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ESTROGEN AND ARYL HYDROCARBON RECEPTOR SIGNALING
CROSSTALK MECHANISMS

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ABSTRACT

The biological effects of estrogens are mediated by the two estrogen receptor (ER) isoforms, ERα and ERβ. The ERs are ligand-inducible transcription factors that belong to the nuclear receptor (NR) superfamily. Ligand-activated ERs bind to specific DNA elements in promoters of target genes, thereby inducing transcription. Due to the promiscuous ligand-binding cavity, ERs are prone to interference by environmental pollutants. Chemicals that disturb hormonal systems are known as endocrine disruptive chemicals (EDCs). Many EDCs are ligands for the aryl hydrocarbon receptor (AhR) that regulates the cellular response to xenobiotics. 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD; dioxin) is one example of an extremely toxic contaminant with endocrine disruptive properties that binds with high affinity to AhR. AhR and its heterodimerization partner AhR Nuclear Translocator (ARNT) belong to the bHLH-PAS superfamily, another class of transcription factors. Members of this family serve as sensors of various environmental stimuli, such as low oxygen tension and xenobiotic insult.

Crosstalk between the ER and AhR systems leads to inhibition of estrogenic signaling both in vitro and in experimental animals. In addition, reports of human exposure to dioxin describe adverse endocrine effects, such as alterations in sex ratio in children of exposed parents.

In this thesis, we have studied molecular mechanisms of crosstalk between ER and AhR signaling pathways. We have identified ARNT, the binding partner of AhR, as a coactivator of the ERs. ARNT coactivation function relies on the N-terminal region of ARNT, and the mechanism differs from that described for the classical p160 coactivators. In addition, ARNT shows a preference for ERβ. We could also show that the antiestrogenic effect of TCDD is partly due to competition between ER and AhR for its common cofactor ARNT and that ERβ activity is more susceptible to TCDD.

Furthermore, we have compared the biological effects of two prototypical AhR ligands, 3-methylcholanthrene (3-MC) and TCDD, on ER transcriptional activation. We show that these compounds exert distinct effects on ER signaling. TCDD and 3-MC differ in structure and stability: while TCDD is refractory to biotransformation, 3-MC is metabolized by enzymes in the exposed cells. We describe that 3-MC is converted into metabolites with estrogenic properties depending on cell type. The metabolites are active via the ligand-binding pocket of ERα.

The differences between 3-MC and TCDD have also been characterized on a genome-wide level in HepG2 cells. The results show that 3-MC, in contrast to TCDD, induces several known estrogen target genes. Gene induction is correlated with recruitment of ERα to the respective promoters. Furthermore, the estrogenic activities exerted by these compounds depend on ERα and do not function through ERβ, suggesting an ER isoform-specific mechanism of action.

In summary, we have identified a novel point of convergence between ER and AhR signaling. Our data also suggest that the antiestrogenic effects of TCDD exposure might be influenced by ER isoform expression. The studies of 3-MC highlight the importance of taking metabolism into account when evaluating the endocrine disruptive potency of chemicals.
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<tr>
<td>3-MC</td>
<td>3-Methylcholanthrene</td>
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<tr>
<td>AF</td>
<td>Activation function</td>
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<tr>
<td>AhR</td>
<td>Aryl hydrocarbon receptor</td>
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<tr>
<td>AP-1</td>
<td>Activator protein 1</td>
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<tr>
<td>AR</td>
<td>Androgen receptor</td>
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<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
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<tr>
<td>BFR</td>
<td>Brominated flame retardant</td>
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<tr>
<td>bHLH-PAS</td>
<td>Basic helix-loop-helix Per-ARNT-SIM</td>
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<tr>
<td>bMAL</td>
<td>Brain and Muscle ARNT-like protein</td>
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<td>Bisphenol A</td>
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<td>DBD</td>
<td>DNA-binding domain</td>
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<tr>
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<td>E2</td>
<td>17β-estradiol</td>
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<td>EDC</td>
<td>Endocrine disruptive chemical</td>
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<td>ER</td>
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<tr>
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<td>Estrogen response element</td>
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<tr>
<td>GREB1</td>
<td>Gene regulated by estrogen in breast cancer 1</td>
</tr>
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<td>HAH</td>
<td>Halogenated aromatic hydrocarbon</td>
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<tr>
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<td>Histone acetylase transferase</td>
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<tr>
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<td>Hypoxia-inducible factor</td>
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<tr>
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<td>Polycyclic aromatic hydrocarbon</td>
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<tr>
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<tr>
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<td>Persistent organic pollutant</td>
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<td>2,3,7,8-Tetrachlorodibenzo-(p)-dioxin</td>
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<tr>
<td>TIF2/SRC2</td>
<td>Steroid receptor coactivator 2</td>
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<tr>
<td>TR</td>
<td>Thyroid hormone receptor</td>
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<tr>
<td>XRE</td>
<td>Xenobiotic response element</td>
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1 INTRODUCTION

One feature that all living organisms share is the absolute requirement to adapt to a constantly changing environment. These changes are for example characterized by low or high availability of nutrients, temperature fluctuations or by the presence of compounds that disturb the normal functions of the cells. To survive, organisms must decipher the current environmental restrictions and adapt their physiological status accordingly. Therefore, diverse systems that sense changes in the surroundings have evolved, for example different receptors that convey the information from the outside to the inside of the cell. Cell surface receptors and intracellular receptors are parts of these systems.

One way for cells to respond to environmental changes is by increasing or decreasing the intracellular concentrations of protein, which is achieved mainly by altering the transcriptional rate of the corresponding mRNA. In eukaryotes, this is a highly controlled process. Inducible transcription factors that are switched on in response to a specific cue are key molecules in this control. Examples of sensory systems include ligand-inducible transcription factors belonging to the nuclear receptors or to the bHLH-PAS superfamily, which will be discussed below.

1.1 NUCLEAR RECEPTORS - OVERVIEW

1.1.1 NR structure

Nuclear receptors (NRs) are ligand-dependent transcription factors that belong to a large superfamily of proteins (reviewed in [2]). There are 48 known NRs in humans regulating a wide range of vital biological functions such as fetal development, homeostasis, reproduction, immune function and metabolism. The NRs mediate the biological effects of endogenous compounds such as sex hormones, cholesterol metabolites, bile acids, fatty acids, as well as exogenous substances like vitamins and a number of drugs and chemicals. However, for some of these receptors the ligand(s) are not yet identified, hence, they are referred to as orphan receptors.

NRs are evolutionarily conserved and have a modular structure. They can be classified into subgroups depending on their dimerization and DNA-binding properties [3, 4]. NRs consist of three main domains: the variable A/B-domain in the amino terminus, followed by the central DNA binding domain (DBD) containing the two characteristic zinc-finger motifs [5] and the carboxy-terminal ligand binding domain (LBD).

NRs contain two activation functions (AFs), which play crucial roles in NR activity. The ligand-independent AF-1 in the A/B domain can be of variable length and sequence among different NR family members and is bound by e.g. cell-specific coactivators or other transcription factors. AF-2 in the LBD is activated upon ligand-binding and is critically involved in transcriptional regulation.
1.1.2 NR signaling

In the absence of ligand, the NRs reside either in the cytoplasm or in the cell nucleus in a complex with chaperones and/or corepressors (reviewed in [6]). Transcriptional activation is initiated by ligand-binding, which triggers profound conformational changes that lead to dissociation of the repressive complex and in turn, to receptor dimerization and recruitment of transcriptional coactivators.

The coactivators of the p160 family (e.g. SRC1, TIF2, and SRC3) do not themselves appear to have DNA binding activity but are recruited to NR target gene promoters via protein-protein interactions with NRs [4]. P160 coactivators interact with a hydrophobic groove formed on the surface of the agonist-bound LBD of the receptor, and involve direct contact via the conserved LxxLL motif (NR-box) present in the coactivator [7]. The p160 family has been shown to possess intrinsic histone acetyltransferase (HAT) activity [8, 9] and they also recruit other chromatin-modifying factors such as cyclic AMP response element-binding protein (CBP)/p300, p300/CBP-associated factor (pCAF), and mediator proteins like Thyroid Receptor Associated Protein (TRAP) 220 that link this complex to the basal transcription machinery. Acetylation of the histones relaxes the chromatin structure creating increased access of the receptor-coactivator complex to response elements, which leads to transcription of NR target genes (reviewed in [10]). Alternatively, some NRs (e.g. TRs, RARs) are bound to their recognition sites on DNA at any time. In the absence of ligand, they are found in a complex with transcriptional corepressors, thus repressing target gene expression. Ligand binding triggers exchange of these corepressors with coactivators, which in turn leads to transcriptional activation.

Upon activation, the NRs bind to so-called response elements in the regulatory regions of target genes. For example, upon binding to estradiol, ER homodimers will associate with specific estrogen response elements (EREs) and thereby regulate the expression from estrogen target genes. The NR family is functionally versatile and the members may act by binding to DNA either as monomers (e.g steroidogenic factor-1), homodimers, or heterodimers with the common partner retinoid X receptor (RXR). NRs can activate gene expression both in the presence and absence of ligand and, in addition, some NRs may mediate non-genomic effects by interacting with kinases like phosphoinositide (PI) 3-kinase and extracellular signal-regulated protein kinase (ERK).

1.2 ESTROGEN RECEPTORS

1.2.1 ER biology

The physiological actions of estrogens are mediated by the two estrogen receptor (ER) isoforms ERα and ERβ that both belong to the NR superfamily. In the late 60’s, it was discovered that a receptor protein mediated the biological effects of estrogen, when ERα was isolated and characterized for the first time [11, 12]. Cloning of the human ERα cDNA was described in 1985 [13] and in 1996, a second estrogen receptor was cloned from rat prostate, termed ERβ [14].

The two ERs are not alternative splice variants of the same gene but are transcribed from different genes located on separate chromosomes and display discrete expression
patterns as well as distinct ligand specificities. In humans, ERα is expressed in the reproductive tissues (uterus, testis, breast, e.g.), kidney, bone, white adipose tissue and liver. ERβ has been found to be expressed in the ovary, prostate, lung, gastrointestinal tract, bladder, and hematopoietic and central nervous systems (reviewed in [15, 16]).

Generation of ER knockout (KO) mouse models has provided important information about ER signaling [17, 18]. The ERα KO phenotype reflects the many actions of estrogen in female reproduction as ERα deficiency leads to a complex phenotype with severe disturbances in several reproductive organs and infertility in both sexes. In ERβ KO mice, females are subfertile due to reduced ovarian efficiency, whereas the males are fertile (reviewed in [15]).

1.2.2 ER structure

Similar to other NRs, the ERs have a modular structure consisting of six functional domains labeled A-F. The amino terminal A/B domain displays only 17% homology between the human ER subtypes [19]. The highly conserved C-domains, containing the DNA-binding function, are 97% homologous at the amino acid level and both receptors can bind to the consensus ERE. Adjacent to the conserved DBD region lies the flexible D-domain, which acts as a “hinge” between the C and E domain and also comprises a nuclear localization signal (NLS). Finally, in the carboxy terminus of ER resides the F-domain, which seems to have a complex regulatory role [20, 21] (Figure 1).

The ligand-binding domains (E-domains) are regulated by ligand-binding and contain the AF-2, a dimerization interface and an additional nuclear localization signal. Although the E-domains of ERα and ERβ are only 56% identical at the amino acid level [22], both ERs bind estradiol with high affinity [23]. However, the existing differences provide a basis for differential responses to certain ligands, i.e. isoform-selective ligands [24].

On the subcellular level, the ERs are localized predominantly in the nucleus both in the unliganded and liganded state, due to the nuclear localization signals within the D- and E-domain [25, 26].

Both ERs display two transactivation domains, AF-1 and AF-2, and synergy between these two functions leads to full transcriptional activity. However, whereas the ligand-dependent AF-2 of the ER subtypes are equally potent, the ERβ AF-1 appears weaker and, thus, ERβ activity relies more on the AF-2 function [27].

![Figure 1. Schematic representation of the human ERs. Homology at the amino acid level is indicated (%).](image-url)
1.2.3 ER signaling

Upon ligand binding, the LBD of ER undergoes a structural change to provide a binding surface for cofactors such as p160 coactivators [28]. ERs exist as multiprotein complexes with coregulator molecules. The coregulators can either stimulate or inhibit transcription; hence, they are referred to as coactivators (e.g. SRC1, TIF2) or corepressors (e.g. NCoR, SMRT), respectively. The specific set of coregulators recruited in response to ligand binding, the relative abundance of certain key factors, as well as tissue-specific cofactors, will determine the transcriptional response to ligands. For instance, the synthetic ligand tamoxifen may act either as agonist or antagonist depending on promoter context. For instance, the activity at ERE sites is repressed by both ERα and ERβ in the presence of tamoxifen; however, Activator Protein 1 (AP-1) elements are activated by both receptors [29]. Cell type, the distinct set of recruited coregulators, as well as the architecture of the promoter, will influence the effects of tamoxifen.

ER signaling can be divided into two discrete modes of action, often referred to as genomic and non-genomic activity (Figure 2). The classical, genomic ER signaling occurs through direct binding of ligand-bound ER dimers to ERE sequences in the regulatory regions of estrogen responsive genes (Figure 2; 1A). The ERE consensus sequence consists of two half-sites, two 5-base pair inverted repeats, separated by any three base pairs, GGTCAnnnTGACC. However, most of the well-studied EREs from ER target genes are located at the proximal promoter regions and differ from the consensus sequence by 1-3 base pairs [30]. They also exhibit reduced binding affinity for ER [31]. Some target genes seem to be regulated by EREs situated far away from the transcriptional start site. For instance, both the TFF1/pS2 and GREB1 genes appear influenced by distal EREs [32-34].

Another type of genomic activity occurs through indirect association with promoters by protein-protein interactions with other transcription factors, such as AP-1 or Sp1 [35] (Figure 2; 1B). This non-classical model of ER signaling takes place at AP-1 or Sp1 binding sites, and possibly involves differential coactivator recruitment [36]. Both ERα and ERβ are able to modulate gene expression by either classical ERE-mediated signaling, or by interacting with other transcription factors like AP-1 and Sp1.

The second mode of action, referred to as non-genomic activity, has been shown to involve interactions with cytoplasmatic signal transduction proteins, such as Stat5, Stat3 and members of the Src family of tyrosine kinases [37, 38] (Figure 2; 2). This activity is characterized by rapid effects in response to E2, and membrane-localized ERs have been reported to mediate this signal transduction [39, 40].

1.2.4 ER ligands

Estrogens are hormonally active, structurally related, signaling molecules that control a wide range of key biological functions, such as reproduction, metabolism, and bone homeostasis. Estrogens are active in the central nervous system as well as in the cardiovascular and immune system (reviewed in [41]). The ovaries of women with active menstrual cycles produce 17β-estradiol (E2), the most active hormone, which can be converted into estrone and estriol [42]. Estradiol is converted from androgens
(e.g. testosterone) by the enzyme aromatase (CYP19). Outside the gonads, sites such as adipose tissue, bone and brain express aromatase and thus have the capacity to synthesize estrogens. In postmenopausal women, estrone is the predominant form of estrogen. Compared to E2, estrone binds to the ERs with lower affinity [42].

Figure 2. ER signaling mechanisms. 1A) “Classical” ER activity through direct binding to estrogen response elements (EREs). 1B) Activated ERs signal through protein-protein interactions with other transcription factors, such as AP-1 or Sp1. 2) Non-genomic activity involves other signal transducers such as Stats and kinases and causes rapid responses.

In addition to endogenous estrogens, the ERs also show affinity for environmental pollutants, such as certain polycyclic aromatic hydrocarbons, phthalates, and pesticides, thus termed xenoestrogens. Moreover, dietary plant-derived compounds like flavonoids and lignans, so called phytoestrogens, have been found to induce ERs and their influence on human health is believed to be mainly beneficial [43-46].

The promiscuous ligand-binding cavity of the ER makes both receptor isoforms important targets for pharmaceuticals. For instance, the synthetic estrogen tamoxifen has been in clinical use for 30 years for treatment of breast cancer. Tamoxifen is a partial agonist-antagonist, as it acts as an antagonist in breast but as an ER agonist in certain key tissues such as uterus and bone [47].
1.3 BHLH-PAS PROTEINS - OVERVIEW

Another class of transcription factors is the basic helix-loop-helix Per-ARNT-SIM (bHLH-PAS) superfamily. bHLH-PAS proteins are key regulators in many essential physiological processes, controlling adaptive responses to environmental changes [48, 49]. The members of this superfamily can be classified as either sensors that detect and respond to environmental cues, or as heterodimerization partners for other bHLH-PAS members resulting in functional transcriptional units. The term PAS is an acronym of the three founding members of this family: Per, ARNT and SIM. Per and SIM were identified in the fruit fly Drosophila as products of the Period and the Single-minded loci, respectively [50-52]. SIM is a key player during neural development, whereas PER together with Clock and bMAL, additional bHLH-PAS members, are involved in control of circadian rhythms. Other important members are the sensors of low oxygen tension, Hypoxia-Inducible Factors HIF1α and HIF2α.

Among the partner factors within the bHLH-PAS family, the AhR nuclear translocators (ARNT) 1-3 are found. These proteins are important as dimerization partners for several bHLH-PAS sensory factors and thereby critical for the transcriptional output of these factors [48]. ARNT-1 (hereafter referred to as ARNT) and ARNT-2 are highly homologous and serve as partners for AhR and the HIFs [53], whereas ARNT-3 (also known as bMAL) is selectively recruited by Clock [54].

The bHLH-PAS family has a conserved structural arrangement with the bHLH domain located in the N-terminal part of the protein (Figure 3). The basic region is required for specific DNA binding to target regulatory DNA and the HLH part is the main dimerization interface of the bHLH-PAS proteins. The PAS domain, located immediately after the bHLH domain, harbors two conserved subdomains termed the A and B repeats. In mammals, the PAS domain functions as interaction surface for both other family members, as well as cellular chaperones such as the hsp90 molecular chaperone complex (reviewed in [55]). Furthermore, most bHLH-PAS members have transcriptionally active domains in the C-terminal end of the protein.

![Figure 3. Schematic representation of some members of the bHLH-PAS family of transcription factors.](image-url)
1.4 ARYL HYDROCARBON RECEPTOR

1.4.1 AhR biology

The aryl hydrocarbon receptor (AhR; sometimes referred to as the dioxin receptor, DR) is an evolutionary well-conserved inducible transcription factor belonging to the bHLH-PAS proteins. AhR is expressed in diverse mammalian species, in lower vertebrates e.g. zebrafish (zfAhR2; [56]) and in invertebrates like the fruit fly Drosophila (spineless; [57]) and the nematode Caenorhabditis elegans (CeAHR; [58]). In humans, AhR is ubiquitously expressed but displays highest expression levels in lung and placenta and lowest in kidney, brain and skeletal muscle [59].

AhR serves as a sensor molecule and is mainly described as a regulator of the cellular responses to xenobiotic substances. It is the only bHLH-PAS family member known to bind ligands. However, it is clear that the AhR is involved in many other physiological processes, such as modulation of the immune system, and that interference with these functions causes toxicity (reviewed in [60],[61]). It is believed that the ancestral function of AhR may have been as a developmental regulatory factor, which later in evolution acquired the ability to induce cytochrome P450 1A1 (CYP1A1) and other metabolizing enzymes. This adaptation led to the ability to bind PAHs, thus eventually forming the basis for the toxic actions of dioxin and related compounds [62, 63].

To gain insight into the physiological roles of AhR, AhR null mice and transgenic mouse models have been generated by several groups [64-66]. Apart from resistance to the diverse toxicities exerted by TCDD [67], AhR-deficient mice display reduced weight, reproductive problems and liver abnormalities, such as failure of ductus venosus closure [68-70]. The spectrum of developmental defects observed in AhR-/- mice in combination with the fact that the AhR system is well conserved suggests that AhR has a major function for the developing organism.

Recently, AhR function in the immune system has been investigated in regulatory T cells and in the mouse model for multiple sclerosis (MS) [71-73]. Human autoimmune diseases such as MS are influenced by many factors, for instance environmental factors. The results from these studies point toward an important regulatory role for AhR in T cell differentiation. Interestingly, AhR regulates distinct populations of T cells in a ligand-dependent fashion, making it a promising target for pharmaceutical therapy. Furthermore, it provides a link between environmental pollutants and the immune system.

1.4.2 AhR signaling

The unliganded AhR is found in a complex consisting of cellular chaperones and chaperone-like proteins [74]. The complex, which resides mainly in the cytoplasmatic compartment, serves to anchor AhR in the cytoplasm and to protect the receptor from degradation [76, 77]. Also, the role of this complex is to keep AhR in a conformation capable of binding ligand [75].

Ligand-binding leads to translocation of AhR into the nucleus where it dimerizes with its partner protein ARNT. Formation of the AhR/ARNT heterodimer converts the complex into its high affinity DNA binding form, which then binds to specific
regulatory DNA sequences known as xenobiotic response elements (XREs) located within the promoters of target genes. The core XRE is defined as TNGCGTG and it has been reported that AhR binds to the first part of the XRE (TNGC) while ARNT interacts with the second half site (GTG) [78, 79]. Binding to the XRE leads to recruitment of specific coactivators, e.g. CBP/p300, coactivators of the p160 family, BRG1, Thyroid Receptor Associated Protein TRAP220 and nuclear receptor coactivator 4 [80, 81]. Finally, the basal transcription machinery is recruited to the promoter.

AhR activity leads to increased gene expression from a battery of AhR target genes like CYP1A1, CYP1A2, CYP1B1, glutathione-S-transferase (GST), NADPH-quinone oxidoreductase and xanthine oxidase [82, 83]. The products of these AhR-regulated genes are involved in metabolism of xenobiotic compounds, as well as of many endogenous substances, like steroid hormones.

Although the best described AhR signaling is through functional XREs, which have been found in numerous genes, its activity also depends on phosphorylation status and cell- and promoter context. Moreover, AhR ligands have been reported to induce degradation of the AhR through the proteasome [84-86], and to upregulate the AhR repressor protein (AhRR), thus repressing AhR action [87, 88].

1.5 ARYL HYDROCARBON RECEPTOR NUCLEAR TRANSLOCATOR

1.5.1 ARNT function

Aryl hydrocarbon receptor nuclear translocator (ARNT), the obligatory binding partner for AhR, also belongs to the bHLH-PAS proteins. Among the other partners for ARNT are the hypoxia-inducible factor HIF1α, SIM and Per. In order to form functional transcriptional complexes, these proteins need to form heterodimeric complexes with ARNT.

The role for ARNT as a general partner factor for bHLH-PAS members is well characterized. Whether or not ARNT homodimers have a regulatory role, is not fully elucidated. However, ARNT has been shown to form functional homodimers and activate an E-box regulated reporter in vitro [113, 114]. Given ARNT’s wide spread tissue distribution, its tendency to form homodimers and its nuclear localization, there are indications for a function of ARNT in regulation of transcriptional activity [115]. The structure of ARNT conforms to the consensus for the bHLH-PAS members (see Figure 3). The N-terminal part contains the bHLH and a nuclear localization signal. The PAS domain comprises two subdomains, PAS A and PAS B. The C-terminal part harbors a strong transactivation domain, functionally discrete from the DNA binding and heterodimerization functions [116].

The transactivation capacities of AhR versus ARNT display differences in their modes of action. The transactivation domain of AhR is only functional when AhR is heterodimerized with ARNT, or in vitro when isolated from the rest of the protein, whereas ARNT’s transactivation domain is constitutive and expressed in the context of the intact protein [108].
1.5.2 ARNT biology

In rat, ARNT is expressed in all tissues tested [117]. Also in the developing mouse embryo, there appears to be ubiquitous expression of ARNT mRNA [118]. Disruption of the Arnt allele in mice leads to an embryonic lethal phenotype, which results in severe defects in angiogenesis and placental development [119, 120]. These findings are consistent with the function of ARNT in the response to hypoxia where ARNT-HIF1α dimers regulate VEGF signaling thus promoting angiogenesis. Conditional Cre-loxP mutants have been generated to study the effects of ARNT loss, for instance in liver and in T cells [121, 122]. These studies showed that mice lacking ARNT expression were inhibited in AhR and HIF1α signaling and they also appeared resistant to TCDD-induced thymic involution.

Interestingly, ARNT has been identified to be heavily downregulated in patients with diabetes type 2. When further analyzed in mice, it could be demonstrated that ARNT suppression severely impairs the insulin release in response to glucose, as well as other features resembling those observed in diabetic humans [123]. These data indicate a novel regulatory role for ARNT.

1.6 AHR LIGANDS

1.6.1 Exogenous and endogenous substances

AhR activities can be induced by a spectrum of structurally diverse synthetic and naturally occurring chemicals (Figure 4). However, the best-characterized high affinity ligands for the AhR are planar hydrophobic molecules including ubiquitously present environmental pollutants, such as halogenated hydrocarbons (HAHs) and the non-halogenated polycyclic aromatic hydrocarbons (PAHs) (reviewed e.g. in [89]). The HAH group includes dibenzofurans (PCDF), biphenyls (PCB) and polychlorinated dibenzo-p-dioxins (PCDDs). Most HAHs are lipophilic and resistant to biodegradation due to their halogenation. The PAH group contains members like benzo(a)pyrene, 3-methylcholanthrene, benzoflavones, rutacarpine alkaloids, and aromatic amines. Generally, the PAHs have lower affinity for AhR than the HAHs; the most potent HAH congener, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD; dioxin) has the highest affinity for the AhR among all known synthetic ligands [90]. Some HAHs have been used for commercial purposes (e.g. PCB), however, most of these chemicals are by-products generated by combustion of carbon-containing materials such as wood, coal, diesel, household waste and tobacco [91].

The AhR ligands also include a wide array of natural compounds. The majorities of identified AhR ligands that do not have anthropogenic origin are in fact diet-derived and represent perhaps our greatest exposure source. The presence of AhR ligands in different vegetables, fruits, herbs and teas has been reported [92-94]. For instance, flavonoids, carotinoids and phenolics can activate the AhR pathway. Additionally, some endogenous activators for AhR have been found. Conversion of dietary indoles (e.g. idole-3-carbinol and tryptophan) in the digestive system results in very potent AhR agonists. Moreover, the biological activity of AhR is strongly activated by
photoproduts of tryptophan, like 6-formylindolo[3,2-b]carbazole (FICZ) in vitro [95-97] and in vivo [98]. It has been suggested that tryptophan photoproduts may function as light sensors as they induce changes in the expression of genes regulating the circadian rhythm [98]. Thus, AhR may play a role in the response to light-darkness cycles and expression of AhR target genes would therefore follow a diurnal/nocturnal pattern. Interestingly, it was recently shown that the bHLH-PAS family member Clock contains intrinsic HAT activity ([99]. This finding implicates that histone acetylation and chromatin remodeling are events required for normal circadian function.

![Figure 4. Examples of AhR ligands.](image)

1.6.2 2,3,7,8-Tetrachlorodibenzo-p-dioxin

2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is a prototypical AhR ligand and the most potent HAH congener. TCDD is an omnipresent environmental pollutant, due to its chemical stability and persistency. The positioning of the chlorine atoms in TCDD inhibits oxygenation by the metabolizing enzyme CYP1A1, thus, TCDD is metabolized extremely slowly. In humans, the biological half-life of TCDD is estimated to 7-9 years [100]. As it is highly lipophilic, TCDD tends to accumulate in tissue. It has been proposed that the main basis for the toxicities resulting from TCDD exposure is the capacity to cause sustained activation of the AhR-mediated pathway. Due to its chemical persistency, TCDD will be bioconcentrated in the environment, which results in high levels in the end of the food chain.

TCDD became known as contaminant in Agent Orange, a defoliant used in the Vietnam War. It has also been released into the environment during industrial accidents, such as the Seveso accident in Italy in 1976. TCDD is present in combustion
emissions of various sources, such as incineration of wastes, power plants that use coal or oil, wood burning and home heating systems. The compound has been a contaminant in the manufacture of various pesticides and as a result, has been directly spread in the environment during the use of these pesticides. TCDD is also formed in car and diesel exhaust and during the synthesis and combustion of polyvinylchloride. Occupational exposure to TCDD may occur through inhalation and dermal contact to fire fighters and cleanup workers involved with PCB transformer fires or with incineration operations. It may also affect workers handling pesticides.

Biomonitorg data indicate that the human population is continuously exposed to low doses of TCDD primarily through ingestion of contaminated food. Foods high in fat from animal origins, like milk and other dairy products, beef, fish and eggs, comprise 95% of the estimated total daily exposure to TCDD. TCDD has been found in human blood, serum, adipose tissue and breast milk. Of major concern is the fact that breast-fed children are the most highly exposed group, and data indicate that 50% of the total dioxin burden is ingested during the first years of life [101-103].

Within the European Union, the estimated Tolerable Daily Intake (TDI) for PCDD, PCDF and PCB-like dioxins has been set to 2 pg TEQ/kg bodyweight [105]. Notably, large parts of the European population are exposed to daily doses of up to 3.0 pg TEQ/kg bodyweight, i.e. levels higher than the TDI.

The extreme toxicity of TCDD is reflected by the plethora of biological effects manifested following exposure ranging from teratogenic effects, immunosuppression through thymic involution and tumor promotion. Numerous animal studies have been conducted demonstrating remarkable inter-species differences in susceptibility. For instance, the guinea pig is extremely sensitive whereas a hamster tolerates more than 1000-fold as much, as measured by their lethal dose (LD$_{50}$) values.

There are human exposure data from accidental and occupational exposure events [106]. One of the hallmarks of high TCDD exposure in humans is the dermal condition chloracne, which has been reported from soldiers and residents in Vietnam following Agent Orange release, as well as in children and adults after the Seveso accident. In 2004, the Ukraine president Viktor Yushchenko was supposedly poisoned with TCDD and developed severe chloracne.

1.6.3 3-Methylcholanthrene

3-Methylcholanthrene (3-MC) is a prototypical AhR ligand and a potent mutagen. 3-MC belongs to the group of PAHs, comprising over 100 chemicals. Some of the most commonly occurring environmental PAHs are anthracene, benz(a)anthracene, benzo(a)pyrene, benzo(b)fluoranthene, chrysene, fluoranthene, fluorene, naphthalene, and pyrene, to mention a few. PAHs are formed through burning of organic material such as oil, wood, and tobacco. Many are classified as human carcinogens.

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1Toxic Equivalents (TEQ). Potencies of PCDD, PCDF and PCB-like dioxins are usually estimated according to the Toxicological Equivalent Factor (TEF) system, where the toxicity of each congener is attributed a TEF relative to TCDD, the most toxic PCDD. To calculate the total TEQ of a dioxin mixture, the amounts of each compound are multiplied with their TEF and then added together. 104. Van den Berg, M., et al., The 2005 World Health Organization reevaluation of human and Mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol Sci, 2006. 93(2): p. 223-41.
PAHs generally require metabolic activation in order to exert genotoxicity (reviewed in [107]). In mammals, the PAH metabolism primarily occurs in the liver by cytochrome P450 enzymes. CYP1A1 can metabolize a wide range of PAH molecules (reviewed in [108]). CYP1A1 is present in many tissues but the background levels are relatively low, however, it is strongly induced following exposure to PAH or certain other chemicals. In addition, CYP1A2 and the CYP1B subfamily can metabolize PAHs. Upon metabolism, the PAHs become more polar and water-soluble, hence they can be more easily excreted. However, metabolism of PAHs generates bioactive derivatives, such as arene oxides, that may covalently bind to and form adducts with DNA, thus leading to mutations.

3-MC is not considered an environmental pollutant, although it is not clear to what extent 3-MC is present in tobacco smoke, industrial emissions, and various other PAH emissions. Due to the mutagenic properties of 3-MC, it has frequently been used to study carcinogenesis. In contrast to TCDD, 3-MC is a so called procarcinogen, meaning that the parental compound may be biotransformed into various reactive derivatives of which several have carcinogenic potential [109-112].

### 1.7 ENDOCRINE DISRUPTION – GENERAL ASPECTS

#### 1.7.1 Molecular mechanisms of endocrine disruptive chemicals

Endocrine disruption, the phenomenon that exogenous substances interfere with the endocrine system, has raised worldwide concern during the last decades. Endocrine disruptive chemicals (EDCs) pose a documented risk to wild life and ecosystems. For instance, eggshell thinning in birds of prey, reduction of the Baltic seal population and sex reversal in aquatic snails have been observed and linked to EDCs in the environment [124]. In addition, EDCs have been shown to exert numerous hormone disruptive activities in experimental animals. These factors, in combination with the increased incidence in certain endocrine-related human diseases, have led to the assumption that EDCs are potential health threats also in humans.

EDCs can act at multiple sites via multiple mechanisms of action. However, for most associations reported between exposure to EDCs and a variety of biological outcomes (e.g. TCDD in the Seveso accident), the mechanisms of action are poorly understood. This makes it difficult to distinguish between direct and indirect effects, and primary versus secondary effects of EDCs exposure.

Although EDCs could affect every possible hormonal pathway, most is known about interference of EDCs with the hormone receptors of the NR family, i.e. the steroid hormone receptors and the thyroid hormone receptor. There are several possible modes of action (Figure 5), such as binding to the hormone receptor like an agonist/antagonist (A), disturbing the receptor activity by activating alternative pathways (B) and altering cellular availability of the circulating hormone by interfering with its metabolism (C).
1.7.2 EDCs and human disease

Considering the continuous low-level exposure of the general population, it has been difficult to establish a direct causal association between a particular EDC and adverse health outcomes. Human studies are often conducted under different experimental conditions, which make comparisons impossible, and exposure data are often lacking.
In the global assessment report by WHO, several human health effects are discussed such as reproductive effects (sperm counts, spontaneous abortions, sex ratios, gonadal abnormalities), cancers and immune function. It is concluded that there are strong indications of human health problems caused by EDCs, which makes this area a high research priority [124].

One recently debated example of an EDC is bisphenol A (BPA), a monomer with estrogenic properties. It is used in its polymeric form as lining of food and beverage containers, baby bottles, medical tubing and dental fillings. BPA has been shown to leak from the plastic, both when heated and in room temperature [125, 126], and human exposure is considered substantial [127]. It is a lipophilic compound and can accumulate in fat, and it has also been found in human plasma, urine and breast milk (reviewed in e.g. [128]). Recently, an epidemiological report describe a link between high urinary levels of BPA and cardiovascular disease, liver abnormalities and diabetes in a US adult population cross-sectional study, establishing a clear correlation between high BPA exposure and disease in humans [129].

Another example is a study of persistent organic pollutants (POPs) in a cohort of Swedish fishermen’s families with high lifetime consumption of POP contaminated Baltic Sea fish. POP burden was correlated to adverse health effects like increased incidence of diabetes type 2, and increased risk of osteoporosis and breast cancer within this cohort [130-134].

1.8 AHR-ER CROSSTALK

1.8.1 In vivo data

Several studies have shown intricate physiological relationships between the AhR and estrogen receptor systems. Animal models, such as the AhR null mice, have provided significant evidence for AhR in reproduction. For instance, it has been demonstrated that female AhR null mice have reduced reproductive success and that the AhR regulates expression of the ovarian P450 aromatase, CYP19, a key enzyme in estrogen synthesis [68].

Several studies report adverse effects of TCDD in the reproductive tract of mice and rats, for instance inhibition of uterine wet weight increase, progesterone receptor binding and c-fos mRNA levels [135-137]. Long-term, low-dose TCDD exposure of female rats was demonstrated to reduce the rates of mammary and uterine tumors, suggesting that TCDD inhibits tumor formation in certain tissues [138]. Moreover, women exposed in Seveso display lower incidence of breast cancer suggesting inhibitory AhR-ER crosstalk [139].
1.8.2 Molecular mechanisms

Some possible mechanisms that have been implicated in AhR-ER crosstalk and experimental data that support them are summarized below (reviewed in [140]).

a. **Competition at promoter level.**

ER transactivation is repressed by the binding of liganded AhR to XREs, which blocks the ER binding or formation of the ERα/Sp1 or ERα/AP-1 complex. This mechanism has been reported to occur at the c-fos, pS2, cathepsin D and Hsp27 promoters [141-145].

b. **Competition for common regulatory proteins.**

Simultaneous induction of ER and AhR leads to competition for common regulatory factors or coactivators, thus inhibiting full transcriptional activity of the receptors by squelching [146-149].

c. **Degradation of ER.**

TCDD has been shown to enhance degradation of ERα by targeting it to the proteasome, thus lowering the cellular levels of ER [150]. In addition, AhR-ARNT has been reported as part of an ubiquitin ligase complex involved in targeting proteins to the proteasome. The assembly of this complex seems to be AhR ligand-dependent [151].

d. **Accelerated metabolism of estradiol.**

TCDD and other AhR ligands activate the CYP1A1 and 1A2. Given that circulating estrogens are metabolized in the liver, predominantly by 1A1 and 1A2, TCDD exposure could lead to altered hormonal balance [152].

Estrogenic effects of AhR ligands have also been reported. Ohtake and coworkers proposed that the estrogenic effects exerted by 3-MC, demonstrated in MCF-7 and Ishikawa cells, as well as on mouse uterine wet weight, was due to recruitment of liganded AhR to the ERα. AhR thus acted as a coactivator of ERα [153].

In contrast to these findings, Abdelrahim et al showed that the prototypical AhR ligands 3-MC and 3,3’,4,4’,5-pentachlorobiphenyl (PCB, or PCB126) were able to induce ERE-regulated activity in MCF-7 breast cancer cells. The mechanism of the estrogenic activity was proposed to be dependent on, and directly mediated by, ERα as evidenced by ligand-binding assays. 3-MC also induced increased uterine wet weight and estrogen-responsive cyclin D1 mRNA in both AhR^{+/+} and AhR^{-/-} mice demonstrating that the estrogenic effects were not depending on AhR [154]. The authors suggest that the discrepancies between theirs and the Ohtake study could be explained by different 3-MC concentrations.
\section{AIMS OF THE STUDY}

The general aim of this thesis has been to characterize possible crosstalk mechanisms between ER and AhR signaling pathways. In particular, our objectives have been to:

\begin{itemize}
  \item Analyze the function of the bHLH-PAS protein ARNT in the estrogen signaling pathway (\textit{Paper I}).
  \item Characterize the ARNT-ER interactions, with special focus on the molecular mechanisms and putative influence on the antiestrogenic effects of TCDD (\textit{Paper II}).
  \item Study the effects of the AhR ligands TCDD and 3-MC on the ER signaling in different cell lines (\textit{Paper III}).
  \item Compare the effects of TCDD and 3-MC in HepG2 cells on a genome-wide level (\textit{Paper IV}).
\end{itemize}
3 MATERIALS AND METHODS

The results presented in this thesis are based on some experimental procedures that were used frequently. In this section, I will shortly discuss the methods that were used in more than one of the included papers (transfections, luciferase assays, RNA interference, ChIP, RT-PCR and real-time PCR). Moreover, I will briefly describe the cell lines that were used throughout these studies.

3.1 CELL LINES

The experimental data presented in this thesis were all obtained from cells in culture. Although animal models are an essential part of medical science, the need to replace animals due to ethical and economical reasons is increasing. Immortalized cell lines derived from various tissues and species are useful tools as they are versatile, can be propagated infinitely, and can be manipulated for diverse mechanistic studies.

In this thesis, various cell systems were employed:

• COS-7 and CV-1 cells, which are derived from kidney cells of the African green monkey [155]. These cell lines were transiently transfected with various plasmid constructs, such as ER or ARNT expression plasmids, or luciferase reporter constructs.

• HeLa cells (human cervical carcinoma; described in e.g. [156, 157]). Stable HeLa cell lines expressing either ERα or ERβ together with an ERE-regulated reporter (HELN ERα and HELN ERβ), were obtained from the lab of Patrick Balaguer (INSERM, Montpellier) and have been useful tools in this work (described in [158]).

• HepG2 liver cells (derived from human hepatoma) have high metabolic capacity [159, 160]. We also obtained stable HepG2 cell lines expressing either ERα or ERβ together with an ERE-regulated reporter (HepELN ERα and HepELN ERβ) from Patrick Balaguer.

• A human breast cancer cell line, T47D, which has a high endogenous expression of ERα and contains all essential components for functional estrogen signaling was used [161].

• MCF-7 cells; also derived from human breast cancer, and with high endogenous expression of ERα [162, 163]. Furthermore, MCF-7-ERβ cells were obtained from the lab of Anders Ström, and these cells express the short ERβ isoform (ERβ 485) with a flag-tag and under the control of an inducible tet-off system.

• HC11, a mouse mammary epithelial cell line that has the potential to differentiate into lactating tissue, and expresses both ERα and ERβ [164]. This cell line has low metabolic capacity. We have also frequently used a variant HC11 cell line, HC11-ERE, which contains a 3xERE-TATA-luciferase reporter construct stably incorporated (described in [165]).
3.2 TRANSIENT TRANSFECTION ASSAYS

Transient transfections have been frequently used in this thesis. Certain cell lines are refractory or sensitive to the transfection procedure, but this method is a useful tool for introducing exogenous DNA into a cell. It leads to a transient transformation of the transfected cells while they are transcribing and expressing the foreign construct. The transformation is not stable; hence, the effect will be diluted in dividing cells. Depending on doubling time of the cell line, the effect will last for some days.

As transfection method, we have generally used Lipofectamine reagent (Invitrogen), which relies on the lipidfection principle. This method delivers DNA by liposomes that are fused to the cell wall and thus introduce foreign DNA into the cell. It is a rather mild transfection method that most mammalian cells can support, in contrast to for instance electroporation that disrupts the cell wall by electricity.

3.3 LUCIFERASE ASSAYS

Luciferase reporter assays have been used to assess the transactivation capacity of for instance the ERs. Typically, we have transfected cells with a promoter-reporter gene construct (e.g. an ERE-regulated luciferase construct), allowing the construct to be expressed for 24 hours (e.g. in the presence of E2), and then measured the accumulated luciferase. This method relies on transcription of the luc gene, and subsequent translation of the luciferase protein. Luciferase is an enzyme that catalyzes the substrate (luciferin) in a reaction that emits bioluminescence (i.e. light), which can be measured. Luciferase is a non-toxic, stable protein and will be accumulated within the cells, and its activity can be measured and quantified by a luminometer. The light emission correlates linearly with transcriptional activity. Generally, this is an easy, sensitive and reproducible method to assess the activity of a transcription factor. In recent years, it has become more common to assess transcriptional activity of a certain transcription factor by measuring mRNA production of its target genes using real time PCR (sections 3.6 and 3.7). However, luciferase assays still have some advantages over real time PCR. For instance, RNA preparations can be influenced by RNA stability and are also more laborious.

As internal control, we have cotransfected β-galactosidase together with the luciferase reporter used. This is a second reporter that can be used to normalize for transfection efficiency or cell lysate recovery between treatments. A constitutive promoter drives its expression, and the β-galactosidase activity in each sample can be measured in a separate assay.

3.4 RNA INTERFERENCE

RNA interference (RNAi) is a sequence-specific gene silencing [166-168]. The process is mediated by short (21-28 nucleotides) double-stranded RNA molecules (siRNA), where the anti-sense strand of the siRNA serves as a template for an enzymatic complex that recognizes and subsequently cleaves complementary RNA (mRNA). This mRNA is then rapidly degraded. The siRNA method was used in this thesis as a tool to “knockout” or reduce the protein levels of, for instance, ARNT or AhR in a cell. The
drawbacks of this approach are mainly that the success relies on the delivery into the cells (i.e. transfection), in contrast to for instance viral transduction methods and that the effect of the siRNA is diluted in dividing cells.

### 3.5 CHROMATIN IMMUNOPRECIPITATION

Chromatin immunoprecipitation (ChIP) was used to assess the binding of various proteins to a region of the DNA, e.g. a promoter sequence. Following treatment with ligand of interest, the cells are treated with formaldehyde to induce crosslinks between the chromatin and DNA-bound proteins. Thereafter, the chromatin is sonicated to cleave the DNA into smaller fragments (preferably around 200 bp). The DNA fragments are incubated with a specific antibody directed against the protein of interest, which then may be immunoprecipitated, still cross-linked to DNA. The crosslinks are then reversed and the DNA can be eluted. The eluted DNA is enriched for the regions that the protein of interest binds to.

The DNA can be analyzed by conventional PCR or by real-time PCR (discussed below). By real-time PCR it is possible to reliably quantify the recruitment to the promoter region. As a control, non-treated samples can be used. However, it is extremely important to include a mock sample without antibody and a non-specific IgG control in the immunoprecipitation step in order to calculate the background binding to the beads.

### 3.6 REVERSE TRANSCRIPTION PCR (RT-PCR)

One way to estimate the output of a treatment is to measure the mRNA that is transcribed from a gene of interest. This can be achieved by extracting total RNAs from tissues or cells following treatment and reversely transcribe the RNAs into complementary DNA (cDNA). Reverse transcription is done with a reverse transcriptase enzyme, which is a DNA polymerase enzyme that converts single-stranded RNA into double-stranded DNA. The relative amount of the particular cDNA of interest can then be assessed by PCR using specific primers and the resulting PCR product is proportionate to the target mRNA.

In general, there are three priming methods used in RT-PCR (review in e.g. [169]): gene-specific primers, random hexamers or oligo-dT primers. Oligo-dT primers anneal to the polyadenylated (poly A+) mRNA tails, which results in only one cDNA per mRNA. This leads to specificity since ribosomal RNA will not be transcribed. However, it is not possible to normalize to the ribosomal 18S RNA when using this approach. In addition, only full-length mRNAs will be transcribed which is a major drawback since the RNA may be fragmented. In this thesis, random hexamers were used. The hexamers align to multiple sites along the mRNA, which results in several copies of the desired cDNA leading to less specificity but also higher yields, as well as least bias in the resulting cDNA [170].
3.7 REAL-TIME PCR

Real-time PCR is a very sensitive method for quantification of specific mRNA targets. Whereas conventional PCR measures the build-up of amplification products at the end of the cycling, real-time PCR measures amplification from each transcript continuously, in real-time.

One approach within this technology uses non-specific DNA intercalating dyes, such as SYBR Green, that bind to any double-stranded DNA generated during the PCR. By binding, fluorescence is emitted. The specificity of the primers must be thoroughly tested by melt curve analysis to confirm one single PCR product. The other approach, using gene-specific probes such as TaqMan probes, is more specific but also very expensive. In this work, SYBR Green was utilized and melting curves have been made for each new primer pair.

Of special concern is the choice of an endogenous reference gene. Preferably, this gene shall remain stable and not be regulated by the treatments, and therefore house-keeping genes such as actin or GAPDH have frequently been used. However, actin and GAPDH have been shown to be regulated by various treatments and in different cell lines, while 18S rRNA remained stable e.g. [171, 172]. In this thesis, 18S rRNA was used as an internal standard. According to my own measurements, this gene was stable during the treatments and, thus, functions as an acceptable normalization gene.
4 RESULTS AND DISCUSSION

4.1 THE BASIC HELIX-LOOP-HELIX-PAS PROTEIN ARNT FUNCTIONS AS A POTENT COACTIVATOR OF ESTROGEN RECEPTOR-DEPENDENT TRANSCRIPTION (PAPER I)

The fact that the p160 family of NR coactivators has structural similarities with the bHLH-PAS transcription factors prompted us to investigate the effects of the bHLH-PAS protein ARNT on the estrogen receptors.

To analyze whether ER and ARNT were associated through protein-protein interactions, co-immunoprecipitation assays were carried out. The results showed that both ER subtypes interact with ARNT, independently of hormone. In transfection experiments, we could establish that ARNT strongly potentiates ER activity to the same extent as the classical coactivator TIF2. In addition, ARNT displayed ER-isoform selective properties with a marked preference for ERβ. TIF2, which belongs to the p160 family, interacts with the ERα through LxxLL motifs in a ligand-dependent manner [173, 174]. Hence, the mechanism of coactivation seems to differ between TIF2 and ARNT.

In light of these results we wished to test the activity of other bHLH-PAS members on ER activity. Two closely related proteins, ARNT-2 and bMAL, were investigated. The three family members are highly homologous in their bHLH and PAS domains but bMAL differs considerably from the others in the region C-terminally of the PAS domain. The experiments showed that bMAL failed to coactivate ER transcriptional activity while ARNT-2 and ARNT were equally potent, indicating that the C-terminal part containing a transactivation domain may be the key domain for coactivation. Using deletion mutants, the C-terminal was demonstrated to be indispensable for ARNT’s coactivating function of ER. This observation is in contrast with ARNT function when in complex with AhR or HIF1α, where the C-terminal transactivation domain is dispensable [175]. Instead, the bHLH and PAS domains have been shown essential for AhR and HIF1α function [176, 177], suggesting that ARNT has at least two distinct modes of action.

Using a similar approach, the involved domains of ERs were determined by transient transfections with various ER constructs. The results showed that ARNT was still able to potentiate ER transcriptional activity when the A/B domains were absent. Hence, it was concluded that the LBDs of ERs, not the A/B domains, are involved in the interaction with ARNT. However, these experiments revealed differences between the ER subtypes. When comparing the effects of ARNT on either full-length ERs or on the isolated LBDs, ARNT’s coactivating effect on ERβ was apparent only in the context of the full-length ERβ. This suggests that ARNT functions more effectively on the ERβ subtype depending on the AF-1. Again, this indicates a novel mechanism of coactivation since it has been reported that p160 coactivators fail to enhance ERβ AF-1 [27].

To substantiate the involvement of ARNT in ER signaling in a physiological setting, chromatin immunoprecipitations of endogenous ER and ARNT were performed in T47D breast cancer cells. We decided to investigate the recruitment to the promoter of the classical estrogen-responsive gene pS2. Association of both ERα and ARNT to the
pS2 promoter was observed in response to E2, verifying the mechanistic data. The finding that ARNT is required for both functional AhR signaling in response to TCDD and for ER activity indicates a more important physiological role for ARNT than previously believed. Moreover, the preference for ERβ is interesting in light of the differential expression patterns of the two ERs.

4.2 THE TRANSCRIPTION FACTOR ARYL HYDROCARBON RECEPTOR NUCLEAR TRANSLOCATOR FUNCTIONS AS AN ESTROGEN RECEPTOR β-SELECTIVE COACTIVATOR, AND ITS RECRUITMENT TO ALTERNATIVE PATHWAYS MEDIATES ANTIESTROGENIC EFFECTS OF DIOXIN (PAPER II)

The finding that ARNT is not only an obligatory heterodimerization partner for the AhR and HIF1α, but is also involved in estrogen signaling, prompted us to investigate whether competition for a common pool of cellular ARNT might contribute to the antiestrogenic consequences of TCDD exposure.

We co-treated cells with estradiol and TCDD and measured the resulting estrogenic activity on an ERE-regulated reporter gene. Additionally, we exposed the cells to hypoxic environment in order to trigger the formation of ARNT-HIF1α complexes. The results showed that both exposure to TCDD and to low oxygen levels severely compromised ER function. Furthermore, the added inhibition on ER activity when both AhR and HIF1α were active simultaneously was not significant, which indicates that the inhibitory mechanism is similar in both cases. In line with our findings, it has been shown that competition with the ligand-activated AhR for recruitment of ARNT inhibits HIF1α activity [178, 179]. On the other hand, it has also been reported that competition for ARNT cannot fully account for inhibitory AhR-HIF1α crosstalk, suggesting that alternative mechanisms exist [180, 181].

The functionality of ERα and ARNT interaction was assessed by their association with the estrogen-responsive c-fos promoter in the presence of both E2 and TCDD. ERα was found on the c-fos promoter both in the absence and presence of hormone. In response to E2, strong recruitment of ARNT was also observed. Interestingly, the inclusion of TCDD inhibited ARNT binding, which was in line with our hypothesis that sequestering of ARNT may influence the activity of ER-regulated genes.

In cells treated with siRNA to lower the cellular levels of ARNT protein, the E2-induced transcriptional activities of ERs, in particular of ERβ, were significantly compromised. However, the siRNA was designed to target a sequence of ARNT that is highly homologous to ARNT-2. Therefore we cannot exclude that functional ARNT-2 is enough for proper ER signaling. To get conclusive answers, ER signaling would have to be studied in ARNT knockout mice. Since disruption of ARNT results in a lethal phenotype, a conditional knockout would be necessary. Conditional ARNT-1 knockout mice where ARNT is deficient in mammary gland have been reported [182]. The results indicate that ARNT is dispensable for ER signaling. Still, ARNT-2 may be present in mammary gland, given that they have partially redundant roles and distribution [53].

As we had observed a preference of ARNT for ERβ, we were curious to know how ARNT affects ERα/β heterodimers. This was done using single-chain ERs constructs.
Again, ARNT had a stronger impact on ERβ homodimers, as evidenced by the increase in ERE-regulated transcription. Moreover, when ER mutants with swapped A/B domains were utilized, the results showed that the key function for the enhancing effects exerted by ARNT is located within the ERβ A/B domain. Interestingly, the mutant with ERα AF-1/ERβ AF-2 was not responsive to ARNT coactivation. It is not fully clear how ERβ AF-1 is regulated. For instance, it has been reported that CBP/p300 binds to AF-1 and mediates synergy between AF-1/AF-2 domains, and that p300 interacts with the N-terminal region of ERβ [184]. Interestingly, the same region was shown to be crucial for ARNT coactivating mechanism of ERβ. Moreover, p300 has been reported to mediate the interaction between ARNT and HIF1α [185]. These findings are in line with our data and suggest a role for p300 in mediating ARNT coactivation of ERβ.

The preference of ARNT for ERβ was verified in ChIP experiments, which demonstrated that ARNT is recruited mainly by ERβ, and to a lesser extent by ERα. In addition, re-ChIP experiments verified that ARNT and ERβ are present in the same complex on estrogen-responsive promoters and that recruitment of the complex is impaired by TCDD.

If competition for ARNT between ERs and AhR is involved in the antiestrogenic effects of TCDD, then ARNT’s isoform selectivity suggested that also TCDD would have a stronger effect on ERβ than on ERα. To test this, we compared the inhibitory effects of TCDD in different cell lines that either expressed one or the other isoform, or in which ER activity was induced by isoform-selective agonists. In all the systems, we could see a much stronger effect of TCDD on ERβ than on ERα transcriptional activity.

In summary, we could show a novel molecular mechanism for the anti-estrogenic effects of TCDD whereby activated AhR sequesters ARNT away from the ER signaling pathway. Most interestingly, ARNT coactivation function as well as the anti-estrogenic effect of TCDD was much more pronounced on ERβ. Considering the differences in biological functions of ERα and ERβ, this could have major implications when evaluating the impact of dioxin on human health and suggest that tissues expressing ERβ are the most sensitive to TCDD-mediated interference.

4.3 3-METHYLCHOLANTHRENE DISPLAYS DUAL EFFECTS ON ERα AND ERβ SIGNALING IN A CELL-TYPE SPECIFIC FASHION (PAPER III)

In paper III we investigated the differences between two prototypical AhR agonists, TCDD and 3-MC, with respect to their capacity to disrupt estrogenic signaling.

The adverse effects of TCDD on ER signaling are well documented and were verified also in our experiments. 3-MC however, had distinct impact depending on the cell context. In the mouse mammary cell line HC11, we could see similar response to 3-MC as to TCDD resulting in impaired ER activity. However, in liver HepG2 cells, 3-MC rather exhibited estrogenic properties by inducing robust ERE-regulated activity. Further, in CV-1 kidney cells, the same dose-dependent increase in transcription was observed.
Since it has been reported that 3-MC-bound AhR interacts with and activates ERα, we speculated that this could explain our results [153]. However, the differences in response between the cell lines (i.e. the antiestrogenic effect in HC11), in spite of a similar reporter, were puzzling. The fact that 3-MC dose-dependently induced ER activity impelled us to test whether 3-MC acts through a *bona fide* agonist-like mechanism. Involvement of the ER ligand binding domains was confirmed using various ER LBD constructs. The 3-MC-induced ERE activity was inhibited by the ER antagonists tamoxifen and ICI182,780, which also substantiated the involvement of the LBD. However, a ligand-binding assay clearly showed that neither 3-MC nor TCDD have ER ligand-binding properties. These findings were in line with other reports, which showed that 3-MC activates ERα in a manner independent of the AhR [154, 186], but still did not explain the discrepant effects in the cell lines following 3-MC exposure.

The impact of TCDD and 3-MC at gene expression level was measured by RT-PCR of classical estrogen- and TCDD-responsive genes, pS2 and CYP1A1, respectively. The gene induction was determined in HepG2 cells with stable expression of ERα and in HepG2 wildtype cells after incubation with 3-MC, E2 or TCDD. In both cell types, CYP1A1 was highly induced in response to 3-MC as well as to TCDD. Cells lacking ERα failed to induce the pS2 gene in the presence of E2 and 3-MC while ER-containing cells responded to both these compounds with increased pS2 expression. When the putative involvement of the AhR was investigated in cells with reduced AhR protein levels, the 3-MC-induced pS2 expression was significantly impaired. In summary, these results indicated that the estrogenic activity of 3-MC is dependent on both ERα and a functional AhR.

Results so far showed that 3-MC activated ER-dependent transcription in HepG2 and CV1 cells but not in HC11 cells by an agonist-like mechanism. TCDD on the other hand behaved as an antiestrogen in all cell lines tested. Furthermore, the 3-MC activities rely on the ER ligand-binding pocket, and depend on a functional AhR signaling. Yet, 3-MC itself did not display ligand-binding properties. Taken together, these findings suggested that the metabolic capacity of the cell system could determine the outcome of 3-MC exposure. 3-MC is a polycyclic aromatic hydrocarbon with a relatively short biological half-life. To compare the metabolic profiles of HC11 versus HepG2 cells, HPLC analysis of medium collected from the respective cell lines following 3-MC exposure was performed. Strikingly, HC11 cells displayed low metabolic capabilities while HepG2 cells generated a vast number of metabolites. With the aim of testing the estrogenicity of these generated metabolites, fractions of HepG2 eluates were used to treat HC11 cells. Consistent with previous results, the parental compound 3-MC did not activate the ERE-regulated reporter. However, two of the fractions tested showed significant ERE activity. The ligand binding properties of the fractions were substantiated by the fact that the ER antagonist ICI182,780 inhibited their actions.

In conclusion, we demonstrated that metabolism of 3-MC gives rise to compounds with estrogenic properties. These findings underline the importance of taking metabolism into account when investigating biological effects of putative EDCs.
4.4 MOLECULAR AND GENOMIC CHARACTERIZATION OF ESTROGENIC EFFECTS OF 3-METHYLCHOLANTHRENE (PAPER IV)

TCDD and 3-MC are used as prototypical AhR agonists and P450 inducers. They are used as interchangeable ligands of AhR both in vitro and in vivo. However, the results from Paper III prompted us to question that practice. In paper IV, we applied a genome-wide approach to compare these compounds.

To determine the gene networks regulated by TCDD and 3-MC, microarray experiments of total RNA from HepG2 cells expressing ERα were conducted. As a control, the well-characterized synthetic estrogen diethylstilbestrol (DES) was included. The cells were treated 24 hours, in order to allow for possible metabolism of 3-MC to occur. Results showed that these three chemicals regulate distinct sets of genes with only minor overlap between the treatment groups. Classifying the upregulated genes according to biological function corroborated this notion. Furthermore, 3-MC altered the expression of around 700 genes, which was more than twice as many affected by TCDD and three times as many altered by DES. We speculate that this reflects the biodegradation of 3-MC, which produces numerous biologically active metabolites and their actions.

The microarray data were verified by RT-PCR of selected upregulated genes. In order to evaluate the effect of 3-MC on the ERs, HepELN cells expressing either ERα or ERβ were used. Known ER target genes, such as pS2 and IGFBP4, were demonstrated to be upregulated by 3-MC in ERα-, but not in ERβ-expressing cells, while DES acted via both receptors.

We also identified ER regulation of three novel genes: follistatin (FST), utrophin (UTRN) and plasminogen inhibitor-1 (PAI-1).

When putative promoter sequences of the upregulated genes were extracted and analyzed by in silico methods, estrogen-responsive elements were identified in several 3-MC-induced genes. Additionally, the genes identified as novel ER targets, all contained ERE sequences within their promoters. However, the functionality of these EREs remains to be investigated.

The regulation of IGFBP4 in HepG2 cells has not been characterized but EREs have been identified upstream of the transcriptional start site. We examined ERα, AhR and ARNT binding to this region by chromatin immunoprecipitation (ChIP) assays. As controls, the classical estrogen target GREB1 promoter and the CYP1A1 enhancer region were included. The results showed that ERα, but not AhR or ARNT, is recruited to the IGFBP4 ERE in response to DES and 3-MC confirming the notion that these compounds regulate IGFBP4 via ERα.

In summary, we show that the prototypical AhR ligands TCDD and 3-MC regulate separate sets of genes in HepG2 cells. Furthermore, 3-MC seems to induce estrogenic responses through an ERα-specific mechanism, which calls for more thorough investigations.
5 CONCLUSIONS AND FUTURE PERSPECTIVES

The general aim of this thesis has been to characterize molecular mechanisms of ER and AhR crosstalk. Many questions still remain regarding the complex interplay between these pathways. However, according to results within our group, a previously not recognized role for the bHLH-PAS factor ARNT, namely as coactivator of ERs, has been extended to include also other nuclear receptors, LXR and RXRβ (unpublished results). The exact mechanism of ARNT-ER interactions and their physiological consequences for estrogen signaling is still under investigation. In particular, the preference of ARNT for ERβ-mediated activity is intriguing and has major implications for the interpretation of the endocrine disruptive effects of TCDD. Conditional ARNT/ARNT-2 double knockout mice, or cells with downregulated ARNT and ARNT-2 levels would provide useful tools to study ER signaling in the absence of ARNT.

In paper III, we have demonstrated that metabolism of 3-MC gives rise to compounds with estrogenic activities. Experiments aiming to identify the metabolite(s) are ongoing. Also, the metabolic enzymes involved are yet to be determined. To investigate this further, siRNA experiments targeting the CYP1A1, as a first candidate, are under way. Although 3-MC exposure is not considered a hazard for the general public, similar chemicals (PAHs) are ubiquitously present in the environment and have been found in humans. The increased prevalence of e.g. autoimmune disease, allergies, metabolic disorders and certain cancers is of great concern and exposure to environmental contaminants has been suggested as a risk factor [187-189]. Therefore, it is important to outline the molecular mechanisms of PAH interactions with the estrogen signaling machinery. Moreover, we have observed ER isoform-specific activities of the 3-MC-derived compound. These findings are intriguing in light of the ongoing efforts to synthesize selective estrogen receptor modulators (SERMs) in the pharmaceutical industry.

Results from our microarray study show that TCDD and 3-MC have discrete activities in the cells. Due to strict national regulations controlling the use of TCDD, many laboratories have instead used 3-MC both in vivo and in vitro. However, it is clear that the effects induced by TCDD, which is extremely stable and causes sustained activation of AhR, cannot be compared to those exerted by 3-MC, and vice versa. That metabolism of various PAHs results in bioactive derivatives has been reported previously, and highlights the difficulties in risk assessment of human exposure.
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7 REFERENCES


