Gal4 can bind (Backlund and Ingelman-Sundberg, 2004).

2.6 USE OF INHIBITORS

NFκB inhibitors SN50 and Bay 11-7085. In paper III, SN50 and Bay 11-7085 were used as inhibitors for the NFκB pathway. SN50 is a cell-permeable inhibitor peptide containing the NLS of the NFκB subunit p50 coupled to the hydrophobic region of the signal sequence of the Kaposi fibroblast growth factor to assure import into the cell

(Lin et al., 1995). Bay 11-7085 blocks the release of NFκB from IκBα by inhibiting IκBα phosphorylation and subsequently degradation (Pierce et al., 1997).

PKC inhibitor Bisindolylmaleimide GF 109203X. In paper IV, the competitive inhibitor bisindolylmaleimide (Bis) was used. Bis inhibits PKC activity by preventing ATP binding to its catalytic site (Toullec et al., 1991).

2.7 MELANOGENIC ASSAY

Tyrosinase activity. For tyrosinase activity measurements (Paper IV), tyrosinase enzyme was obtained from supernatants of melanocyte lysates after centrifugation. Enzyme activity was measured spectrophotometrically at 492nm at 37°C for one hour as the formation of dopachrome in a reaction mix consisting of supernatant, L-Dopa (2mg/ml) and 50mM phosphate buffer at neutral pH. Neutral pH is required for optimal tyrosinase activity and L-dopachrome stability. Tyrosinase activity was calculated over the first 20 min (excluding the lag-phase) and normalized to protein concentrations. This assay may underestimate tyrosinase activity, because (i) only ½ of L-dopa is converted into L-dopochrome by tyrosinase, while the other ½ is reduced back to L-dopa and (ii) L-dopachrome is concomitantly catalyzed by TYRP2 into DHICA (Nordlund et al., 2006).

Melanin. To determine the melanin content of melanocytes (Paper IV), cells were harvested, centrifuged and pellets were solubilized in 1M NaOH for 1h at 60°C. Melanin was measured spectrophotometrically at 450nM.

3 AIMS OF THE PRESENT STUDY

The overall aim of the present study was to elucidate the molecular mechanisms regulating AhR function and signaling upon activation by different stimuli including xenobiotics, potential physiological ligands and UVB. A further aim was to investigate the functional consequences of AhR activation by these different types of activators on normal physiological cell signaling.

The specific aims of this study were:

- To further characterize pathways for the formation and metabolic degradation of the suggested physiological AhR ligand FICZ as well as to identify the presence of FICZ metabolites in human material.
- To gain insights into the regulation of the transcription factor and ubiquitin ligase function of the AhR (i) under physiological, xenobiotic-free conditions and (ii) in the presence of xenobiotics i.e. AhR activation and signaling upon indolocarbazole (FICZ and ICZ) vs TCDD treatment.
  Moreover, to investigate the functional consequences of differences in the regulation of AhR signaling under these two conditions.
- To analyze the activation and regulation of AhR signaling upon UVB irradiation using the AhR-target gene CYP1A1 as an example
- To elucidate AhR-mediated mechanisms underlying xenobiotic-induced hyperpigmentation and to define a novel role of the AhR in modulating human melanogenesis in the skin.

4 RESULTS AND DISCUSSION

4.1 PROPERTIES OF 6-FORMYLINDOLO[3,2-*b*]CARBAZOLE (FICZ) – A SUGGESTED PHYSIOLOGICAL AhR LIGAND (PAPER I & II)

AhR signaling and its regulation have mainly been studied upon activation by TCDD. This man-made and non-metabolizable compound has been intensively used as a prototypical ligand for the AhR and most of our understanding of AhR signaling is based on studies using TCDD or structurally similar xenobiotics as stimuli. Because these compounds are not endogenously produced or present in cells, xenobiotic-induced AhR signaling may rather reflect the de-regulation of normal signaling than signaling under physiological conditions. In order to learn more about the role of the AhR in normal cellular functions, many attempts have been made to identify the endogenous ligands of this receptor. We used the tryptophan-derived photoproduct 6-formylindolo[3,2-*b*]carbazole (FICZ) as an example of a physiological ligand and characterized its properties (Paper I). Later on, we investigated AhR activation and function under xenobiotic-free conditions by using FICZ and indolo[3,2-*b*]carbazole (ICZ) as ligands (Paper II).

4.1.1 Characterization of FICZ

4.1.1.1 FICZ - a bona fide ligand for the AhR

Competitive binding studies in the late 1980´s showed that photoproducts of the amino acid tryptophan, but not tryptophan itself, were able to bind and activate the AhR (Helferich and Denison, 1991; Rannug et al., 1987). One of this photoproducts was identified as 6-formylindolo[3,2-*b*]carbazole, shortly termed FICZ (Rannug et al., 1995). FICZ is a high affinity ligand and powerful AhR activator (Rannug et al., 1987; Wei et al., 2000) and by performing AhR-ELISA (Paper I) and dose-response curves at 3 hours in HepG2-XRE-Luc cells (Paper II) we confirmed its potency. In HepG2-XRE-Luc cells, FICZ activated the XRE-luciferase gene reporter with an EC_{50} value (EC_{50} FICZ: $1.5(\pm 0.61)\cdot 10^{-10}$M) that was 19.3 fold lower than the EC_{50} value for TCDD (EC_{50} TCDD: $2.9(\pm 0.86)\cdot 10^{-9}$M) indicating that FICZ is more powerful in activating the AhR than TCDD, the prototypical high affinity ligand.

4.1.1.2 The light-dependent formation of FICZ and its metabolic fate

Tryptophan seems to serve as a common precursor for many naturally-occurring AhR ligands including indolocarbazoles, indigoids, tryptanthrins, malassezin, trypthamine, indoleacetic acid and kynurenic acid. Many of these compounds are derived from tryptophan by enzymatic activity in both microorganisms and fungi as well as plants and higher organisms (reviewed in Denison and Nagy, 2003). In contrast to the enzymatic formation of these compounds, FICZ was formed upon irradiation of tryptophan with visible light and ultraviolet radiation, as also indicated in previous studies (Diani-Moore et al., 2006; Oberg et al., 2005). Because tryptophan is one of the amino acids that is able to absorb light in the range of UVB, UVB was very effectively in producing FICZ, whereas only low amounts of FICZ could be detected after UVA (320-400nm) irradiation of tryptophan solutions. Light and UVA-dependent FICZ

formation was enhanced by the photosensitizer riboflavine. In a study on keratinocytes, AhR activation was mainly observed after UVB irradiation and nearly no responses were obtained upon UVA irradiation (Fritsche et al., 2007). As shown here, FICZ can be effectively produced by irradiation of tryptophan. Whether FICZ can also be formed enzymatically like other tryptophan-derived AhR ligands has to be elucidated.

FICZ is rapidly degraded in biological systems (Bergander et al., 2003; Bergander et al., 2004). A number of mono- or dihydroxylated metabolites, which were less efficient AhR activators, were produced when FICZ was metabolically degraded. The hydroxylation of FICZ was mainly catalyzed by CYP1A1, CYP1A2 and CYP1B1 enzymes and FICZ was shown to be an extraordinary good substrate for these enzymes, especially for CYP1A1. The significance of the catalytic activity of CYP1A1 in FICZ metabolism was proven in the murine hepatoma cell line (Hepa-1 c37), containing non-functional CYP1A1 enzyme. Hepa-1 c37 cells showed almost no FICZ metabolism, confirming that metabolism of FICZ occurs mainly through the CYP1A1 enzyme (Wei et al., 2000). To a lesser extent, FICZ was also converted by aldehyde oxidases to the indolo[3,2-*b*]carbazole-6-carboxylic acid (CICZ) which can be further hydroxylated by CYP1 enzymes. The hydroxylated metabolites of FICZ were very good substrates for sulfotransferases that catalyze the conjugation of sulfo-groups to form water-soluble and excretable sulfuric acid esters. In fact, sulfoconjugated metabolites of FICZ were identified in human urine samples by using LC/MS/MS (Paper I). The identification of FICZ-derived sulfoconjugates in biological samples suggests the presence of FICZ or its metabolites in biological systems and underlines the physiological relevance of this compound and its metabolites.

4.1.2 AhR activation by FICZ

FICZ is a high affinity AhR ligand, inducing a transient CYP1A1 expression at pM to nM concentrations (Wei et al., 1998). A rapid and transient increase in AhR activation, measured as the induction of luciferase reporter activity, was also observed in HepG2-XRE-Luc cells treated with low nM concentrations of FICZ. Time-course experiments revealed a maximal induction at around 3 hours, after which levels decreased. Control levels were reached at around 12 hours (Paper I and II). This transient induction pattern, which is also observed after ICZ treatment, can be abrogated by stimulation with higher concentrations (μM-mM) (Wei, unpublished data; Chen et al., 1995). Endogenous gene expression of CYP1A1 and the AhR repressor, both AhR-target genes, showed a similar transient induction pattern in response to low concentrations of FICZ, peaking consistently with the reporter gene activity at around 3 hours before declining to basal levels (Paper II). These transient induction patterns of AhR activation and target gene expression correlated well with intracellular FICZ levels. Intracellular FICZ level declined rapidly over the first hours and were below detection limit after 3 hours (Paper I). With the disappearance of ligand, also AhR activation tapered off.

4.1.3 Autoregulatory loop between AhR signaling and CYP1A1 – FICZ induces its own metabolism

AhR signaling and CYP1A1 seems to be linked to each other by an autoregulatory loop since high constitutive CYP1A1 mRNA levels have been observed in the absence of exogenous ligands in cells with impaired CYP1A1 enzyme activity (Chang and Puga,

1998; RayChaudhuri et al., 1990; Wei et al., 2000). Like many other naturally-occurring compounds which are both AhR ligands and substrates for CYP1 metabolizing enzymes (Chen et al., 1995; Schaldach et al., 1999; Sinal and Bend, 1997), FICZ induces its own metabolism through the transcriptional up-regulation of AhR-controlled CYP1 genes. When CYP1A1 enzyme activity was impaired by α-NF treatment and FICZ metabolism was slowed down, luciferase reporter activity was not longer transient, but showed a rather sustained response to FICZ treatment (Paper I). Similar observations were also obtained from Hepa-1 c37 cells, deficient in functional CYP1A1. In these cells, FICZ treatment evokes a prolonged up-regulation of CYP1A1 mRNA levels, an induction pattern similar to the one observed after stimulation with the non-metabolizable ligand TCDD (Wei et al., 2000). Moreover, CYP1A1 inhibition using chemical inhibitors resulted in cell cycle arrest in rat hepatoma cells, a response rather expected upon TCDD treatment (Levine-Fridman et al., 2004). It therefore seems that the autoregulation of ligand availability is essential for normal cell signaling and the maintenance of homeostatic functions.

4.2 COMPARSION OF AhR SIGNALING UNDER PHYSIOLOGICAL AND NON-PHYSIOLOGICAL CONDITIONS (PAPER I & II)

In order to understand the regulatory mechanisms controlling AhR signaling under physiological, xenobiotic-free conditions, we investigated AhR activity and function following activation by indolocarbazoles and compared it to the activation by TCDD. The indolocarbazoles FICZ and ICZ are naturally-occurring (Bjeldanes et al., 1991; Rannug et al., 1987) and rapidly metabolized through CYPs 450 (Paper I; Chen et al., 1995), thereby fulfilling two properties that seems to be important for physiological ligands. TCDD, in contrast, is exclusively produced by anthropogenic activities and is due to its physiochemical properties resistant to biological degradation (Poland and Knutson, 1982).

4.2.1 The regulation of the transcription factor and ubiquitin ligase function of the AhR by indolocarbazoles and TCDD

In study II, we analyzed the transcription factor and ubiquitin ligase function of the AhR in HepG2 cells in response to indolocarbazoles and TCDD treatment. Indolocarbazole treatment evoked a transient induction of luciferase reporter gene activity and target gene expression, which was in contrast to the sustained transactivation of TCDD-activated AhR. These two classes of ligands may not only exert temporal different responses in gene expression, but may also induce different AhR target gene profiles as shown by Laub and colleagues (Laub et al., 2010). Differences between indolocarbazole and TCDD treatments were also observed with regard to the intracellular distribution and DNA-binding capacities of the activated receptor. Localization studies using a GFP-tagged mouse AhR, transiently transfected into HepG2 cells, revealed a rapid translocation of activated receptor from the cytoplasm into the nucleus upon FICZ, ICZ and TCDD activation. Yet, only TCDD-activated AhR was still enhanced in the nuclear compartment after 6 hours, whereas indolocarbazole-activated receptor showed an equal distribution between cytoplasm and nucleus as also observed in vehicle treated cells. Studies using a mutant mAhR that

bears an alanine to isoleucine substitution at amino acid 375 in the LBD and shows impaired ligand recognition (Murray et al., 2005), confirmed that the observed differences were due to ligand activation of the receptor. Consistently with the subcellular compartmentalization data and the target gene expression, DNA-bound receptor was isolated from the CYP1A1 promoter of TCDD-treated cells after 16 hours. Upon indolocarbazole treatment, the AhR was only transiently recruited to XREs and was clearly not detectable after 16 hours. Interestingly, in comparison to TCDD, recruitment of the indolocarbazole-activated receptor was more rapidly (within 10 minutes), a trend that was also observed in target gene expression and substrate degradation. Taken together, profound differences in the regulation of the transcriptional activation properties of the AhR by these two types of ligands exist.

Even the ubiquitin ligase function of the receptor was distinctly regulated by indolocarbazoles and TCDD. AhR and ERα levels were down-regulated in response to both classes of ligands, suggesting that the ligase function is activated upon ligand binding independently on a specific type of ligand. Total AhR levels were rapidly and transient decreased via the 26S proteasomal pathway in response to indolocarbazole treatment, but remained persistent depleted upon TCDD exposure. Recovery of indolocarbazole-activated receptor was blocked by cycloheximide (CHX) co-treatment, indicating that new synthesis of receptor is necessary to restore basal levels. Whether ERα levels are restored after indolocarbazole treatment could not be assessed by immunoblotting, because the protein synthesis inhibitor CHX was required in all experiments due to the fast turnover of ERα (Reid et al., 2003). However, because MCF7 cells pre-treated with indolocarbazoles remained estrogen-responsive, a transient down-regulation of the ERα can be assumed.

ERα regulates the growth of hormone-responsive breast cancer cells (Brunner et al., 1989). An increasingly attractive strategy in breast cancer treatment, but also prostate cancer treatment, is the depletion of steroid receptor levels in tumor cells (Rodriguez-Gonzalez et al., 2008). Selective ER downregulators (SERDs), e.g. the ERα ligand ICI-182780, have been used to promote the degradation of activated receptor. An alternative approach to induce ERα degradation may be via selective, non-toxic AhR ligands, which may also be effective in cases where ERα became resistant to hormone therapy. Recently, I3C, a precursor of ICZ, has been shown to induce ERα degradation via the AhR E3 ligase function thereby disrupting GATA3/ER crosstalk and subsequently ERα expression in breast cancer cells (Marconett et al., 2010). Moreover, in $APC^{min/+}$ mice, a mouse model for colon carcinogenesis, I3C has been shown to reduced the incidences of small intestine tumors by promoting AhR-mediated β-catenin degradation (Kawajiri et al., 2009). Thus, by inducing the ubiquitin ligase function of the receptor, non-toxic, physiological ligands may have important therapeutic implications for cancer prevention.

4.2.2 Dynamic regulation of the AhR pathway is required for receptor responsiveness and physiological signaling

The two functions of the AhR, the transcription factor and ubiquitin ligase function, were dynamically regulated upon activation by physiological ligands of the indolocarbazole type. Due to their fast metabolism (Paper I and Chen et al., 1995), these ligands activated AhR signaling in a transient manner and AhR activity could be rapidly reset to basal levels following the metabolic clearance of ligand and the

degradation of ligand-activated receptor. Rapid metabolism of activating ligands by ligand-induced CYPs and other drug-metabolizing enzymes has also been suggested as an underlying mechanism to obtain normal nuclear receptor signaling (reviewed by Nebert, 1991). Even degradation of activated receptor via the proteasomal pathway is a common mechanism for the regulation of nuclear receptors to limit receptor activity and retain ligand responsiveness (Wijayaratne and McDonnell, 2001). In case of the AhR, receptor levels are not only reduced in response to ligands, but also after stimulation by UVB (Paper III). By using an experimental design, in which cells were pre-treated with ligands for 5 hours, before new ligand stimulation after 14 hours of rest, we analyzed ligand responsiveness. Cells remained only ligand-responsive when pre-treated with indolocarbazoles, suggesting that a transient activation of this pathway and a rapid restoration of constitutive levels are important for normal cellular signaling. Transient receptor activation and degradation are also observed after treatment of cells and experimental animals with ITE (Henry et al., 2006). Animals treated with single or repeated doses of these metabolizable, naturally-occurring compounds, i.e. ICZ, FICZ and ITE, show no signs of toxicity (Henry et al., 2006; Mukai and Tischkau, 2007; Pohjanvirta et al., 2002) indicating that these ligands will not disturb physiological signaling.

4.2.3 Dysregulation of dynamic AhR signaling - a mode of action of toxicity

In contrast to indolocarbazole treatment, TCDD treatment resulted in prolonged AhR activation and persistent depletion of AhR and ER levels. As a functional consequence, cells became refractory to new ligand stimulation. Thus, TCDD rendered cells into a state in which normal dynamic cell signaling was interrupted. Disturbed signaling and impaired ligand responsiveness can also be observed in cells and mice in which AhR activity is artificially prolonged through the introduction of a constitutive active AhR, lacking parts of its LBD (Andersson et al., 2002; McGuire et al., 2001). AhR-CA mice show signs of TCDD toxicity (Brunnberg et al., 2006) suggesting that continuous AhR activation is unfavorable. Many regulatory mechanisms including the AhR repressor, metabolic clearance of ligands and ligand-induced receptor degradation seems to have evolved to prevent continuous AhR activation and the pleiotropic toxic effects linked to it (reviewed in Mitchell and Elferink, 2009). Disturbance of these processes and subsequently dysregulation of dynamic cell signaling may therefore be part of the mode of action of dioxins and other metabolically inert, synthetic compounds and account for their widely observed toxicity.

4.3 STIMULATION OF THE AhR PATHWAY BY UVB USING THE EXAMPLE OF THE AhR TARGET GENE CYP1A1 (PAPER III)

AhR activation has not only be observed after ligand treatment, but also after stimulation with UVB radiation (Fritsche et al., 2007). In zebra fish (Behrendt et al., 2010), murine (Goerz et al., 1996; Goerz et al., 1983) and human skin (Katiyar et al., 2000) as well as in keratinocytes (Fritsche et al., 2007; Gonzalez et al., 2001; Villard et al., 2002; Wei et al., 1999) AhR target gene expression, in particular CYP1A1, CYP1A2 and CYP1B1, is induced following UVB irradiation. UV radiation affects

many signal transduction pathways (reviewed in Bender et al., 1997) and in paper III we analyzed its impact on AhR activation and CYP1A1 gene regulation. Due to the availability of the sensitive XRE-luciferase reporter in HepG2-XRE-Luc cells, we performed this study in human hepatoma cells (HepG2) instead of keratinocytes, which are clearly the physiological more relevant cell type. Most of the studies in paper III were performed with the reporter gene system and not on endogenous CYP1A1 gene expression. Because the half-lives of both luciferase and CYP1A1 mRNA and protein are quite similar ($t_{1/2\ mRNA}$~ 2-2.4 hours; $t_{1/2\ protein}$ ~ 3 hours) (Lekas et al., 2000; Marderosian et al., 2006; Thompson et al., 1991; Werlinder et al., 2001), the reporter gene assay was used as an appropriate surrogate for gene expression analyses. The UVB doses used in most of the experiments ranged between 10-20mJ/cm^2. These doses are significantly lower than the minimal erythema dose (MED) in humans and can therefore be considered as physiologically relevant.

4.3.1 AhR activation by UVB irradiation

HepG2-XRE-Luc cells responded to UVB irradiation with a slow, belated increase in luciferase reporter activity that could be blocked by treatment with the partial AhR antagonist MNF. Studies performed on HaCaT AhR KO cells, showing extremely low AhR protein levels, and Hepa-1 c12 cells lacking the AhR, confirmed the AhR as the mediator of UVB responses (Fritsche et al., 2007; Wei et al., 1999). In concordance with the reporter studies, endogenous CYP1A1 gene expression was also up-regulated by UVB at late time points. However, the temporal induction pattern observed after UVB irradiation was distinct from the induction by FICZ. As described earlier (Paper I and II), FICZ treatment at low nM concentrations resulted in a rapid and transient induction peaking at around 3-6 hours. On the contrary, UVB-induced reporter gene activity and CYP1A1 expression occurred first after 9 hours and was suppressed below control levels, i.e. levels obtained in sham-irradiated cells receiving new medium at treatment start, at earlier time points. It has been proposed that the inducing effects of UVB on AhR signaling are mediated by FICZ (Rannug et al., 1987), of which intracellular formation was shown in UVB-irradiated HaCaT cells (Fritsche et al., 2007). However, the differences observed in kinetics in this study rather suggest the involvement of additional signaling molecules and factors.

4.3.2 Interactions between the AhR and nuclear factor κB in the regulation of UVB-induced CYP1A1 gene expression

As described above, UVB irradiation initially suppressed reporter gene activity and CYP1A1 gene expression in a dose-dependent manner. Suppression of CYP1A1 expression has been noted after H_2O_2 treatment. This suppression is mediated by the nuclear factor I (NFI/CTF) (Barker et al., 1994; Morel and Barouki, 1998; Morel et al., 2000), which undergoes redox cycling of cysteine residues in its TAD in response to oxidant treatment (Morel and Barouki, 2000). In TCDD-induced cells, suppression of CYP1A1 expression has also been observed after co-treatment with TNF-α, IL-1 β and LPS (Ke et al., 2001; Tian et al., 1999). These proinflammatory cytokines and microbial endotoxins have been shown to suppress AhR activity and CYP1A1 expression through activation of the NFκB pathway (reviewed in Tian et al., 2002). Because NFκB can be activated through UVB-generated ROS (reviewed by Cooper

and Bowden, 2007), we investigated whether crosstalk between the two transcription factors underlies the modulation of UVB-induced CYP1A1 gene expression. Upon irradiation, the NFκB subunit p65 underwent nuclear translocation, but was mainly cytoplasmic at later time points when CYP1A1 expression was not longer suppressed. Two different NFκB inhibitors, the inhibitory peptide SN50 and the IκBα phosphorylation inhibitor Bay 11-7085 (Lin et al., 1995; Pierce et al., 1997), abolished the initial suppression suggesting that NFκB and AhR mutually regulate their activities in response to UVB. Despite their inability to bind to the AhR, as tested in the Gal4-AhR fusion protein assay, both inhibitors themselves increased luciferase reporter gene activity. Whether this is due to an involvement of NFκB in the modulation of constitutive levels remains to be elucidated.

p65, which is able to directly interact with the AhR (Tian et al., 1999), suppresses CYP1A1 gene expression through competition for SCR-1 and p300/CBP and blockage of histone acetylation (Ke et al., 2001). Which mechanism is involved in mediating the inhibitory effects of NFκB activation on AhR signaling following UVB irradiation remains unknown. The use of the XRE-luciferase reporter construct, which does not resemble the chromatin architecture of endogenous genes, allows no conclusions to be drawn about the inhibitory mechanisms of NFκB.

4.3.3 AhR signaling as part of the UV stress response

UVB irradiation restricted not only the expression of the CYP1A1 gene at early time points via the activation of NFκB, but decreased also the catalytic activity of the CYP1A1 enzyme. Upon UVB irradiation, CYP1A1 enzyme activity was damped as measured by the clearance of intracellular FICZ levels (see also Paper I). The reduction of CYP1A1 expression and activity may be a protective precaution of cells to limit ROS-generation by high CYP1A1 activity (Barouki and Morel, 2001). Moreover, AhR signaling became refractory to repeated UVB irradiation and oxidant treatment, but remained responsive to ligand treatment. In contrast to the observed refractoriness in TCDD-pre-treated cells, reduced receptor levels seemed not clearly account for the receptor unresponsiveness after UVB and oxidant treatment. In response to oxidant exposure, proteins can undergo redox cycling of their cysteine residues. This may impair their ability to interact with DNA and other proteins and reduce their transactivation properties. Whether such changes affect upstream regulators of the AhR e.g. kinases or account for the inability of the AhR itself to respond to repeated UVB or oxidant treatment has to be investigated.

The observed restriction of AhR signaling and CYP1A1 enzyme activity following UVB irradiation may be part of protective mechanisms of cells against environmental stressors. Stressors such as UV, oxidative stress and heat shock have also been shown to regulate the activity of many E3 ubiquitin ligases (reviewed by Ohtake et al., 2009). For instance, the CUL3 ubiquitin ligase complex is inactivated upon oxidative stress resulting in the stabilization of the transcription factor NRF2 involved in antioxidant responses (for review see Nguyen et al., 2009), whereas the CUL4A ligase is activated by UV targeting the xeroderma pigmentosum group C protein (XPC) to degradation thereby allowing nucleotide excision repair (reviewed by Jackson and Xiong, 2009). The down-regulation of AhR levels upon UVB irradiation may suggest that the AhR is part of a stress-responsive ubiquitin ligase complex that can be regulated by UVB and oxidative stress. However, it remains to be elucidated how this complex affects other

substrate proteins and contribute to the stress response system. Cellular responses to UV or oxidants are not only observed in skin cells. Even other cells e.g. hepatocytes, lymphocytes and HepG2 possess a functional antioxidant defense and UV stress response system and the AhR may be part of it.

4.4 A NOVEL FUNCTION OF THE AhR IN THE SKIN (PAPER IV)

As implicated in Paper III and reviewed in Ranug and Fritsche (Rannug and Fritsche, 2006), AhR signaling can be stimulated by UVB and may be part of the UV stress response. Like many other cells, skin cells undergo adaptive changes to protect themselves against the deleterious effects of UVB radiation and UV-induced ROS production (Herrlich et al., 2008; Muthusamy and Piva, 2009). These changes include cell cycle arrest, DNA repair and, in the case of melanocytes, an enhanced formation of photon-absorbing melanin. An increase in pigmentation, a pathological condition called hyperpigmentation, can also be observed in humans after exposure to xenobiotics such as PAHs, PCBs and dioxins, compounds well-known to be AhR ligands (Axell and Hedin, 1982; Haresaku et al., 2007; Hsu et al., 1985; Lu and Wu, 1985; Urabe and Asahi, 1985; Yamashita and Hayashi, 1985). Even in other organisms than humans, enhanced melanogenesis has been reported after exposure to AhR ligands of xenobiotic origin (Iwata et al., 1981; Kleeman et al., 1988; Zodrow and Tanguay, 2003). These results and the data on AhR activation by UVB, the major physiological stimulus for the production of melanin, suggest a role of the AhR in melanogenesis.

4.4.1 AhR signaling in melanocytes

In order to study the role of the AhR in melanogenesis, we first characterized the AhR pathway in human melanocytes, the pigment-producing cells of the skin. Human primary melanocytes expressed low basal levels of AhR and ARNT mRNA compared to their cancerous counterparts in which AhR overexpression can clearly be linked to their invasive potential (Villano et al., 2006). Treatment of these cells in culture with TCDD and FICZ resulted in an up-regulation of AhR target genes e.g. CYP1A1, CYP1B1, the AhR repressor and the AhR itself. In agreement with the observations in Paper II, FICZ treatment provoked a transient induction, whereas expression levels were still up-regulated after 3 days by TCDD treatment. Even in melanocytes, ligand-induced AhR degradation was detected confirming that the AhR pathway is fully functional in these cells. AhR protein levels varied in melanocytes from different donors, but could not clearly be coupled to the pigmentation status of the cells.

4.4.2 The AhR mediates xenobiotic-induced hyperpigmentation

The production of melanin is a very complex process that requires organelle biogenesis, gene expression, protein sorting and organelle transfer. However, the key and rate-limiting step in the biochemical formation of melanin polymers is the oxidation of L-tyrosine to L-dopa, a reaction that is solely catalyzed by the melanosomal tyrosinase enzyme. Tyrosinase enzyme activity, as measured by a spectrophotometrical absorbance-based enzyme assay, was up-regulated in an AhR-dependent manner in melanocytes upon TCDD treatment (10nM). The sustained up-regulation of tyrosinase activity was followed by an increase in intracellular melanin. Neither enhanced

tyrosinase activity nor melanin content could be attributed to the stimulation of melanocyte proliferation. Our observations are supported by histopathological analyses of hyperpigmented human skin which showed normal numbers of melanocytes, but elevated melanin accumulation (Hashiguchi et al., 1987; Pastor et al., 2002).

The biosynthesis of melanin polymers is mainly determined by the activity of the tyrosinase enzyme, which can be posttranslational modified by phosphorylation. Phosphorylation of serine 505 and 509 by PKC-β enhances the catalytic activity of tyrosinase (Park et al., 1999) and promotes interactions with tyrosinase-related protein 1 and 2 in the melanogenic complex, which further stabilizes tyrosinase (Wu and Park, 2003). Co-treatment of melanocytes with the PKC-β inhibitor bisindolylmaleimide I (50nM) had no effect on TCDD-induced tyrosinase activity, excluding altered PKC-β signaling as the underlying mechanisms for the observed induction of tyrosinase activity, even though TCDD has been shown to interfere with this pathway in other cells. Interestingly, several potential XREs were identified in the 5´-flanking region of melanogenic genes. Three, respectively two XREs with the core nucleotide sequence of GCGTG exist in the promoter region of tyrosinase and tyrosinase-related protein 2 and expression of both melanogenic genes was up-regulated upon TCDD-treatment. Co-treatment with MNF abolished the TCDD-induced tyrosinase gene expression, but had only a marginal effect on TYRP2 mRNA levels indicating a complex transcriptional regulation of this gene. Both melanogenic genes can therefore be considered as novel transcriptional targets for the AhR. Several other genes TYRP1, MCR1 and MITF, which are also involved in the regulation of melanogenesis, were tested upon ligand treatment, but none of them was induced at the tested time points.

Taken together, this study revealed the molecular mechanisms underlying xenobiotic-induced hyperpigmentation. As we show here, xenobiotic exposure induces melanogenesis in human melanocytes via direct activation of the transcription factor function of the AhR and the modulation of melanogenic genes.

4.4.3 A possible role of the AhR in UVB-induced melanogenesis

We showed that xenobiotic-induced hyperpigmentation, as studied after TCDD exposure, is mediated through the AhR signaling pathway. However, an up-regulation of tyrosinase enzyme activity was also noted after FICZ treatment. As expected, tyrosinase tyrosinase activity was early and transiently induced in FICZ-treated cells and higher concentrations (up to 100nM) were required for a successful induction probably due to the rapid metabolic degradation of FICZ. Thus, it seems that tyrosinase regulation is also under the influence of AhR signaling activated by physiological ligands. The major physiological stimuli of melanogenesis is UVB radiation (reviewed in Kadekaro et al., 2003). Because the AhR can be activated by UVB (Fritsche et al., 2007; Paper III), it is tempting to speculate that this receptor is also involved in the regulation of UVB-induced melanogenesis. Under the growth conditions (melanocyte growth medium M2) used in this study, melanocytes were extremely susceptible to UVB-induced apoptosis and showed decreased tyrosinase activities even at very low doses of irradiation. Melanocytes grown in M2 have high pheomelanin to eumelanin ratios (Duval et al., 2002) and may therefore be highly sensitive to UVB irradiation. Down-regulation of tyrosinase activity, growth cycle arrest (Im et al., 1998) and UVB-induced apoptosis (Bohm et al., 2005) have been observed in melanocyte cultures grown in the absence of α-MSH suggesting that the observed cytotoxic effects of UVB

on our cultures may be an effect of the medium composition. Therefore it was not possible, under the experimental conditions used here, to elucidate whether UVB-activated AhR mediates the stimulation of melanogenesis in irradiated human melanocytes. However, studies on AhR -/- mice revealed a reduced tanning response when compared to WT mice. These mice failed to enhance melanogenesis in response to UVB irradiation and showed impaired melanocyte proliferation and differentiation (B. Jux, personal communication). Whether AhR signaling mediates tanning in human melanocytes through similar mechanisms remains to be elucidated.

In conclusion, these data suggest that the AhR does not only mediate xenobiotic-induced hyperpigmentation, but also contribute to UVB-induced melanogenesis.

4.4.4 The AhR, a novel factor involved in the regulation of human melanogenesis

Many different factors and signal transduction pathways have been implicated in the regulation of melanogenesis (reviewed in Park et al., 2009). In this study, we identified the AhR as a novel regulator of human melanogenesis, a role that was greatly unknown to date. By regulating both adaptive responses e.g. the production of protective melanin and skin cell proliferation and differentiation, it seems that this receptor has important functions in maintaining skin homeostasis under normal conditions and in response to environmental stressors such as damaging UVB irradiation.

In this study we investigated the direct effects of AhR activation on the melanogenic pathway in melanocyte monocultures. However, melanocytes are greatly stimulated to proliferate and produce melanin by paracrine factors secreted from surrounding cells including dermal fibroblasts, Langerhans cells and keratinocytes (Archambault et al., 1995; Hirobe, 2005), which also express a functional AhR pathway. Especially keratinocytes, which form together with melanocytes functional epidermal melanin units, secret a number of different mitogenic and melanogenic factors. No differences in tanning were observed between conditional knock-out $K5Cre^{+}$ mice, deficient in AhR in keratinocytes, and WT mice, suggesting that keratinocyte-derived factors involved in the regulation of murine melanogenesis may not necessarily be under the control of the AhR (B. Jux, personal communication). Experiments using co-culture systems or organotypical cultures of human skin cells may clarify whether AhR signaling in keratinocytes, in cooperation with its observed directs effects on melanogenic genes in melanocytes, contribute to the regulation of human melanogenesis.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

As an approach to study AhR signaling in a more physiological context, two naturally-occurring substances of the indolocarbazole type, FICZ and ICZ, were used in this study. Both compounds elicited very transient responses in terms of activation of the transcription factor and ubiquitin ligase function of the AhR allowing thereby the receptor to keep its ligand responsiveness. Rapid resetting of signal transduction pathways into non-induced, basal states is clearly required for viable cells to respond to new stimulation. An important prerequisite for these transient activation patterns is the rapid degradation of activated receptor and the rapid metabolic clearance of activating ligands. As shown with FICZ, these ligands are both AhR activators and targets for AhR-controlled CYP1 metabolizing enzymes. Thus, these ligands induce their own metabolic breakdown. The autoregulatory circuit between CYP1A1 activity and AhR signaling is therefore an important mechanism to restrict the activating potential of ligands. Disturbance of this regulatory loop, for instance by impairment of CYP1A1 activity as observed after UVB irradiation or chemical inhibitor treatment, resulted in prolonged, TCDD-like responses. Continuous activation of the AhR, as observed in TCDD-treated cells, abolished dynamic cell signaling of both the AhR and other signal transduction pathways. It seems therefore that the disruption of dynamic regulation of AhR signaling may be the underlying mode of action for the toxicity of most non-metabolizable xenobiotics including TCDD and structurally similar compounds.

Besides xenobiotic exposure, normal cell signaling can also be disrupted upon UVB radiation. Cells try to compensate for the damaging effects of this physiological stimulus by initiating adaptive responses such as cell cycle arrest and DNA repair. As many other signaling pathways, the AhR is also activated upon UVB irradiation. AhR activity and CYP1A1 expression were restricted following irradiation by another UVB-activated transcription factors, NFκB. The modulation of AhR signaling and CYP1A1 expression may therefore be part of the UV stress response, protecting cells against the generation of more oxidative stress by elevated CYP1A1 levels and activity.

Especially skin cells are frequently exposed to UVB irradiation and chemical factors, including xenobiotics, due to their position as the outermost barrier of the body. Like in other tissues, AhR signaling seems to be also important for the maintenance of the homeostasis in the skin. The production of protective pigment is induced in melanocytes in response to both UVB and xenobiotic exposure. As identified here, AhR signaling mediated the induction of the melanogenic pathway through the transcriptional control of genes encoding enzymes involved in the biochemical formation of melanin. The regulation of melanogenesis is clearly a novel function of the AhR in the skin.

Normal cell signaling depends on the functioning of hundreds of signal transduction pathways, including the AhR signaling pathway. Numerous complex molecular mechanisms have evolved to control AhR activity and function as well as crosstalk with other pathways thereby ensuring proper signaling. As shown here, profound differences in AhR regulation exist between non-physiological and physiological ligands. Studies on AhR activation by xenobiotics may allow us indirectly to draw

conclusions about its involvement in physiological processes, but so far we have only got a glimpse of these. Thus, more has to be learned about the function and regulation of AhR signaling in a xenobiotic-free context. The use of naturally-occurring compounds as ligands will be extremely helpful to mimic the physiological situation under experimental conditions. In the end, this will help us to fully understand the physiological role of the AhR and to identify the physiological stimuli controlling this pathway.

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