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AH RECEPTOR MEDIATED TOXICITY - STUDIES *IN VITRO* AND IN A TRANSGENIC MOUSE MODEL

Sara Brunnberg



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**TILL MAMMA, PAPPA
OCH JONATHAN**

ABSTRACT

The polychlorinated dibenzo-*p*-dioxins, dibenzofurans and biphenyls are ubiquitously present in the environment. Humans are exposed mainly through diet to continuous low levels which on average are in close proximity to those considered tolerable. The foetus and newborn infant are considered as a particular risk group. From exposure studies of laboratory animals, exposure during early life-stages leads to the most sensitive effects and the male reproductive organs are among the most susceptible target tissues for *in utero* and lactational TCDD exposure. The aryl hydrocarbon receptor (AhR) mediates most, if not all, of the toxic effects of dioxins. To further improve risk assessment, mechanistic understanding is required.

Previous studies of the Constitutively Activated-AhR (CA-AhR) mouse model have shown that some well-known effects of dioxins appear in the CA-AhR mouse, that the CA-AhR is expressed in numerous tissues and that the functional activity of the mutated Ah receptor corresponds to a relatively low dose of TCDD, resembling the human exposure situation.

The purpose of the present thesis work was to characterize the phenotype of the CA-AhR with regard to vital organs, reproductive organs and bone tissue, a non-reproductive tissue under hormonal regulation, and to further explore the molecular interaction of AhR and Estrogen Receptor (ER) dependent pathways. A persistent increase in the heart weight was seen only in the male CA-AhR mice possibly indicating a gender specific susceptibility to effects of an activated AhR in the heart. Bone tissue of both male and female CA-AhR mice were also shown to be affected and the effects of CA-AhR were different with regard to the two different types of bone, cortical and trabecular and females seemed to be more affected than males in contrast to the result from the heart. Several endpoints of male reproductive toxicology such as decrease in ventral prostate weight and epididymal sperm count, regarded as sensitive signs of *in utero*/lactational TCDD exposure were affected in the CA-AhR mice. The weight of uterus was decreased in adult and increased in immature CA-AhR mice possibly indicating both anti-estrogenic and estrogenic properties of the CA-AhR in relation to the status of the ERs. This may be associated with the result from *in vitro* studies showing the AhR partner factor Arnt efficiently coactivating ER dependent transcription. Most of the effects observed in the CA-AhR mouse model in the present thesis could be considered modest indicating a relatively low activity of the CA-AhR resembling the human exposure scenario.

This thesis present data showing a novel finding of the function of Arnt and that the CA-AhR mouse can serve as a useful model for continuous low exposure to AhR ligands, where the mechanistical understanding of the most sensitive effects on early development can be further studied and which, in the future may help improving risk assessment of dioxin-like AhR ligands.

LIST OF PUBLICATIONS

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* These authors contributed equally to this study

CONTENTS

Ah Receptor mediated toxicity - studies *in vitro* and in a transgenic mouse model ii

1	Introduction.....	1
1.1.1	TEF	2
1.2	Toxicity in reproductive and endocrine regulated organs in animals.....	3
1.2.1	Male reproductive tract	3
1.2.2	Female reproductive tract.....	3
1.2.3	Bone.....	4
1.3	Toxicity in humans	4
1.4	Arylhydrocarbon Receptor (AhR).....	5
1.4.1	AhR ligands	8
1.5	Aryl hydrocarbon receptor nuclear translocator (Arnt)	8
1.6	Estrogen receptor (ER)	9
1.7	Crosstalk of ER and AhR	10
1.7.1	AhR act antiestrogenic	10
1.7.2	AhR act estrogen-like.....	11
1.7.3	ER has been shown to modulate AhR dependent activity ..	12
1.8	AhR-/-mice	12
1.9	CA-AhR	13
2	Methodological considerations	15
2.1	Mouse model	15
2.2	Uterotrophic assay	15
2.3	Chromatin immunoprecipitation assay	15
2.4	RNA isolation	16
2.5	Northern blot, RT-PCR and qRT-PCR.....	16
2.6	Immunohistochemistry	18
2.7	Histopathology.....	18
2.8	Sperm analysis	18
2.9	Peripheral quantitative computed tomography	18
2.10	Biomechanical testing.....	19
2.11	Serum measurements of a bone degradation marker (CTX)	19
2.12	Statistics.....	20
3	Aim of the present study	21
4	Results and Discussion.....	22
4.1	Heart.....	22
4.2	Bone	22
4.3	Gene expression.....	23
4.4	CYP1A1	24
4.5	Male reproductive tract.....	24
4.6	Female reproductive tract.....	25
4.7	Crosstalk ER and AhR/Arnt.....	25
5	General conclusions.....	28
6	Future perspectives	29
8	Acknowledgements	31
9	References.....	32

LIST OF ABBREVIATIONS

AF	Activation function
AhR	Aryl hydrocarbon receptor
AhRE	Arylhydrocarbon response element
Arnt	Aryl hydrocarbon receptor nuclear translocator
bHLH	Basic helix-loop-helix
bMAL	Brain and muscle Arnt-like protein
BMD	Bone mineral density
CTX	Carboxyterminal telopeptide
E2	17 β -estradiol
ERs	Estrogen receptors (α and β)
CA-AhR	Constitutively activated Ah receptor
CYP	Cytochrome P450
DR	Dioxin receptor
DRE	Dioxin response element
HAH	Halogenated aromatic hydrocarbons
HIF-1 α	Hypoxia inducible factor 1 α
Hsp 90	Heat shock protein 90
IHC	Immunohistochemistry
3-MC	3-methylcholanthrene
Opn	Osteopontin
PAS	Per-Arnt-Sim homology
PCB 126	3,3',4,4',5-pentachlorobiphenyl
PCDD	Polychlorinated dibenzo- <i>para</i> -dioxins
PCDF	Polychlorinated dibenzofurans
PCR	Polymerase chain reaction
RT-PCR	Reverse transcription- polymerase chain reaction
SRC-1	Steroid receptor coactivator
TCDD	2,3,7,8-tetrachlorodibenso- <i>p</i> -dioxin
TIF-2	Transcriptional intermediary factor-2
XAP2	Hepatitis B virus X-associated protein 2
XRE	Xenobiotic response element

1 INTRODUCTION

Since World War II a large number of synthetic compounds have been released into the environment. Several of these compounds are resistant to chemical and biological degradation and persist in our environment for a long time and are (thus) called *Persistent Organo chlorine Pollutants (POPs)*. Of particular concern is that some of these chemicals can disturb the development of the endocrine system and the organs responding to endocrine signals. The awareness of their deleterious effects started with observations of wildlife populations, such as the eggshell thinning of wild birds of prey. This was described in the 1960's and was later linked to the exposure of an organochlorinated pesticide, dichloro-diphenyl-trichloroethane (DDT).

Also, the polychlorinated dibenzo-*p*-dioxins (PCDD), polychlorinated dibenzofurans (PCDF), and dioxin-like polychlorinated biphenyls (dioxin-like PCB) belong to the POPs. Every group contains a large number of congeners displaying widely varying toxic potencies.

configuration with lateral chlorine substitution. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic congener and has been used as a prototype compound to investigate the toxic potential of PCDDs, PCDFs and coplanar PCBs.

The group of dioxins consists of 210 different congeners comprising of 75 PCDDs and 135 PCDFs. The molecule is built up by two benzene rings connected by either a single (furan) or a double oxygen bridge (dioxin) and contains one to eight chlorine atoms (Fig 1). Among the 210 theoretically possible congeners, 17, containing 4-8 chlorines, are of major toxicological concern. Dioxins are formed as contaminants in the production of various chlorinated compounds, e.g. chlorinated phenols and phenoxy acetic acid herbicides and in combustion processes such as waste incineration (Ahlborg et al, 1995). Since PCDDs have a high thermal stability it is important that the waste is burned under very high temperature over 800 °C.

PCBs were introduced for commercial use during the 1930s as mixtures of the various 209 congeners. This class of compounds contains 1 to 10 chlorine atoms attached to a biphenyl. PCBs were used in hundreds of industrial and commercial applications because of their chemical stability, non-flammability and electrical insulating properties. They were also used in building materials in the 1960s. As for the dioxins, it is the location of the chlorine atoms that make the PCBs more or less planar in its configuration and thus more or less dioxin-like in structure and toxicity. PCBs also may contain small amounts of dioxin contamination, some of which are much more toxic than the main chemical.

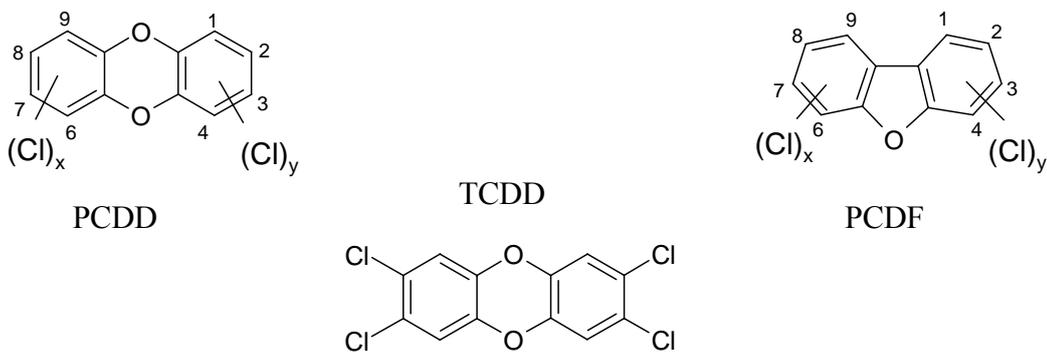


Figure 1.

Chemical structures of polychlorinated dibenzo-*p*-dioxin (PCDD), polychlorinated dibenzofuran (PCDF), and the most toxic dioxin; 2,3,7,8-tetrachloro dibenzo-*p*-dioxin (TCDD).

The environmental levels of dioxins and PCBs have been declining since the 1970s when organo-chlorinated compounds were recognized as highly toxic chemicals and governments and industry took actions to stop further environmental pollution. However, the concentrations are still unacceptably high. The risk assessments of dioxins and dioxin-like PCBs performed by WHO (WHO-ECEH/IPCS 2000) and EU (EC-SCF 2000, 2001) show that the average exposure of dioxins and dioxin-like PCBs for the general population today is very close to the tolerable daily intake (TDI) of 2 pg TEQ/kg bw (EC-SCF 2000, 2001). Dioxins and PCBs are fat-soluble and thus tend to bioaccumulate in tissue lipid and in the food chain. Food is therefore the major source for human exposure to dioxins and PCBs, especially fatty foods of animal origin, such as fish, dairy products and meat.

A particular risk group is the sensitive foetus and the newborn infant, who get higher amounts of dioxins and PCBs from breast milk (per kg body weight) than adults get from food (Ahlborg *et al.* 1995; Pohjanvirta and Tuomisto 1994).

1.1.1 TEF

It is generally believed that most, if not all effects of dioxins and dioxin-like are mediated by activation of the arylhydrocarbon receptor (AhR). However, the toxic potencies of congeners within the dioxin-like category vary over several orders of magnitude. Also the concentrations of the different dioxin-like congener vary considerably in the environment. In order to estimate potential health risk from the total exposure to “dioxin-like” compounds the “toxic equivalent factors” (TEF) concept was developed (Ahlborg *et al.* 1994). The background for the assignment of TEFs for the dioxin-like compounds is their structure–activity relationship for binding and activation of the aryl hydrocarbon receptor (AhR). In this approach, 2,3,7,8-TCDD, the most potent congener, is assigned a TEF of 1, and all other congeners are assigned TEFs that reflect their toxic potency relative to that of TCDD. The TEFs are then further used to calculate the “toxic equivalent” (TEQ) which is equal to the sum of the concentrations of the individual congeners present in the mixture multiplied by their individual TEF values.

1.2 TOXICITY IN REPRODUCTIVE AND ENDOCRINE REGULATED ORGANS IN ANIMALS

Exposure to dioxin elicits a plethora of biologic responses ranging from biochemical alterations such as enzyme induction, through overtly toxic responses including tumor promotion and a lethal wasting syndrome. However, the most sensitive adverse effects observed in multiple species appear to be on foetal development, including effects on the developing reproductive (especially males), immune and nervous systems (EC-SCF 2000, 2001; WHO-ECEH/IPCS 2000).

1.2.1 Male reproductive tract

The toxicity of the reproductive organs has been extensively investigated. In the male rat, *in utero* and lactational exposure to TCDD decreases the weight of seminal vesicle and prostate gland (Hamm *et al.* 2000; Lin *et al.* 2002b). The rodent prostate is multilobular and consists of ventral, dorsolateral, and anterior prostate lobes. Prostate development in mice is inhibited by *in utero* and lactational TCDD exposure and the nature and severity of the effects varies from lobe to lobe, but the ventral prostate is the most severely affected (Ko *et al.* 2002; Lin *et al.* 2002a). TCDD inhibits prostate development in the absence of any consistent decrease in postnatal plasma androgen concentrations or postnatal testicular androgen content (Ko *et al.* 2002; Lin *et al.* 2002b). The effect on testis weight are conflicting, a significant decrease of testis weight was shown, in mice after *in utero* to TCDD (Lin *et al.* 2001) however in another study much higher doses of TCDD did not result in a decrease of testis weight at adult age (Theobald and Peterson 1997). Also in rats, exposure to TCDD results in either decreasing or no effect at all on testis weight (Faqi *et al.* 1998; Ohsako *et al.* 2002). However, there seems to be a critical window when dioxins affect testis weight (Ohsako *et al.* 2002).

The epididymis is responsible for the sustenance, protection, transport, maturation and storage of spermatozoa (Foley 2001). The reduced levels of epididymal sperm reserve is considered one of the most sensitive end points after *in utero*/lactational exposure to TCDD and there are several reports of decrease in cauda epididymal sperm counts (Gray *et al.* 1995; Mably *et al.* 1992; Theobald and Peterson 1997). This effect was much greater than the observed decrease in the number of testicular sperm of TCDD-treated rats (Gray *et al.* 1995; Mably *et al.* 1992). This has been confirmed by recent studies of exposure to relatively low dose of TCDD which had a minimal or no effect on testicular development and spermatogenesis but negatively influence epididymal sperm count (Ohsako *et al.* 2001; Simanainen *et al.* 2004)

1.2.2 Female reproductive tract

In female rats, exposure to TCDD at GD8 resulted in a reduction of ovarian weight much later in life in association with an early decline in fertility (Gray and Ostby 1995). An increase in persistent vaginal estrus was also observed, at 1 year of age. However, females exposed at GD 15 experienced less functional ovarian toxicity (Gray *et al.* 1997). This was seen also after exposure of young adult female mice (Cummings *et al.* 1996). This indicates that exposure to TCDD early in life may result in premature aging of the ovaries. However, it is not yet clear if this is the result of a direct effect of TCDD on the ovary or by interfering with the hypothalamus-pituitary pathways.

Endometriosis is a condition where tissue similar to the lining of the uterus, the endometrial stroma and glands is found elsewhere in the body. Endometriosis is a common disorder and only menstruating species including humans and nonhuman primates can develop this disease often accompanied by chronic pelvic pain, infertility, and adhesion formation (Rier and Foster 2002). Dietary exposure to TCDD was shown to increase the incidence and severity of spontaneous endometriosis in rhesus monkeys (Rier *et al.* 1993). In rodents, this condition does not develop spontaneously however it can be surgically induced and in mice the endometric sites increase following exposure to TCDD (Cummings *et al.* 1996).

The induction of spontaneous mammary gland tumours was inhibited by chronic treatment to TCDD for two years (Kociba *et al.* 1978). However, when exposed to TCDD *in utero* female rats were more susceptible to chemically induced mammary tumours as adults (Brown *et al.* 1998; Lamartiniere 2002). This was associated with increased number of terminal end buds which is the least mature and most susceptible structure within the mammary gland

1.2.3 Bone

Bone tissue is a non-reproductive tissue under influence of many hormones. Estrogen is the most important hormone in regulation of bone remodelling in adult females and males. Not many studies have investigated the effects of dioxins on bone tissue. However, a small number of experimental studies have demonstrated that TCDD and the dioxin-like PCB congener, 3,3',4,4',5-pentachlorobiphenyl (PCB126), impair bone tissue composition and function. Increase in trabecular volume has been reported in ovariectomized female rats co-exposed to the dioxin-like PCB 126 and 17 β -estradiol, however, in intact female rats PCB 126 and 17 β -estradiol decreased the trabecular volume (Lind *et al.* 2004). This has also been reported after exposure to TCDD, either *in utero*/lactational or during adulthood (Jämsä *et al.* 2001; Miettinen *et al.* 2005). A reduction in cortical bone mineral density was shown in male rats exposed to TCDD *in utero* or via lactation (Miettinen *et al.* 2005) while this was not observed after exposure during adult life of female rats (Jämsä *et al.* 2001). Also teeth are target for dioxin toxicity, both dentin formation and tooth eruption was inhibited in rats exposed to TCDD *in utero* (Gao *et al.* 2004; Kattainen *et al.* 2001; Kiukkonen *et al.* 2002).

1.3 TOXICITY IN HUMANS

There have been occupational and accidental exposures where the levels of human exposure have reach much higher levels than those for the general population. In the 1950s and 1960s occupational exposure occurred with production and use of dioxin contaminated herbicides. Besides regular use, the herbicide Agent Orange was used in the Vietnam war to uncover the trails used by the Vietnamese combatants and was contaminated with TCDD and exposed not only the US air force soldiers but also the Vietnamese Soldiers and the Vietnamese population in large areas (Schechter *et al.* 2006). In Seveso, Italy a chemical factory which produced a trichlorophenol herbicide (TCP) exploded in 1976 and a chemical cloud containing at least 30 kg of TCDD (Bertazzi *et al.* 1998) emerged and contaminated the area around the plant. Domestic animals and birds were seriously affected and many died within a few days. Many children were exposed and quite a few developed chloracne, which has been considered

as one of the hallmarks effect in humans exposed to high doses of dioxin (Schechter *et al.* 2006). The exposed population in Seveso have been followed since the accident and apart from chloracne other effects have been reported (Bertazzi *et al.* 1998). A change in the sex ratio, with an excess of girls, was observed between 1977 and 1984 among children born to parents with high TCDD serum concentrations in 1976 (Mocarelli *et al.* 1996). This has later been linked to the exposure level of the father before and during puberty (Mocarelli *et al.* 2000). In addition, developmental enamel defects were seen in the teeth of adults that were less than 5 years at the time of the accident (Alaluusua *et al.* 2004). In other accidents, in Japan 1968 and in Taiwan 1979, known as “Yusho” and “Yuooheng”, respectively, rice oil used for cooking was contaminated with PCDFs and PCBs and resulted in effects such as chloracne and hyperpigmentation as well as developmental effects of the exposed population (Schechter *et al.* 2006). Recently, the Ukraine president Viktor Yushenko was poisoned with TCDD and developed severe chloracne.

In Sweden a cohort based on Swedish fishermen and their wives, with high consumption of POP-contaminated fatty fish from the Baltic Sea have been extensively investigated for epidemiological evaluations of human health effects of POPs (Svensson *et al.* 1994). From this cohort links between POP exposure and adverse effects, such as an increased risk of type 2 diabetes, increased risk of having a child with low birth weight, semen function, increased risk for breast cancer and an increased risk of osteoporotic fractures in the fishermen’s wives have been reported (Rignell-Hydbom *et al.* 2004; Rylander *et al.* 2005; Rylander *et al.* 1998; Wallin *et al.* 2004). In a later study, from the same exposure groups, no association of POP exposure and bone metabolism markers were found (Wallin *et al.* 2005). A link between high body burden of dioxin and increased risks of type 2 diabetes mellitus has also been reported from another highly exposed cohort (Cranmer *et al.* 2000). Furthermore, other epidemiological studies have been performed with exposure levels closer to that of the average human population. One of them show a mineralization defect in the permanent first molar teeth of a normal child population is suggested to be linked to exposure levels of PCDDs and PCDFs through mother’s milk (Alaluusa *et al.* 1996).

1.4 ARYLHYDROCARBON RECEPTOR (AHR)

Most, if not all of the toxic effects of dioxins are mediated via the AhR (also termed dioxin receptor). This receptor belongs to the family of basic helix-loop-helix-PAS (bHLH-PAS) regulatory proteins/transcription factor.

AhR homologs are found in invertebrates like *Drosophila melanogaster* to vertebrates (Hahn 2002). A single AhR gene has been identified in mammals, whereas many fish species, including the Atlantic killifish *Fundulus heteroclitus* possess two distinct AhR genes (AhR1 and AhR2) (Hahn 2002). However, a wide range of sensitivity to TCDD induced toxicity exists among different vertebrate species e.g. the guinea pig is very sensitive to TCDD whereas the hamster shows a 1000-fold greater resistance (McConnell *et al.* 1978). There also exist intraspecies differences, the inbred DBA mouse strain possess a critical point mutation in the ligand binding domain of the AhR, which leads to a lower ligand affinity and subsequently the DBA mice are about 14-fold more resistant (non-responsive) to the TCDD than the sensitive (responsive) inbred C57BL/6 mouse strain. This intraspecies difference also exist among rat strains

Han/Wistar and Long-Evans rats show the same effects of TCDD treatment in terms of enzyme induction however Han/Wistar rats show a 1000-fold greater resistance to the acute toxic effects and lethality of TCDD than the Long-Evans rats (Pohjanvirta and Tuomisto 1994).

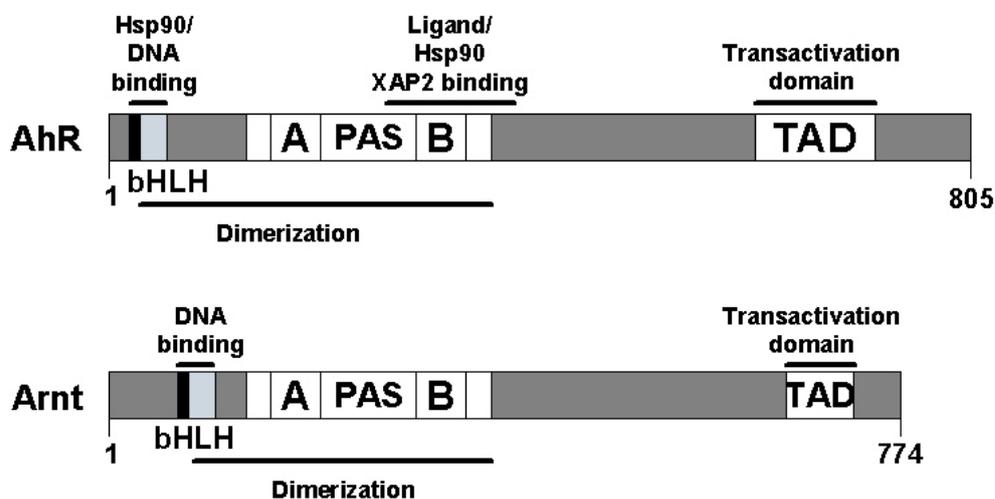


Figure 2. Schematic representation of AhR and Arnt domain structure.

The basic region is mandatory for DNA binding and the HLH part of the protein mediates dimerization with partner proteins which is also important for DNA binding. The bHLH motif is found in several transcription factors important for different biological processes such as proliferation and cell-specific differentiation (e.g. Myc/Max, MyoD (Littlewood and Evan 1995)). The PAS acronym comes from a closely related sequence motif in three different eukaryotic proteins period (Per), Aryl hydrocarbon receptor nuclear translocator (Arnt), and single-minded (Sim). Other members of this family are hypoxia-inducible factor (HIF), neuronal (NPAS), and circadian (Clock) transcription factors. The bHLH-PAS region contains the nuclear localization signal (NLS) and appears to have two nuclear export signals (NES) (Berg and Pongratz 2001; Ikuta *et al.* 1998). The PAS domain is divided in two hydrophobic repeats, PAS A and PAS B. The formation of stable protein-DNA complexes by the AhR/Arnt heterodimer to the xenobiotic response elements (XRE) sequence requires the PAS A domain (Chapman-Smith *et al.* 2004). Ligand binding occurs over the PAS B domain and this region is also important for the association with Hsp 90. Deletion of the minimal PAS B domain resulted in a constitutively activated AhR in transient transfection assays (McGuire *et al.* 2001) and this mutated AhR was later termed CA-AhR (see below). The carboxy (C) terminal half of the AhR contains a transactivation domain (TAD) (Ma *et al.* 1995).

In the absence of ligand, AhR is retained in the cytoplasm as a latent, non-DNA binding heteromeric complex with a dimer of the molecular chaperone heat shock protein (hsp) 90 and other proteins, including an immunophilin-like protein called the hepatitis B virus protein X-associated protein 2 (Xap2) (also termed AIP or ARA9)

(Carver *et al.* 1998; Ma and Whitlock 1997; Meyer and Perdev 1999) and the p23 cochaperone (Nair *et al.* 1996) (Fig. 3).

The Hsp90 complex is important both for the localisation of the AhR to the cytoplasm in the absence of ligand probably by masking the NLS, and also to stabilize the high-affinity ligand-binding conformation of AhR. Moreover, Hsp 90 is important to protect AhR from degradation (Pongratz *et al.* 1992). Recent studies suggest that Xap2 is responsible for the stabilization of the AhR-Hsp90 complex, which in turn reduces proteosomal degradation, influences cellular localization and enhances signalling of AhR (Ma and Whitlock 1997) (Kazlauskas *et al.* 2002; Kazlauskas *et al.* 2001; Meyer and Perdev 1999). The p23 cochaperone may have a role in ligand mediated release of the AhR from hsp90 (Kazlauskas *et al.* 1999).

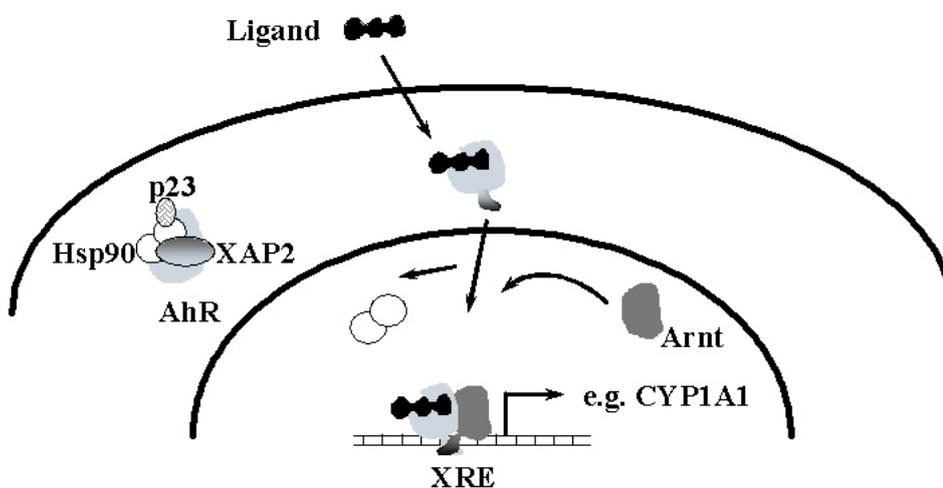


Figure 3. Schematic illustration of AhR activation

Upon ligand binding the AhR complex translocates into the nucleus where it dissociates from Hsp90, Xap and p23 and instead heterodimerizes with the partner factor Arnt (Fig.3). This ligand-bound heterodimer then binds to specific regulatory elements within DNA and thereby modify transcription. These elements with the core sequence, GCGTG, are variously termed the xenobiotic response elements (XRE), the dioxin response element (DRE) or the Ah response element (AhRE). Recently a second, novel AhRE was discovered (AhREII) and shown to respond to 3-methylcholantrene (3-MC) (Sogawa *et al.* 2004) and TCDD (Boutros *et al.* 2004). In contrast to the battery of drug metabolizing enzymes that are regulated by the AhRE, AhREII encompasses a set of genes involved in transport and ion channels. The two motifs are rarely found together in the promoters of the same gene however there might be genes where they exist together but if this leads to different effects on the level of gene expression is not known today (Boutros *et al.* 2004).

Finally, the ligand activated AhR becomes down regulated and this appears to be ubiquitin mediated and occurs via the 26S proteasome pathway following nuclear export of AHR (Davarinis and Pollenz 1999; Ma and Baldwin 2000; Roberts *et al.* 2000)

The liganded AhR complex was found to activate gene expression of a factor designated AhR repressor (AhRR), a new member of the bHLH-PAS family. This protein lacks the activation domain and it was proposed that by competing with AhR for dimerizing with Arnt it inhibits AhR function (Mimura *et al.* 1999). The AhRR mRNA varies across different tissues with high constitutive expression in heart, brain, kidney and testis of mice and rats (Bernshausen *et al.* 2006; Korkalainen *et al.* 2004). The expression of AhRR in heart could be further increased by exposure to TCDD and 3-MC (Korkalainen *et al.* 2004; Mimura *et al.* 1999).

1.4.1 AhR ligands

In addition to environmental pollutants, there exist many other AhR ligands including compounds derived from numerous plant species, such as the bioflavonoids genistein and resveratrol, catechins found in green tea and indole-containing compounds (found e.g. in cruciferous vegetables) and their metabolites which can produce either activation or inhibition of AhR signal transduction (Denison and Nagy 2003) (Casper *et al.* 1999). Interestingly, indolo-[3,2-*b*]-carbinol (ICZ), which can be formed from indole-3-carbinol in the digestive tract, has been demonstrated to have one of the highest affinities to the AhR and is a potent inducer of AhR dependent gene expression *in vitro* (Bjeldanes *et al.* 1991). Indirubin and indigo identified in human urine are tryptophan-derived products that potently enhance AhR activity (Adachi *et al.* 2001). A tryptophan photoproduct 6-formylindolo[3,2-*b*]carbazole (FICZ), also results in a high affinity ligand (comparable to that of TCDD) which in turn induces the AhR target gene CYP1A1 (Rannug *et al.* 1995). Moreover, an endogenous ligand 2-(1'H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester (ITE) have been isolated from the porcine lung (Song *et al.* 2002).

1.5 ARNT

Arnt is general partner factor for members of the bHLH/PAS family of proteins. In contrast to AhR, Arnt does not bind ligand and does not associate with Hsp90 complex (Cox and Miller 2004). Arnt is crucial for normal development and mice lacking Arnt are not viable beyond embryonic Day 10.5. The primary cause of lethality can be attributed to defects in vascularisation of the placenta and/or developing yolk sac (Kozak *et al.* 1997; Maltepe *et al.* 1997).

Three isoforms of Arnt have been identified. Arnt and Arnt-2 are highly homologous proteins whereas brain and muscle Arnt like protein (bMAL) (also known as member of PAS (MOP)3/Arnt-3) shares considerable sequence homology to Arnt and Arnt-2 in the bHLH and PAS domain but is less well conserved in the C-terminal domain. Interestingly, Arnt and Arnt-2 are functionally exchangeable whereas bMAL interacts only with selected members of the bHLH-PAS family of transcription factors such as the circadian regulator, Clock. All three proteins are expressed in embryos. In the adult mouse Arnt is ubiquitously expressed whereas Arnt2 expression is restricted to the brain and kidneys and bMAL/ARNT3 to the brain and skeletal muscle (Gu *et al.* 2000). In addition to forming heterodimers with other bHLH-PAS proteins, Arnt can also form homodimers. This homodimer can bind specific DNA regions termed E-box (Antonsson *et al.* 1995) but the physiological role of the Arnt/Arnt dimer is poorly understood. The N-terminal domain of Arnt contains a nuclear localisation signal

(NLS) which mediates an exclusively nuclear localisation of Arnt. A strong transactivation domain (TAD) is located in the C-terminal domain (Fig 2). Both the first and second alpha helices of the bHLH region are required for dimerization. The basic region is required for XRE binding but not for dimerization (Gu *et al.* 2000).

1.6 ESTROGEN RECEPTOR (ER)

Estrogen is a steroid hormone, which regulates proper development and function of the reproductive organs in both females and males. Moreover, estrogen is important for maintaining bone mass and represents a protective effect against cardiovascular disease. The physiological effects of estrogen are mediated by the ER α or ER β receptor subtypes (Nilsson *et al.* 2001). These proteins belong to the nuclear receptor super family, which among others includes, the glucocorticoid, progesterone, androgen as well as large number of structurally conserved receptors for which no ligand has been identified, namely the orphan receptor group.

These transcription factors are characterized by their conserved structural arrangement which includes the DNA binding domain (DBD) containing two highly conserved Zinc finger motifs (Fig 4). The ligand binding domain (LBD), located C-terminal of the DBD is not as well conserved, not even between the two ER subtypes, and is involved in a multitude of different functions such as dimerization between receptors, recruitment of co-activators or co-repressors (Fig 4). Ligand binding induce a conformational change of the ERs and a crucial event in discrimination of agonist and antagonists is the positioning of helix 12 (Nilsson *et al.* 2001). Upon binding of an agonist such as 17 β -estradiol and diethylstilbestrol the helix 12 becomes located over the ligand-binding pocket leading to interaction with coactivators. When an antagonist, such as tamoxifen and raloxifen binds ER, the helix 12 becomes displaced in another configuration with no surface to interact with coactivators (Nilsson *et al.* 2001).

However, both tamoxifen and raloxifen have been shown to act as partial agonists for ER α , depending on gene promoter and the character and balance of coactivators and corepressors present in different target cells (Katzenellenbogen *et al.* 2000) but they are pure antagonist to ER β (Matthews and Gustafsson 2003). This may partly be explained by the difference in the N-terminus of the two ERs. The ERs contain two distinct transactivation domains, activation function 1 (AF-1) located in the N-terminus and AF-2, found within the LBD. In some cells both AFs are required for maximal transcriptional activity, whereas in others only one is important. The N-terminus of the two ER subtypes and subsequently the AF-1 is structurally different and ER β display a functionally weaker AF-1 (Delaunay *et al.* 2000) or negligible activity (Cowley and Parker 1999) on ERE based reporters. However the activity of AF-2 is similar in the two ER subtypes (Cowley and Parker 1999).

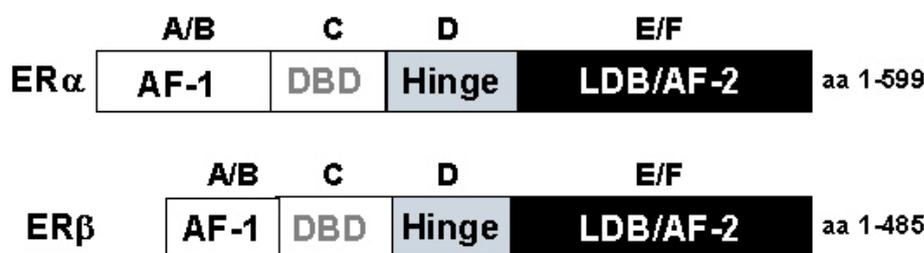


Figure 4. Schematic representation of ER α and ER β , indicating functional domains

In the absence of ligand, the ERs are present in a non-activated, latent conformation in the nuclear compartment of the cell and interact with repressor proteins such as nuclear receptor corepressor (NCoR) or the silencing mediator for retinoid and thyroid hormone receptor (SMRT) (Klinge 2000). Another form of repressor protein is the Short heterodimer partner (SHP) which is member of the orphan nuclear receptor family however it possesses no DNA binding domain and has been shown to interact with ERs and inhibit E2 dependent activation (Klinge *et al.* 2001). The repressor proteins maintain the ERs in a repressed state and inhibit the transcriptional activity of the ERs. Upon agonist binding, these repressor proteins are released and the ERs allow interaction with coactivators of the p160 family like steroid receptor coactivator-1 (SRC-1) or transcriptional intermediary factor-2 (TIF-2) (Klinge 2000). These coactivators belong to the bHLH-PAS domain transcription factor family and possess an LXXLL motif termed nuclear receptor (NR) box which they use to interact with the AF-2 domain of ER (Hall and McDonnell 2005). One of the functions of the p160 coactivator is to recruit other transcriptional coactivators and histone acetyltransferases such as p300 and CREB binding protein (CBP) to ER dependent enhancers in target genes. The acetylation of histones induces alteration in chromatin structure and reduces the inhibitory effect of nucleosomes on transcriptional activation and results in increased gene expression (Hall and McDonnell 2005).

The interaction of ER α with target gene promoters can occur directly, through specific estrogen response elements (ERE) or indirectly through contacts with other DNA bound transcription factors such as AP-1 or SP1 (Nilsson *et al.* 2001). In addition to the classic genomic mechanism, estrogen rapidly induces cellular effects that are independent of ER transcriptional activity. These effects are commonly referred to nongenomic mechanisms or membranes initiated signals and are frequently associated with the activation of various protein-kinase cascades such as the MAPK signalling pathway or phosphoinositol 3-kinase pathway (Bjornstrom and Sjoberg 2005).

1.7 CROSSTALK OF ER AND AHR

1.7.1 AhR act antiestrogenic

The antiestrogenic properties of dioxin are well documented and arise from observations in the long-term study performed in 1978 (Kociba *et al.* 1978). Female adult rats exposed to dioxin in two years exhibited lower incidence of spontaneous mammary gland and endometrial tumours. These observations lead to further investigation of the impact of dioxins on the female hormonal system. TCDD was

shown to inhibit both constitutive and 17 β -estradiol-induced uterine wet weight increase in rats and mice. In addition, TCDD decreases cytosolic and nuclear estrogen and progesteron receptor levels in the uterus. In AhR-responsive MCF-7 human breast cancer cells, TCDD inhibits 17 β -estradiol-induced cell proliferation and causes a rapid down-regulation of the nuclear estrogen receptor binding activity. TCDD also decreases the mRNA levels of some estrogen responsive genes such as Cathepsin D, pS2 and progesteron receptor (Safe and Wormke 2003). For different PCDDs, there is a good correlation between the binding affinities of these congeners for the AhR and their ability to down-regulate uterine and hepatic estrogen receptor levels (Ahlborg *et al.* 1995).

A number of possible mechanisms for the antiestrogenic effects observed by dioxin exposure have been suggested.

1. Antiestrogenic activities of TCDD are related to induction of CYP1A1/1A2 and altered steroid metabolism.

The circulating estrogens are primarily metabolized in the liver and the 2- (predominantly CYP1A1 and CYP1A2) and 4- (CYP1B1) hydroxylated metabolites are considered to be pathways to inactivate the estrogens. The induction of CYP1A1/1A2 by TCDD could then result in altered steroid metabolism. However, treatment of rodents with TCDD did not change the levels of circulating 17 β -estradiol (Ahlborg *et al.* 1995; Safe and Wormke 2003). In addition, a number of antiestrogenic responses induced by different AhR agonists are observed in MCF-7 cells at concentrations not inducing CYP1A1 (Ahlborg *et al.* 1995; Safe and Wormke 2003). On the other hand, in a recent study several gene knock-out mice of AhR, CYP1A1, CYP1A2, CYP1B1 were used to study the antiestrogenic effect of TCDD and surprisingly no inhibitory effect was seen in the mice lacking CYP1B1 (as well as for those lacking AhR) (Takemoto *et al.* 2004) indicating a role for CYP1B1 in the antiestrogenic effects by TCDD.

2. Competition for DNA-binding sites

The ligand activated AhR/Arnt complex may disrupt the binding of ER to specific regions within DNA by competition for DNA-binding sites. An estradiol-responsive enhancer sequence required for formation of a Sp1/ER-DNA complex is located in the promoter region of Cathepsin D. This region of the Cathepsin D promotor also contains a XRE/DRE core binding GCGTG sequence and binding of the AhR/Arnt complex to this sequence (termed inhibitory DRE) disrupts formation of the transcriptionally-active Sp1/ER complex and inhibits estradiol-induced reporter gene activity. A similar mechanism is also suggested for the pS2 gene (Safe and Wormke 2003; Wang *et al.* 1998).

3. Proteosomal degradation of ER

TCDD has been shown to enhance the degradation of ER α in breast cancer cells by activation of the proteosomal machinery (Safe and Wormke 2003).

1.7.2 AhR act estrogen-like

Recently interesting and somewhat contrasting effects have been described. It was first reported that 3-MC activated AhR induced transcription of an ERE reporter construct together with an unbound ER (Ohtake *et al.* 2003). This estrogenic action of 3-MC was also detected *in vivo* by increased uterine wet weight and induction of the ER target gene c-fos in uterus of immature female mice (Ohtake *et al.* 2003). Whereas in the

presence of oestrogen 3-MC exhibited antiestrogenic activities (Ohtake *et al.* 2003). However, 3-MC has lately been shown to activate the estrogen receptor without any interaction with the AhR (Abdelrahim *et al.* 2006; Shipley and Waxman 2005). Moreover, TCDD was reported to elicit a weak estrogenic activity (Watanabe *et al.* 2004) on uterine gene expression and another gene array study also display, even stronger, estrogen-like effects of TCDD on the uterine gene expression in the absence of estrogen (Boverhof *et al.* 2006).

1.7.3 ER has been shown to modulate AhR dependent activity

The two-year chronic study by Kociba in 1978 showed a higher incidence of liver hyperplasia and tumours in the female rats (Kociba *et al.* 1978). However, in the liver of ovariectomized rats TCDD did not induce cell proliferation when compared to intact females (Lucier *et al.* 1991) and 17 β -estradiol increased the TCDD induced CYP1A1 activity in the liver of both intact and ovariectomized rats (Sarkar *et al.* 2000). Cell based studies report modulation of AhR dependent activity by ER α , in some studies ER α inhibits the expression of AhR target genes in the presence of TCDD (Beischlag and Perdew 2005; Kharat and Saatcioglu 1996) and in others activation or no effect is observed (Hoivik *et al.* 1997; Matthews *et al.* 2005).

1.8 AHR-/-MICE

AhR gene knock-out (AhRKO) mice have been developed independently in three different laboratories (Fernandez-Salguero *et al.* 1996; Mimura *et al.* 1997; Schmidt *et al.* 1996). Induction of the liver enzymes CYP1A1, CYP1A2 and UDP-glucuronosyltransferase 1A6 in response to TCDD is non detectable in AhRKO mice (Fernandez-Salguero *et al.* 1996; Nishimura *et al.* 2005; Schmidt *et al.* 1996). In addition, two out of three AhRKO lines exhibit decreased liver size and a smaller body size over the first 4 weeks of age (Fernandez-Salguero *et al.* 1997; Schmidt *et al.* 1996). Reduced liver size is also observed in mice carrying a mutation in the nuclear localization sequence of the AhR (Bunger *et al.* 2003). There was a loss of teratogenic response (cleft palate and hydronephrosis) to TCDD exposure showing the role of AhR behind these effects (Mimura *et al.* 1997; Peters *et al.* 1999). Also other toxic effects observed after TCDD exposure was absent in the AhRKO mice such as wasting syndrome and thymic atrophy as well as reduction in prostate weight (Lin *et al.* 2002a). A marked cardiac hypertrophy and increase of mean arterial pressures have been reported in AhRKO mice (Fernandez-Salguero *et al.* 1997; Lund *et al.* 2003). The cardiac hypertrophy markers such as beta-myosin heavy chain and atrial natriuretic factor were increased already at embryonal stage (Lund *et al.* 2006; Thackaberry *et al.* 2003; Thackaberry *et al.* 2005). Interestingly, all three AhRKO lines display reduced fertility related to impaired function of the female AhRKO mice such as maintaining conceptuses during pregnancy (Abbott *et al.* 1999) and ovarian defects during the period of late folliculogenesis to follicular rupture (Baba *et al.* 2005; Benedict *et al.* 2003).

1.9 CA-AHR

In contrast to the loss-of-function studies of the AhRKO mice the constitutively active AhR (CA-AhR) can be described as a gain-of-function mouse model to examine possible biological functions of an activated AhR without adding any exogenous ligand.

A constitutively active AhR mutant was created by partial deletion of the minimal ligand-binding domain of the AhR (McGuire *et al.* 2001) (Fig 5). This mutant receptor failed to bind ligand but was shown to be active *in vitro* and was subsequently expressed in mice, creating the CA-AhR transgenic mouse model (Andersson *et al.* 2002) (Fig 5).

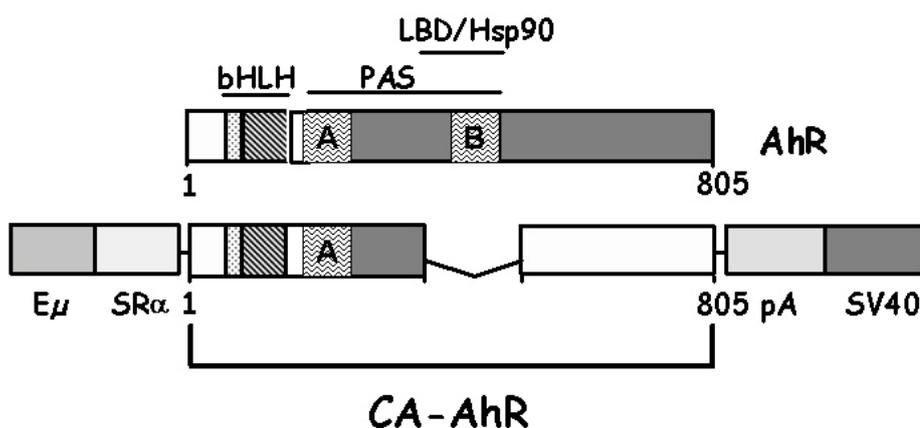


Figure 5. The C-AhR together with AhR for comparison and the expression construct, containing strong promoter (SR α) and the E μ enhancer of the immunoglobulin heavy chain promoter. In addition, the construct contained a poly-adenylation signal (pA) and a splicing region from SV40 large T gene for increased expression.

These mice express the endogenous AhR which resides in the cytosolic compartment as a latent receptor. In addition, the CA-AhR is expressed in every organ examined so far (Andersson *et al.* 2002; Andersson *et al.* 2003) and has been shown to function as a ligand activated AhR in that sense that the well known target gene of dioxin exposure, CYP1A1 is induced in the tissue that express the mutant AhR (Andersson *et al.* 2002; Andersson *et al.* 2003). Some characteristic effects of dioxin exposure such as thymic atrophy and liver enlargement (Andersson *et al.* 2002) are reproduced in the transgenic CA-AhR mice. The degree of induction of hepatic CYP1A1 mRNA and the liver enlargement of the CA-AhR mouse are quite modest. The levels of hepatic CYP1A1 mRNA were comparable to those observed in wild type mice treated with a single dose of 0.3 μ g TCDD/kg body weight indicating a rather low activity of the CA-AhR in the liver (Andersson *et al.* 2002). However, the induction of CYP1A1 expression varies between organs and do not clearly correlate with the expression levels of CA-AhR (Andersson *et al.* 2002). The CA-AhR is also expressed and functionally active in lymphoid tissues and the population of peritoneal CD5⁺ B1 cells is strikingly decreased (Andersson *et al.* 2003).

A similar transgenic mouse expressing a constitutively active mutant of AhR under the regulation of the T cell-specific CD2 promoter was recently generated (Nohara *et al.* 2005). These mice express the CA-AhR mostly in the lymphoid organs and in accordance with the CA-AhR generated in our laboratory this mouse also showed a reduction of the thymus weight in female mice (Nohara *et al.* 2005). In addition, a transgenic mouse expressing a mutant AhR with constitutive activity in keratinocytes and thymus has been developed (Tauchi *et al.* 2005) and the mice develop severe inflammatory skin lesions and many of the inflammation-related genes were up-regulated (Tauchi *et al.* 2005).

The transgenic CA-AhR mice from our laboratory show a reduced life span which coincides with the development of tumors in the glandular part of the stomach, early stages readily detectable at three months of age (Andersson *et al.* 2002). The glandular stomach was further examined with suppression subtractive hybridization techniques to identify genes which might be involved in the stomach tumor development (Kutzenov). The expression of osteopontin, a noncollagenous protein involved in regulation of cytokine production, macrophage accumulation, cell motility and adhesion, was down-regulated starting at the age of 58 days. The CA-AhR has also been shown to have liver tumorigenic potential demonstrated in a tumour promotion study performed with male CA-AhR mice initiated with *N*-nitrosodiethylamine (DEN) (Moennikes *et al.* 2004). This demonstrates an oncogenic potential of the CA-AhR and implicates a role of the AhR receptor in regulation of cell proliferation.

In conclusion, the constitutive activity of CA-AhR appears to mimic a chronic, relatively low dose exposure to TCDD.

2 METHODOLOGICAL CONSIDERATIONS

2.1 MOUSE MODEL

The transgenic CA-AhR mice used in the present thesis work were created by pronuclear injection of C57BL/6 x CBA eggs (Andersson *et al.* 2002). Transgenic CA-AhR and wild-type control animals used in paper I was bred into C57BL/6 mice, resulting in an average genetic background of 87.5% C57BL/6. For paper III and IV the transgenic CA-AhR mice were further backcrossed into C57BL/6 background for at least 10 generations. To discriminate transgenic from wild type mice, polymerase chain reaction (PCR) analysis on genomic DNA from ear biopsies was performed. To verify homozygosity genomic DNA was analysed using DNA (Southern) blotting. The backcrossing resulted in a genetic background very similar to the C57BL/6 strain, >99.8% (Silver 1995) and therefore C57BL/6N were used as control animals in paper III and IV. To avoid environmental discrepancies (food, air and water) during gestation, breeding pairs of C57BL/6 were purchased and breeding were performed in our animal facility next to the transgenic mice.

2.2 UTEROTROPIC ASSAY

The uterotrophic assay is a classic *in vivo* method used for screening of endocrine disrupting substances (Padilla-Banks *et al.* 2001). In order to use controlled estrogen levels and avoid the large fluctuations caused by the cycling levels of endogenous estrogen, adult mice are ovariectomized, or as an alternative, young immature females are used. The use of immature mice has an advantage in avoiding the surgical procedure of removing the ovaries. Moreover, the immature mice are more sensitive to stimulation with estrogens than adult ovariectomized animals (Kang *et al.* 2000). However, the uteri of untreated immature mice are very small making it difficult to prepare enough RNA or protein for analysis. Our choice to use of 20 ng estradiol/day for three days to induce effects has previously been used with sufficient result when using immature mice (Connor *et al.* 1997).

2.3 CHROMATIN IMMUNOPRECIPITATION ASSAY

The chromatin immunoprecipitation (CHIP) assay (Johnson and Bresnick 2002; Weinmann and Farnham 2002) was used to determine if Arnt and ER α bind to the same part of the chromatin, i.e. the promoter of the natural estrogen target gene pS2. After treatment, the cells were fixed with formaldehyd for a short time period to cross-link protein–protein and protein–DNA complexes *in situ*. The DNA was sonicated to generate small uniform fragments. These fragments were mixed with an antibody against the protein of interest, in this case Arnt or ER α . Following immunoprecipitation, cross-linking was reversed and the immunoprecipitated DNA was analysed using PCR with primers designed to amplify a specific region of the pS2 promoter. The immunoprecipitation step takes place when the protein of interest is bound to the DNA which may result in masking of the epitopes and failure of antibody binding. Thus, if a negative result is obtained it is important to test other antibodies which recognize a different epitope on the target protein. In the CHIP assay used in Paper II, the Arnt and ER α were cross-linked to the same region of the pS2 promoter. However, it was not possible to determine if they bound at the same chromatin

fragment or if they had any physical contact on the promoter. To determine this, a re-CHIP must be performed. This means that the fraction of chromatin precipitated by one antibody is subjected again to the CHIP procedure using the other antibody of interest whereafter amplification of the promoter region can be made.

2.4 RNA ISOLATION

To isolate total RNA from fresh-frozen tissue, the samples can be homogenised in an excess of 4M guanidine thiocyanate (GT) solution (Sambrook 1989). The homogenate is ultracentrifuged overnight at 38000 rpm in a CsCl₂ solution (Sambrook 1989). The density of RNA in CsCl₂ (>1.8 g/ml) is greater than that of other cellular components and the RNA forms a pellet on the bottom of the tube. The RNA is then precipitated with 3M NaAc and absolute EtOH. This method resulted in good yield and RNA of good quality, but was time-consuming and had a very low throughput. By switching to homogenisation in liquid nitrogen and isolating the RNA with a commercial phenol-containing reagent, both assay time and throughput were significantly improved. From start until purified RNA it takes no more than two hours. However, my own experience is that the quality is not as good as that obtained from the CsCl₂ gradient centrifugation.

2.5 NORTHERN BLOT, RT-PCR AND QRT-PCR

The gene expression has been analysed with the RNA blot method (Northern blot) and quantitative real-time polymerase chain reaction (qRT-PCR). Usually, 30 µg of total RNA was used for the Northern blots. The filters were hybridized with specific ³²P-labeled cDNA fragments in formamide-containing buffer or a commercial solution (Ambion). Since the latter hybridisation buffer increased the sensitivity of the blot hybridization experiment much less total RNA was required for analysis and subsequent exposure times could also be reduced. This was an important improvement since not much RNA is obtained from small tissues such as ventral prostate (1-3 µg) and the uterus from 21 days old mice (3-10 µg). The radioactive probe could be stripped off the blots with a boiling, low concentration SDS solution which allows the membrane to be reprobbed with different cDNA fragments at least 4-5 times. The hybridized radioactive cDNA fragment can be detected by exposing the filter to autoradiographic film. However, to compare individual RNA levels, the intensity of the band needs to be quantified and differences in loading input corrected for by normalization against the levels of a house-keeping gene. The band intensity was quantified by exposing the radioactive filter to a PhosphorImager screen. Upon laser-induced stimulation, light is emitted from the storage PhosphorImager screen in proportion to the amount of radioactivity in the sample PhosphorImager analysis (Fuji Film). The resulting digital image allows quantification of the bands. These imaging plates are much more sensitive than X-ray film and therefore allow much shorter exposure times to be used. Moreover, the PhosphorImager-based technique offers a linear dynamic range over 5 orders of magnitude compared with 3 for autoradiographic film (Lloyd and Macaskie 1996)

Another method to quantify gene expression is by qRT-PCR (quantitative Reverse Transcription PCR), which was used in Paper III and IV. This method is based on fluorescent reporter molecules that are incorporated into the PCR product which allows monitoring of the levels of amplification product during each cycle of the PCR

reaction. The two major real-time PCR strategies that are commercially established differ in their chemistry. The SYBR Green 1 dye fluoresces upon binding to double-stranded PCR product. The major drawback with this otherwise simple and rather cheap method is that the specificity can be decreased by the amplification of non-specific PCR products or primer-dimers since SYBR Green 1 binds to any double stranded DNA. In contrast, the TaqMan assay uses a fluorogenic nonextendable “TaqMan” probe which is labelled with a reporter dye and a quencher dye. When the probe is intact no signal is emitted. However, during the extension phase of the PCR the 5' endonuclease activity of the Taq polymerase cleaves the probe and the reporter and quencher dye are separated and a fluorescence signal is emitted. If the TaqMan probe is well designed it only binds the specific target and only the amplification of the target is detected. When optimized, both methods require only small amounts of RNA and it is possible to quantify the expression of a large number of genes in a short time period. The qRT-PCR approach used in papers III and IV was that of SYBR Green 1 dye.

There are several considerations to take into account; which method to use when reversely transcribing the RNA, which house-keeping gene to normalize against (always crucial), what primer pairs to use and to ensure that the PCR conditions are optimized. In Paper III and IV the RNA was reversely transcribed using random hexamers. This strategy allows binding of the hexamer primers at multiple sites along the RNA template which may produce more than one cDNA product per original mRNA molecule. Another, more specific method is to prime with oligo-dT which binds the 3' polyA tails of the mRNA molecules. However, with this method it is not possible to normalize against the ribosomal 18S RNA. The gene expression levels can be calculated either by absolute quantification using a standard curve or by relative quantification known as the comparative threshold method (Livak and Schmittgen 2001). The threshold is automatically chosen by the computer, or can be set via the computer. A signal detected above the determined threshold is considered a real signal and is used to define the threshold cycle (C_T), i.e. the number of cycles needed for detection of a true signal. A highly expressed gene results in a low C_T value and genes less expressed get higher C_T values. The threshold cycle should be reached during the early part of the exponential phase of the target amplification. In Paper III and IV the gene expression was calculated using the comparative threshold method. The average C_T values for target genes were normalized with respect to the average C_T values for the house-keeping gene, to yield the ΔC_T (Livak and Schmittgen 2001). The average ΔC_T value obtained from all wild type samples was then subtracted from the average ΔC_T value acquired for each CA-AhR sample, to give the $\Delta\Delta C_T$. The relative copy number was calculated by the expression $2^{(-\Delta\Delta C_T)}$ (Livak and Schmittgen 2001).

Most of the primer pairs in Paper IV were obtained from published data and were therefore considered to be characterized. A melting curve was chosen every time to determine if there were any amplification artefacts. These DNA products often melt at lower temperature and yield a broader peak, and in such cases a new primer pair was chosen. It is, as always, very important to determine a reliable house-keeping gene. Moreover the amplification efficiency of the target and house-keeping genes should be as equal as possible. Therefore were all templates diluted and the ΔC_T values for target and house-keeping genes were compared in a dilution versus ΔC_T series. When a

primer pair was not optimal due to various reasons such as giving rise to primer dimers, a new pair was chosen. Therefore primer pairs obtained from the Harvard PrimerBank were sometimes used (Wang and Seed 2003). Most of the primer pairs from this PrimerBank have not been experimentally validated. However, all primer pairs in this database are identified by using an algorithm with a stringent filter and the primer pairs validated so far (112) have a success rate of 98.2% (Bustin *et al.* 2005; Wang and Seed 2003).

2.6 IMMUNOHISTOCHEMISTRY

The immunohistochemical staining of CYP1A1 was performed on phosphate buffered formalin tissues to study what cells express the CYP1A1 protein, a marker for an activated CA-AhR. Immunohistochemical staining of CYP1A1 was performed on testis fixed in Bouin's solution for 24 hours and then transferred to 70% ethanol until dehydration and embedding. The staining of CYP1A1 in the testis was weak and difficult to interpret. The Bouin's fixative is recommended for histopathological analysis where cellular shrinkage is a problem as for the testis. It preserves the cellular details but it coagulates proteins which can result in artificial protein aggregates and impair immunohistochemical staining. Therefore staining was later performed on testis fixed in phosphate buffered formalin and as a consequence the morphological appearance was not as good however the staining of CYP1A1 became specific.

2.7 HISTOPATHOLOGY

The histopathology was performed in collaboration with AstraZeneca. The tissue was prepared and stained with hematoxylin and eosin according to their standardized good laboratory practice (GLP) protocols. An experienced pathologist, Ivar Paulson was analysing the organ sections and the severity was recorded according to an increasing scale as being minimal, slight, moderate, marked or very marked.

2.8 SPERM ANALYSIS

The mature spermatids, released into the seminiferous tubules of the testis are transported through the rete testis to the epididymis. This organ is divided in different segments caput, corpus and cauda and the spermatids pass through the epididymis until the final storage in the cauda. Therefore sperm storage count is mainly performed by enumerating the spermatids from the caudal part of the epididymis. However, the whole epididymis can also be used to count the spermatids, as in Paper III (Seed *et al.* 1996). The epididymal sperm count can be determined either from fresh or frozen tissue (Paper III), both ways are widely used (Seed *et al.* 1996). The epididymis was minced with a sharp scalpel in the presence of detergent (Seed *et al.* 1996). The sperm count was performed by blindly enumerating head-tail attached spermatids in triplicates on three different occasions using a hemacytometer. This number of replicates should be sufficient to give a high probability that the sample mean was showing the true spermatid population mean (Seed *et al.* 1996).

2.9 PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY

Peripheral quantitative computed tomography (pQCT) are based on the principle that minerals which have a high atomic number compared to soft tissue or air, attenuates the

intensity of an X-ray beam passing through the bone. Thus, high density is associated with a high attenuation of the beam. The pQCT has the ability not only to generate three dimensional data of the bone it can also analyse trabecular (cancellous) and cortical bone separately in contrast to dual energy x-ray absorptiometry (DEXA). In addition to the density measurement, geometric properties such as, area and circumference are also obtained by pQCT. This information can be further used to estimate the strength of the bone (Lind *et al.* 2001). However, they can not substitute a mechanical test which measures the behaviour of the whole bone which also depends on matrix composition (Lind *et al.* 2001; van der Meulen *et al.* 2001). Rat has previously been employed as a standard rodent model for the measurements of density and biomechanical properties while mice have been used far less often. The pQCT machines obtained today are modified to better measure the bones of smaller animals (Beamer *et al.* 1996; Jamsa *et al.* 1998). To discriminate between cortical and trabecular bone, threshold values for density have to be set before the measurements and the measurements are done on specifically determined locations. In Paper IV the diaphysis which is predominantly composed of cortical bone was scanned at a specific point, located 50% of the total bone length. An attenuation threshold value was set to 710 mg/cm³ and all values above that were defined as cortical bone. The metaphyseal part of the bone which consists mostly of trabecular bone, was measured at a point located 5.7% of the total bone length from the proximal tip. Values ranging from 280 to 400 mg/cm³ were considered to be trabecular bone.

The reproducibility of the pQCT measurements was evaluated by using the coefficient of variation (CV) is an evaluation of. In the measurement in Paper IV the CV_r was used which means that the bone was repositioned before each measurement.

2.10 BIOMECHANICAL TESTING

The true bending strength of the bone is dependent not only on the inorganic content which can be densitometrically measured by the pQCT or the geometric properties such as length and area. Also the organic matrix plays a role in bone strength. All these factors interact and contribute to the bending strength. The three point bending test is a common test to measure mechanical properties of the bone however there exist also other tests, such as the four point bending test, compressive test and torsion test. In Paper IV a three-point bending test was performed to determine the bending strength of the tibial diaphysis. The bone was placed horizontally in an anteroposterior direction on two rounded supporting bars located at a distance of 8 mm. At the point where the diaphyseal pQCT measurements were done, the midpoint of the diaphysis, a third bar was lowered towards the bone a loading speed of about 0.5 mm/sec. The third bar is lowered until the bone breaks which is defined as failure. This test generates a load-displacement curve from which energy to failure (area under the curve), stiffness (slope of the curve) and max stiffness can be measured.

2.11 MEASUREMENTS OF A BONE DEGRADATION MARKER (CTX)

In addition to pQCT and biomechanics, changes in biochemical markers of bone turnover can be studied in serum. The main collagen in bone is type I which is synthesized as a procollagen from which N- and C-terminal pro-peptides are cleaved before integration of the mature collagen into the bone tissue (Schaller *et al.* 2005). During osteoclastic bone resorption N- and C-terminal telopeptide fragments of type I

collagen (NTX-I and CTX-I, respectively) are cleaved off and can be detected in both serum and urine (Schaller *et al.* 2005) (Rosenquist *et al.* 1998). CTX-I is very specific of mature degraded bone and was measured in serum with an Enzyme-Linked ImmunoSorbent Assay – ELISA, validated for rat and mouse (RatLaps™ ELISA (Nordic Bioscience Diagnostics A/S, Herlev, DK). This method was convenient because it only needed a small sample volume and was already evaluated by the manufacturer.

2.12 STATISTICS

In the present thesis work most statistical calculations were done by using the parametric method of Student's *t* tests to characterize if the means of two normally distributed populations were equal. To statistically analyse categorized data such as the observations in the histopathological examination the non-parametric chi-square test can be used. A non-parametric test is a rough estimate of confidence and accepts weaker, less exact data as input than the parametric Student's *t* test. However, when the sample size is small as in Paper I, it is more accurate to use Fisher's exact test. The Mann-Whitney *U* test is another non-parametric test used in Paper III to calculate the statistical variation of the uterus weight. The pQCT and biomechanical data were evaluated by ANOVA (one-way analysis of variance) and adjusted for body weight.

3 AIM OF THE PRESENT STUDY

The polychlorinated dibenzo-*p*-dioxins, dibenzofurans and biphenyls are ubiquitously present in the environment. Humans are exposed mainly through diet to continuous low levels which on average are in close proximity to those considered tolerable. The foetus and newborn infant are considered to be at particular high risk. It has been conclusively shown that low doses of dioxin that does not affect adult animals give clear effects if administered during early life-stages. Thus, organs exposed during development are more sensitive to TCDD and the male reproductive organs are among the most susceptible target tissues for *in utero* and lactational TCDD exposure. To further improve risk assessment of dioxin-like compounds, better mechanistic understanding is needed. Previous studies of the CA-AhR mouse model have shown that the CA-AhR is expressed in numerous tissues and the classical effects of dioxins exposure such as liver enlargement and thymus atrophy are reproduced in the CA-AhR mouse. Moreover, the functional activity of the mutated Ah receptor seems to correspond to a relatively low dose of TCDD, thereby resembling the human exposure situation.

The aim of the present thesis work was:

To extend the characterisation of the CA-AhR mice with regard to expression, activity and biological effects of the CA-AhR in more organ systems in order to evaluate its relevance as a model for mechanistic studies for the most sensitive effects (e.g. male reproductive tract) of early life exposure to dioxin-like compounds/ AhR ligands.

To further elucidate the mechanistic role of AhR in the toxic effects of TCDD with specific emphasis on the most sensitive effects, the development of the endocrine and reproductive organs.

To study mechanisms *in vitro* to further understand the interaction between ERs and AhR/Arnt complex and the biological effects observed either by ligand activated AhR or genetically activated AhR (CA-AhR).

4 RESULTS AND DISCUSSION

During the extended characterisation of the CA-AhR phenotype, the main emphasis was put on changes in vital organs (Paper I), reproductive organs (Paper III) and bone tissue which is a hormonally regulated but not reproductive organ (Paper IV). Besides findings in the heart, there were no major effects observed in other vital organs and they will not be further discussed in this section. In addition to the results from the CA-AhR mice, the mechanistic *in vitro* study on interactions between Arnt and the estrogen receptors is also presented and discussed (Paper II)

4.1 HEART

One of the most interesting findings regarding the vital organs was the very consistent heart-to-body weight increase found in male CA-AhR mice. This effect was evident already at relatively early age (58 days) and could be seen in all following age groups up to 12 months of age (Table 1 in Paper I). Previous exposure studies have shown increased heart-to-body weight ratio in mice exposed to TCDD *in utero* (Lin *et al.* 2001; Thackaberry *et al.* 2005). Somewhat contradictory, also AhRKO mice show an increase in the relative weight of the heart together with morphological changes, such as fibrosis and smooth muscle cell hyperplasia of coronary arteries and arterioles (Fernandez-Salguero *et al.* 1997; Lin *et al.* 2001; Lund *et al.* 2006; Lund *et al.* 2005). Thus, it appears that the developing heart is a sensitive target for changes in AhR activity. Interestingly, the increased heart-to-body weight ratio was only seen in male CA-AhR. Such a sex-specific effect has not been discussed in previous papers showing cardiac hypertrophy after TCDD exposure or AhR deficiency (Lund *et al.* 2006; Thackaberry *et al.* 2005). The reason for this could be that it was not observed or that differences between males and females were not analysed. However, many previous studies show that females are protected from developing pathological cardiovascular effects, and this protective effect has been attributed to the presence of estrogen (Mendelsohn and Karas 2005). Thus, the apparent gender specific effect of CA-AhR on the heart to body weight ratio could be caused by modulating estrogen dependent pathways.

4.2 BONE

Bone is a dynamic tissue undergoing constant remodelling. In this context bone is a sensitive target not only during the early development but also throughout the adult life. There are not many studies on the effects of dioxins in bone tissue. CA-AhR mice were investigated to understand if a genetically activated AhR can modify bone tissue. Analysis of trabecular parameters by using metaphyseal pQCT scan revealed changes in both male and female CA-AhR mice. In females, the trabecular area was increased without any changes in trabecular density (Table 1 in Paper IV). In contrast, male trabecular bone mineral density and periosteal circumference were increased (Table 1 in Paper IV). Increase in trabecular volume has previously been reported in ovariectomized female rats co-exposed to the dioxin-like PCB 126 and 17 β -estradiol, however, in intact female rats PCB 126 and 17 β -estradiol decreased the trabecular volume (Lind *et al.* 2004). In contrast, exposure to TCDD during adulthood reduced the trabecular bone volume in female rats (Jämsä *et al.* 2001). The diaphyseal part of the

bone constitutes mostly of cortical bone which is not as active in either bone formation or resorption during adult life. In female CA-AhR mice, the diaphysis showed a reduction in cortical bone mineral density (Table 1 in Paper IV). This has previously been shown in male rats exposed to TCDD *in utero* or via lactation (Miettinen *et al.* 2005) while this was not observed after exposure during adult life of female rats (Jämsä *et al.* 2001). In the biomechanical test, female CA-AhR showed an increased displacement (Table 2 in Paper IV). This may reflect a softer bone tissue composition possibly by structural changes such as altered mineralization or changes in collagen structure. Previous report of PCB126-exposure resulted in an increased osteoid surface of rat tibia, indicating an impairment of the mineralization process (Lind *et al.* 2000). Male Ca-AhR, in contrast to the females, displayed no changes in the diaphyseal part of the bone and neither in the biomechanical test though there was a trend of reduced total bone mineral density in the diaphysis (Table 1 and 2 in Paper IV). This gender difference may be due to males having more bone mass than females (Nieves *et al.* 2005) and males might therefore be less sensitive to the effect of an activated AhR at this age. The observed differences in effects between the cortical bone of the diaphysis and the trabecular bone of the metaphysis may reflect a compensatory mechanism by adding more trabecular bone in the metaphysis during the longitudinal growth (Frost and Jee 1992). Therefore, after the cease of longitudinal growth the CA-AhR may affect bone tissue differently, this may be observed in older CA-AhR mice. Thus, the CA-AhR induces effects in bone tissue in both male and female mice. However, females seem to be more affected than males in contrast to the observed heart-to-body weight ratio.

4.3 GENE EXPRESSION

There was no change in mRNA levels of procollagen 1, a proform of the major collagen type in bone tissue, in the diaphysis of male CA-AhR mice (Table 3, Paper IV). However, the expression of procollagen III which is not particularly abundant in bone tissue was decreased as well as the mRNA levels of osteopontin (Table 3, Paper IV). Osteopontin is not required for normal bone formation and development however, the presence of osteopontin on the bone surface is critical for the remodelling of mature bone. The reduced levels of osteopontin in the male CA-AhR mice may be associated with the increase of metaphyseal bone since bone lacking osteopontin are less readily remodelled (Denhardt and Noda 1998).

Interestingly, expression of osteopontin has also been suggested as a marker gene for cardiac fibrosis and in AhRKO mice, cardiac hypertrophy is associated with increased mRNA expression of osteopontin in the heart (Lund *et al.* 2006). The expression of osteopontin has previously been investigated in CA-AhR mice and was shown to be down-regulated in several tissues (Kuznetsov *et al.* 2005). However, that paper was focused on findings in the stomach and due to very weak expression in the heart it is difficult to draw any firm conclusions about osteopontin changes in the heart. Moreover, in that study osteopontin expression was investigated in female mice, which, in the present work show no sign of heart enlargement (Table 1 in Paper I).

4.4 CYP1A1

Due to the presence of the endogenous AhR in the CA-AhR animals, it has not possible to directly show cell-specific expression of the transgene by e.g. immunohistochemistry. Instead, the sensitive target gene CYP1A1 has been used as a surrogate marker for transcriptional activity of the CA-AhR.

The expression of CYP1A1 was shown to be induced in all of the organs examined indicating activity of the mutant receptor (Paper I, III and IV). When the cell-specific expression was investigated by immunohistochemistry it showed a consistent pattern of expression in endothelial cells in all organs examined. In addition, certain epithelial cells, such as hepatocytes, bile duct and gall bladder epithelium, renal tubular cells as well as spermatids were positively stained (Fig 1 and 2 in Paper I and Fig 3 in Paper III). The staining pattern, particularly of hepatocytes and tubular cells, was uneven, with strong staining of some cells and no visible staining of directly adjacent cells (Fig 1 and 2 in Paper I). The toxicological relevance of this pattern, if any, is unclear. The local induction of CYP1A1 may lead to cellular changes e.g. CYP1A1 is known to metabolize estrogen which in turn may lead to lower cellular levels of estrogens as postulated in regard to the antiestrogenic effects observed after exposure to dioxin-like compounds (Spink *et al.* 1990). Also, the hydroxylated estrogen metabolites generated by CYP1A1 can in turn generate reactive oxygen species and contribute to oxidative DNA damage (Lavigne *et al.* 2001) Thus, the induction of CYP1A1 could lead to effects or may be marker for the possibility of effects on other AhR-related genes of different toxicological significance.

4.5 MALE REPRODUCTIVE TRACT

Exposure to TCDD *in utero* and during lactation has been reported to cause diverse changes in the reproductive system of male rodents, including reduced sperm count, decrease of ventral prostate size and reduced anogenital distance as well as feminized sexual behaviour (Bjerke *et al.* 1994; Faqi *et al.* 1998; Mably *et al.* 1992; Ohsako *et al.* 2001; Sommer *et al.* 1996). The weights of both ventral prostate and testis were decreased in 3 months old male CA-AhR mice (Table 1 in Paper III) which is in agreement with results from previous dioxin exposure studies (Lin *et al.* 2001; Ohsako *et al.* 2002). Of the different prostate lobes, the ventral lobe seems to be the most sensitive to *in utero* exposure of TCDD and it has in some studies been reported to be totally absent. The weight decrease described in Paper III was relatively modest (33%), which may indicate a relatively low activity of the CA-AhR. Several analyses, predominantly by Peterson and coworkers, have shown that TCDD interferes with the formation of the prostate by decreasing the number of prostate buds (Ko *et al.* 2004; Lin *et al.* 2003).

The previously published results on testis weight after *in utero* exposure to TCDD have been somewhat conflicting (Faqi *et al.* 1998; Ohsako *et al.* 2002). This may be due to different experimental protocols since it is likely that there is a critical gestational age window for exposure to produce the testis weight effect. In the present mouse model, the CA-AhR is most likely expressed and active at early foetal life, remain active throughout adulthood and would probably be present in the mice at this critical time-point. In addition, both ventral prostate and testis are sensitive to androgen levels, but the published measurements of this parameter in rodents exposed to TCDD *in utero* has

so far been inconclusive (Faqi *et al.* 1998; Timms *et al.* 2002). Interestingly, TCDD interferes with prostate development without any consistent decrease in the level of androgens either in plasma or locally in the testis when measured postnatally (Roman *et al.* 1995). More than 70 % of the testicular weight is constituted by the seminiferous tubules and therefore a decrease in testicular sperm count may cause a decrease in testis weight (Jahn and Gunzel 1997).

In addition to the effects on prostate and testis, CA-AhR mice seem to mimic *in utero* exposure of dioxins also with regard to the observed decrease in epididymal sperm count (Fig 4 in Paper III). The mechanism behind this effect in the CA-AhR mice is presently unknown. Even though reduced sperm count has been reported in many previous TCDD exposure studies, no mechanistic explanation has yet been experimentally demonstrated although several possible mechanisms are possible (Faqi *et al.* 1998; Gray *et al.* 1995). Reduction in the numbers or function of the Sertoli cells may result in fewer mature sperms. Alternatively, a reduced number of spermatogonial stem cells could result in less mature sperms or there could be increased apoptosis of the germ cells and/or mature that eventually could lead to fewer sperms. In line with this, several investigators believe the effect to be due to changes within the testis but the epididymal sperm count does not prove a toxic effect on spermatogenesis (Blazak *et al.* 1985). It can instead be the effect of an impaired function of the epididymis. This has been studied in the context of adult exposure of TCDD which showed induction of oxidative stress in the epididymal sperms (Latchoumycandane *et al.* 2003). Thus, the decrease in epididymal sperm count observed may be caused by a decreased production in the testis

4.6 FEMALE REPRODUCTIVE TRACT

The female reproductive tract was also affected by the genetically activated AhR. In adult CA-AhR females the weight of uterus was reduced when compared to wild type (Table 1 and Fig 2A in Paper III). This result fits the general belief that an activated AhR act in an antiestrogenic manner (Safe and Wormke 2003). When immature CA-AhR mice were exposed to E2, no antagonizing effect of CA-AhR could be seen on the uterine weight (Fig 2B in Paper III). The lack of effect in this short-term study may reflect that previous studies with the CA-AhR mouse have shown that the activity of the mutant Ah receptor corresponds to a relatively low dose of dioxin (Andersson *et al.* 2002). If this is true also for the uterine activity, the “dose of CA-AhR” might have been too low to counteract the activity of E2 with regard to the uterus weight increase by exogenous E2. Such a dose-dependency has in fact previously been shown in a study with TCDD (Wyde *et al.* 2000). In contrast to these results, in immature CA-AhR mice not co-administered E2 there seem to be a growth promoting effect of the CA-AhR on the uterus to a similar extent as after E2 treatment of wild type mice at the same age (Fig 2B in Paper III). This finding will be further discussed in the following section.

4.7 CROSSTALK ER AND AHR/ARNT

Arnt is the obligatory partner factor for the transcriptional activity of AhR and HIF-1 α . They all belong to the bHLH-PAS family which also includes the p160 steroid receptor co-activators (SRC/TIF-2). Thus, Arnt share structural similarities with the p160 coactivators and it were speculated that it might also sustain transcriptional activity of

the ERs. In coimmunoprecipitation experiments Arnt was shown to interact with both ERs, in contrast to previous results which failed to show an interaction between Arnt and ER α (Klinge 2000). Transient transfection assays showed that Arnt was able to potently enhance ER dependent transcription (Fig 1B in Paper II). Interestingly, Arnt enhanced ER α activity both in the presence and absence of E2. The effect of Arnt on ER β in the absence of E2 was more modest. When the activity of Arnt on ER dependent transcription was compared to that of TIF-2 (a p160 coactivator) the transcriptional activity was fully comparable (Fig 1C in Paper II). TIF-2 interacts with liganded ER in an AF-2 dependent way through contact with the LxxLL motif present in TIF-2 (Heery *et al.* 1997). However, Arnt does not contain any obvious LxxLL sequences which suggest another mechanism of Arnt-ER interaction than that of p160 coactivators and ER. The C-terminal region of Arnt that contains a transactivation function was found to be required for coactivation of ER α and ER β . In contrast, removal of the bHLH or the PAS domains which are crucial for the interaction with AhR, had little effect on Arnt's ability to enhance ER dependent transcription (Fig 3A-D in Paper II).

Also, by using deletion mutants, the domains of the ER proteins most important for interaction with Arnt were studied. In contrast to the full-length proteins where the effects of Arnt were considerable stronger on ER β , both ER α and ER β ligand binding domains (LBD) displayed a similar increase in transactivation in the presence of Arnt (Fig 4A in Paper II). When the AF-1 domain (Δ A/B) of ER α was deleted, Arnt was not able to increase the transcriptional response to the same extent as for the full-length ER α (Fig 4B in Paper II). In contrast, Arnt was capable to co-activate ER β lacking the AF-1 domain and full-length ER β to the same degree (Fig 4C in Paper II) indicating a low or absent activity of ER β AF-1 (Cowley and Parker 1999; Delaunay *et al.* 2000). This qualitative difference between full-length ER and ER Δ A/B might indicate that compared to ER α , Arnt may be more efficient in supporting synergy between AF-1 and AF-2 of ER β , even though the AF-1 of ER β seems weak. In addition, Arnt is recruited to an endogenous E2 regulated promoter (pS2) in an E2 dependent fashion in T47D human breast cancer cells indicating physiological relevance of Arnt co-activating the ERs. Thus, these results may add additional insight into the cellular functions of the bHLH-PAS factor Arnt to include potent coactivating function for ER α and ER β .

In contrast to the well-documented antiestrogenic effects of TCDD there also exist reports of AhR ligands acting in an estrogen-like (Boverhof *et al.* 2006; Ohtake *et al.* 2003; Watanabe *et al.* 2004). One study reported that 3-MC activation of AhR in association with unliganded ER, transcriptionally activated an ERE reporter construct (Ohtake *et al.* 2003). In addition, both 3-MC and TCDD induced mRNA expression of c-fos mRNA in the uterus of ovariectomized mice which was referred to as an estrogen-like effect of both AhR agonists (Ohtake *et al.* 2003). However, 3MC have lately been shown to activate the estrogen receptor without any interaction with the AhR (Abdelrahim *et al.* 2006; Shipley and Waxman 2005). In line with Ohtake, two other studies have reported that TCDD modified the expression of several estrogen dependent genes in the uterus, in the absence of estrogen (Watanabe *et al.* 2004). Although, the estrogen like activity was not in the level of inducing uterine weight increase, they speculated that in the presence of a non ligand bound ER, TCDD might

influence transcription in an estrogen-like manner (Boverhof *et al.* 2006). In accordance with that study, no estrogenic effect of TCDD alone was seen on the uterine weight of immature wild type mice (Fig 2B in Paper III) although CA-AhR and TCDD alone induced the mRNA levels of the ER α target gene Cathepsin D (Fig 2 D in Paper III). In the presence of both 17 β -estradiol and CA-AhR/TCDD induction of Cathepsin D was inhibited (Fig 2 D in Paper III).

The somewhat contradictory results in the uterus of young adult and immature mice suggest that the activation status of the ERs is very important for the outcome of AhR activation. Depending on the estrous cycle in adult mice, the ERs are likely to be more or less bound to ligand whereas in immature mice the ERs are not bound to ligand (Boverhof *et al.* 2006; Ohtake *et al.* 2003). Although less pronounced in male laboratory animals exposed to TCDD, effects such as decreased weight of testis and reduction in epididymal sperm counts have been seen after estrogen exposure in utero (Fielden *et al.* 2002; Spearow *et al.* 1999). Thus, it is tempting to speculate that the mechanism(s) behind the observed effects in the CA-AhR mice is related to activation status of the estrogen receptors in the sexually immature tissues.

The domains of Arnt that are crucial for transactivating AhR were not necessary for Arnt to coactivate the ERs (Fig 3 in Paper II). Yet it is still possible that AhR may be associated with Arnt during this coactivation function. Together with the observation that Arnt was interacting and able to sustain transcription of unliganded ERs, primarily ER α , it is tempting to speculate that Arnt in association with AhR is able to coactivate ER α in an estrogen deprived situation. This supports the idea of TCDD acting estrogen-like in immature tissue and would not conflict with the observed anti-estrogenic activity in presence of estrogen, possibly caused by an abrogated coactivation function of Arnt and/or possibly ligand activated AhR/Arnt complex.

5 GENERAL CONCLUSIONS

This thesis presents data that expression of a constitutively active AhR affected the heart, bone and reproductive organs in the CA-AhR mouse. In Paper I, a persistent increase in the heart to body weight ratio was seen only in the male CA-AhR mice, possibly indicating a gender specific difference in susceptibility to heart effects of an activated AhR. In Paper III the weight of the uterus was decreased in adult and increased in immature CA-AhR mice. This indicates both antiestrogenic and estrogenic properties of the CA-AhR depending on the activation status of the ERs. Moreover, several endpoints of male reproductive toxicology that are regarded as sensitive signs of *in utero*/lactational TCDD exposure, such as decrease of ventral prostate weight and epididymal sperm count, were demonstrated in the CA-AhR mice. Thus, the genetically activated AhR seem to interfere with normal reproductive organ development and/or function. In paper IV, bone tissue of both male and female CA-AhR mice were shown to be affected. Interestingly, CA-AhR affected the two different types of bone, cortical and trabecular in different ways.

In paper II, a novel function of Arnt as being able to efficiently co-activate ER dependent transcription was demonstrated. It was further shown that endogenous Arnt present in T47D human breast cancer cells was recruited in an E2-dependent fashion together with ER α to the promoter of the natural ER target gene pS2.

In conclusion, the effects of CA-AhR and TCDD on the ER dependent pathways may in part, depend on the activation status of the ERs. AhR activation by e.g. TCDD and CA-AhR seem to produce estrogen-like effects in absence of estrogen, while they act antiestrogenic when estrogen is present. Although highly speculative, these interactions could be underlying the observed effects in the reproductive tract of both male and female CA-AhR mice.

Compared to most previous TCDD exposure studies, the effects observed in the CA-AhR mouse model in the present thesis work would be considered relatively modest. This indicates a relatively low activity of the CA-AhR which resembles the human exposure scenario. Thus, the CA-AhR mouse can serve as a good model for continuous low exposure to AhR ligands, where the mechanisms underlying the most sensitive effects on early development can be further elucidated, which hopefully in the future will help improving risk assessment of dioxin-like AhR ligands.

6 FUTURE PERSPECTIVES

Since endogenous levels of Arnt could sustain both AhR and HIF-1 α signaling, it has been suggested that Arnt is expressed at relatively high, non-limiting levels. This aspect needs to be further studied in the context of the estrogen receptor regulated pathways. The role of Arnt and AhR in absence of estrogen (e.g. in sexually immature animals and humans) should be further studied. Is AhR associated with Arnt when Arnt is coactivating the ERs? This could be addressed with a chromatin immunoprecipitation assay on immature CA-AhR uterus, studying if CA-AhR/Arnt and ERs can be detected on a promoter of an ER target gene *in vivo*, and possibly comparing this with adult uterine tissue.

The effects in the reproductive organs of the male CA-AhR mice are crucial to follow up. A careful histopathological evaluation of both testis and epididymis may add more information to the reason for the weight decrease and the reduced epididymal sperm count. An extended gene and protein expression analysis should also lead to a better understanding of the observed effects. A protein that could play a role is WAPL, which is the mouse homologue of the *Drosophila* wings apart-like protein, which is important for regulating heterochromatin structure. It is highly abundant in testis and is required to hold sister chromatids together during meiosis. TCDD has previously been shown to down regulate the expression of WAPL (ref). Thus, a down regulation of WAPL by TCDD may negatively affect spermatogenesis, possibly leading to decreased sperm count.

Expression CA-AhR results in several effects on bone tissue that were different with regard to cortical and trabecular bone types. Gene expression analysis was performed in cortical tissue and therefore it would be interesting to complement these studies by also analysing the epiphyseal part of the bone. Since females show slightly more pronounced effects in the pQCT and biomechanical analysis, bones from female mice should also be subjected to gene expression analysis. Since the biomechanical data suggests a more flexible composition of the female cortical bone which could be caused by changes in the collagen structure such as modified crosslinks, it would be interesting to study these aspects further. Histomorphometric analysis can also add important information regarding cellular changes such as the numbers of osteoblasts and osteoclasts. Measurements of serum levels of a bone formation marker, alkaline phosphatase (ALP) should be included and related to the levels of CTX. In Paper IV the bone tissue was investigated in young adult animals but since the bone tissue is constantly remodelled it would be interesting to also study effects in older animals.

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