Merja Korkalainen

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Academic dissertation

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ABSTRACT

Dioxins are a group of environmental contaminants that raise concern because of their potency, widespread presence and persistence within the food chain. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and other dioxins bring about a wide variety of biochemical and toxic effects, most of which are mediated by the AH receptor (AHR). The AHR functions as a ligand-activated transcription factor and binds to DNA as a heterodimeric complex with the AHR nuclear translocator (ARNT) or its homologue in the brain, ARNT2. The AHR repressor (AHRR) negatively regulates the dioxin signalling by competing with AHR for dimerizing with ARNT. All these principal proteins in dioxin signal transduction, AHR, ARNT and AHRR, belong to the bHLH/PAS superfamily of transcriptional regulators.

A characteristic feature of TCDD toxicity is its wide variation in sensitivity among animal species and strains, which complicates dioxin risk assessment. For the guinea pig, TCDD is the most toxic synthetic compound known, while the resistant hamster tolerates over 1000-fold higher doses. A similar difference exists between two rat strains, the sensitive Long-Evans *(Turku A/B)* (L-E) and the resistant Han/Wistar *(Kuopio)* (H/W). Cloning of H/W rat AHR revealed changes in the structure of C-terminal transactivation domain, which appeared to be the principal reason for TCDD resistance in H/W rats. Because the reason for sensitivity difference between hamsters and guinea pigs was unknown, their AHRs were cloned. Sequencing of hamster AHR revealed a restructured transactivation domain as compared with that in guinea pigs. The results imply that AHR structure, especially in the C-terminal transactivation region, may be an important or even crucial factor to the sensitivity differences in TCDD toxicity.

Although the AHR appears to be the major reason for TCDD resistance in H/W rats, there is also another, currently unknown factor involved. Therefore, the primary structures of the ARNT, ARNT2 and AHRR were compared between L-E and H/W rats to determine whether some of these proteins could be this auxiliary factor participating in the strain-specific differences in TCDD toxicity. The *AHRR* gene had not been cloned earlier in rats, and thus its time-, dose- and tissue-dependent expression was also determined using quantitative RT-PCR. However, no marked differences were found between H/W and L-E rats in the structure or expression of ARNT, ARNT2 or AHRR, suggesting that these bHLH/PAS proteins do not contribute to strain differences in TCDD sensitivity. Moreover, several novel splice variants were discovered in the structures of ARNT and ARNT2, but none of these variants appeared to be related to TCDD resistance in this rat model.

The effects of TCDD on the hypothalamic expression of several genes encoding bHLH/PAS proteins participating in dioxin signal transduction and possibly in wasting syndrome were also determined using quantitative RT-PCR. The wasting syndrome is a TCDD-induced response that shows a clear sensitivity difference between L-E and H/W

rats. However, although some minor changes were detected, they may not account for the wasting syndrome.

Thus, among all the studied proteins involved in dioxin signal transduction, only the structure of the AHR stood out as an essential determinant of dioxin sensitivity in the rat model. The AHR structure also appeared to be a critical factor in the species model.

Merja Korkalainen, Dioksiinien signaalinvälitykseen osallistuvien proteiinien rakenteen ja ekspression merkitys dioksiiniherkkyydessä.
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TIIVISTELMÄ

Dioksiinit ovat ympäristösaasteita, jotka herättävät yleistä huolestuneisuutta voimakkuutensa, levinneisyytensä ja ravintoketjussa pysyvyytensä takia. 2,3,7,8-Tetraklooridibentso-*p*-dioksiinilla (TCDD) ja muilla dioksiineilla on suuri määrä erilaisia biokemiallisia ja toksisia vaikutuksia, joista suurin osa välittyy AH-reseptorin (AHR) kautta. AHR on ligandeilla aktivoituva transkriptiofaktori ja se sitoutuu DNA:han heterodimeerisenä kompleksina ARNT- tai ARNT2-proteiinin kanssa. AHR-repressori (AHRR) säätelee negatiivisesti dioksiinien signaalinvälitystä kilpailemalla AHR:n kanssa ARNT:iin sitoutumisesta. Kaikki nämä keskeiset dioksiinien signaalinvälitykseen osallistuvat proteiinit kuuluvat transkriptionaalisten säätelijöiden muodostamaan bHLH/PAS proteiiniperheeseen.

TCDD:n toksisille vaikutuksille luonteenomaisia ovat suuret herkkyyserot eri eläinlajien ja kantojen välillä, mikä hankaloittaa dioksiinien riskinarviointia. Marsu on erittäin herkkä TCDD:n toksisille vaikutuksille, kun taas hamsteri kestää yli 1000-kertaa suurempia TCDD-annoksia. Samansuuruinen herkkyysero löytyy kahden rottakannan, herkän Long-Evans-rotan *(Turku A/B)* (L-E) ja kestävän Han/Wistar-rotan *(Kuopio)* (H/W) väliltä. Pääasiallisin syy H/W-rottien dioksiinikestävyyteen ovat muutokset AHR:n karboksi-terminaalisen transaktivaatioalueen rakenteessa. Koska syytä hamsterin ja marsun väliseen herkkyyseroon ei tiedetty, niiden AHR:t kloonattiin. Myös hamsterin AHR:n transaktivaatioalueen rakenteesta löydettiin poikkeavuutta marsun AHR:n rakenteeseen verrattuna. Näiden kloonaustöiden tulokset korostavat AHR:n rakenteen ja erityisesti sen C-terminaalisen transaktivaatioalueen rakenteen merkitystä dioksiinitoksisuuden herkkyyseroissa.

Vaikka AHR näyttää olevan merkittävin syy H/W-rottien dioksiinikestävyyteen, myös toinen, tällä hetkellä tuntematon tekijä vaikuttaa siihen. Siksi ARNT:n, ARNT2:n ja AHRR:n primäärirakenteita verrattiin H/W- ja L-E –rottien välillä, jotta saataisiin selville, voisivatko nämä proteiinit osaltaan selittää kantojen välillä olevia herkkyyseroja. *AHRR*-geeniä ei oltu aiemmin kloonattu rotalta ja siksi myös sen aika-, annos- ja kudosspesifisiä vasteita määritettiin kvantitatiivistä PCR:ää käyttäen. Tulokset eivät kuitenkaan osoittaneet eroja ARNT:n, ARNT2:n ja AHRR:n rakenteessa tai ilmentymisessä, mistä voitiin päätellä, etteivät nämä proteiinit ole osasyynä rottakantojen väliseen herkkyyseroon. Sen sijaan ARNT:n ja ARNT2:n rakenteista löydettiin useita uusia rakennevaihteluita, mutta minkään niistä ei havaittu liittyvän TCDD-resistenttiyteen.

TCDD:n vaikutusta useiden bHLH/PAS-proteiineja koodittavien geenien ilmentymiseen rotan hypothalamuksessa tutkittiin kvantitatiivisella RT-PCR:llä. Tutkittavaksi valittiin geenejä, jotka osallistuvat dioksiinien signaalinvälitykseen ja mahdollisesti dioksiinien aiheuttamaan näivetysoireyhtymään, jossa myös on selvä herkkyysero L-E- ja H/W-rottien välillä. Vaikka tutkimuksessa havaittiinkin joitakin pieniä muutoksia näiden rottakantojen välillä, mitkään niistä eivät olleet riittäviä selittämään herkkyyseroa näivetysoireyhtymässä.

Yhteenvetona voidaan todeta, että kaikkien tutkittujen, dioksiinien signaalinvälitykseen osallistuvien proteiinien joukossa ainoastaan AHR:n rakenteella näyttää olevan merkitystä dioksiiniherkkyyden määrittelijänä rottamallissamme. AHR:n rakenne lienee kriittinen tekijä myös eri eläinlajien välisissä herkkyyseroissa.

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Kuopio, June 2005

Merja Korkalainen

CONTENTS

LIST OF ABBREVIATIONS

AHR	aryl hydrocarbon receptor, AH receptor, also called dioxin receptor
AHR	the gene encoding AHR in human and other species (except mouse and Drosophila)
Ahr	the gene encoding AHR in mouse and Drosophila
AHREII	AH receptor responsive element II in DNA, also called DREII or XREII
AHRR	aryl hydrocarbon receptor repressor
ARNT	AH receptor nuclear translocator
bHLH/PAS	basic helix-loop-helix/PAS (homologous region found in PER, ARNT and SIM)
CNS	central nervous system
C-terminus	carboxyterminus of protein
CYP1A1	cytochrome P4501A1
DRE	dioxin responsive element in DNA; called also XRE, xenobiotic responsive element or AHRE, AH receptor responsive element
ER	oestrogen receptor
EROD	ethoxyresorufin-*O*-deethylase
FICZ	6-formylindolo[3,2-*b*]carbazole
HAH	halogenated aromatic hydrocarbon
HIF-1	hypoxia inducible factor
hsp90	heat shock protein 90
H/W	Han/Wistar (*Kuopio*) strain
IARC	International Agency for Research on Cancer of the World Health Organization
ICZ	indolo[3,2-*b*]carbazole
LD50	lethal dose 50%
L-E	Long-Evans (*Turku/AB*) rat strain
MAP	mitogen-activated protein
NcoA	nuclear coactivator
NES	nuclear export signal
NF-κB	nuclear factor kappaB
NLS	nuclear localization signal

N-terminus	aminoterminus of protein
PAH	polycyclic aromatic hydrocarbon
PAS	PER-ARNT-SIM; a homologous region found first in PER, ARNT and SIM proteins
PCB	polychlorinated biphenyl
PER	product of *Drosophila Period* gene
PKC	protein kinase C
pp60^{scr}	a tyrosine kinase encoded by *c-src* gene
Q-rich	glutamine-rich
Rb	a retinoblastoma tumour suppressor protein
RIP140	receptor interacting protein 140
RT-PCR	reverse transcriptase-polymerase chain reaction
SIM	product of *Drosophila Single-minded* gene
SMRT	a silencing mediator of retinoic acid and thyroid hormone receptor
SRC-1	steroid receptor coactivator 1
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEF	toxic equivalency factor
TEQ	toxic equivalent quantity or TCDD equivalent quantity
TGF-β	transforming growth factor-beta
TNF-α	tumor necrosis factor-alpha
USEPA	United States Environmental Protection Agency
WHO	World Health Organization
XAP2	immunophilin-like X-associated protein 2, called also ARA9 or AIP1

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original articles referred to in the text by their Roman numerals:

I Korkalainen, M., Tuomisto, J., and Pohjanvirta, R. (2000) Restructured transactivation domain in hamster AH receptor. Biochem. Biophys. Res. Commun. 273: 272-281.

II Korkalainen, M., Tuomisto, J, and Pohjanvirta, R. (2001) The AH receptor of the most dioxin-sensitive species, guinea pig, is highly homologous to the human AH receptor. Biochem. Biophys. Res. Commun. 285: 1121-1129.

III Korkalainen, M., Tuomisto, J. and Pohjanvirta, R. (2003) Identification of novel splice variants of ARNT and ARNT2 in the rat. Biochem. Biophys. Res. Commun. 303: 1095-1100.

IV Korkalainen, M., Tuomisto, J. and Pohjanvirta, R. (2004) Primary structure and inducibility by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) of aryl hydrocarbon receptor repressor in a TCDD-sensitive and a TCDD-resistant rat strain. Biochem. Biophys. Res. Commun. 315: 123-131.

V Korkalainen, M., Lindén, J., Tuomisto, J. and Pohjanvirta, R. (2005) Effect of TCDD on mRNA expression of genes encoding bHLH/PAS proteins in rat hypothalamus. Toxicology 208: 1-11.

1 INTRODUCTION

Humans and most animals are exposed daily to a number of chemicals in the air, water or food. One class of these chemicals are the environmental contaminants, specifically dioxins and related halogenated aromatic hydrocarbons (HAHs). This group of chemicals raises concern because of their potency, widespread presence in the environment and persistence within the food chain. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most famous and most toxic dioxin.

Dioxins bring about a wide spectrum of biochemical and toxic effects, including reproductive and developmental defects, immunotoxicity, thymus atrophy, chloracne, wasting syndrome, liver toxicity and cancer (Pohjanvirta and Tuomisto, 1994). Practically all of these effects are mediated via the aryl hydrocarbon receptor (AHR), which is a ligand-activated transcription factor (Okey *et al.*, 1994). The AHR regulates the expression of target genes by heterodimerizing with ARNT, the AH receptor nuclear translocator and then interacting with dioxin response elements (DREs, also known as xenobiotic responsive elements [XREs] or aryl hydrocarbon responsive elements [AHREs]) in DNA (Whitlock, 1999). The AHR mediates not only the toxic effects of dioxins but also the adaptive effects, including the induction of many xenobiotic metabolizing enzymes such as cytochrome P4501A1 (CYP1A1) (Nebert *et al.*, 2004).

One characteristic feature of TCDD toxicity is its wide variability in sensitivity between strains and species, which complicates dioxin risk assessment. In our laboratory, a 1000-fold sensitivity difference to acute lethality of TCDD was established between sensitive Long-Evans (*Turku/AB*) (L-E) and resistant Han/Wistar (*Kuopio*) (H/W) rat strains (Pohjanvirta and Tuomisto, 1994). The reason for TCDD resistance in H/W rat was found to be in the AHR, which had an abnormal carboxyterminal (C-terminal) transactivation domain due to a critical point mutation (Pohjanvirta *et al.*, 1998; Tuomisto *et al.*, 1999). Despite the restructured AHR some endpoints of dioxin toxicity, such as induction of CYP1A1, are similar between these rat strains (Pohjanvirta *et al.*, 1988). However, the deleted transactivation domain of H/W AHR affects the expression of those target genes that are the key to dioxin toxicity, such as wasting syndrome and acute TCDD lethality. Therefore, our rat model is useful not only in studying dioxin sensitivity differences but also in elucidating the molecular mechanisms of dioxin toxicity.

Another interesting animal pair used in dioxin sensitivity studies is hamster and guinea pig, which show the same kind of sensitivity difference in acute lethality of TCDD to that occurring between L-E and H/W rats (Pohjanvirta and Tuomisto, 1994). Although TCDD-resistant hamsters and H/W rats are known to share selective responsiveness to TCDD, it is not known whether their resistance is based on a similar phenomenon. In the present study, these two animal pairs were utilized to elucidate the mechanisms determining the

exceptionally large differences present among species and strains in sensitivity to TCDD toxicity. Moreover, this study addressed the question of whether other principal proteins in dioxin signal transduction, in addition to AHR, could be potential factors involved in dioxin sensitivity differences.

2 REVIEW OF THE LITERATURE

2.1 Dioxins

2.1.1 Background

Dioxins (polychlorinated dibenzo-*p*-dioxins) and related HAHs (e.g. dibenzofurans and dioxin like polychlorinated biphenyls [PCBs]) are ubiquitous and persistent environmental contaminants. Their potential hazard to humans and animals is increased by the fact that they are fat-soluble and thus tend to bioaccumulate in tissue lipids and in the food chain. TCDD is a prototype of dioxins (Fig.1). It is the most potent of the halogenated environmental organic pollutants and thus often called the most toxic synthetic compound.

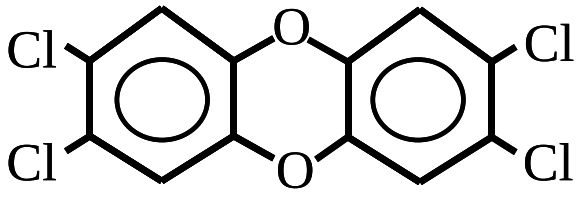

Figure 1. The structure of TCDD

TCDD was introduced as a toxic compound to the general public most notably by an industrial accident in Seveso, Italy in 1976. Due to an explosion in a chemical plant producing trichlorophenol, a toxic cloud containing large quantities of TCDD was released to the environment. Many domestic and wild animals were killed, but no life-threatening health effects were reported among the thousands of exposed inhabitants; only a few hundred chloracne cases were observed during the period of acute exposure (Bertazzi *et al.*, 1998; Mocarelli, 2001). TCDD was classified as a human carcinogen (Group I) by the International Agency for Research on Cancer (IARC) of the World Health Organization (WHO) (IARC, 1997). However, this classification, based on limited evidence of epidemiologic studies, has been somewhat controversial and was both challenged and supported in the literature (Cole *et al.*, 2003; Steenland *et al.*, 2004).

The term dioxin refers to a large family of compounds thought to have similar mechanisms of toxicity. Nevertheless, their toxicities vary greatly and to facilitate the risk assessment of these complex mixtures the concept of toxic equivalency factors (TEFs) was developed by the WHO (van den Berg *et al.*, 1998, 2000; Finley *et al.*, 2003). The TEF values for each dioxin congener was evaluated in relation to the most toxic congener, TCDD. In the

mixture, the measured concentration of each congener is multiplied by its TEF value [= toxic equivalent (TEQ) for this congener]. The sum of all these component TEQs gives the total toxicity of the mixture in TEQs (van den Berg *et al.*, 1998).

While dioxins have never been intentionally manufactured for purposes other than scientific research, they are formed as unwanted by-products during industrial production of some chemicals such as chlorophenols and phenoxyacetic acid herbicides. For example, herbiside 2,4,5-trichlorophenoxyacid (2,4,5-T), which was used as an antifoliant agent (Agent Orange) during the Vietnam War, contained TCDD at relatively high concentrations (Michalek *et al.*, 1996). Dioxins are also formed when organic compounds are burned incompletely in the presence of chlorine. In fact, the current major source of dioxin in the environment comes from combustion processes, such as metal smelting and refining as well as waste-burning incinerators of various sorts (e.g. municipal, hazardous and hospital waste) and also backyard burn-barrels. From all these sources, dioxins are released into air, land and water (Hays and Aylward, 2003).

Food is the major source of human exposure to dioxins, especially fatty foods. In central Europe, the main sources are dairy products and meat, while in Finland most of the exposure comes from fish and fish products. In particular, Baltic herring alone accounts for 52% of the total intake (Kiviranta *et al.*, 2001, 2004). Currently, the average daily intake of dioxins is about 1-2 pg/kg/day in most European countries. This is the actual or lower level that most authoritative agencies and scientific organizations have considered as a tolerable daily intake level (1-4 pg/kg/day) (Hays and Aylward, 2003). On the other hand, the United States Environmental Protection Agency (USEPA) suggested that even far lower levels may pose a significant health risk (USEPA, 2000). Although TCDD emissions and consequently TCDD intake levels have dramatically decreased over the past 30 years, the body burdens of dioxins do not change as rapidly due to the relatively long elimination half-life of TCDD (Päpke, 1998; Aylward and Hays, 2002).

2.1.2 Effects in humans and experimental animals

TCDD produces a variety of adverse biological responses in laboratory animals, including immunotoxicity, reproductive and endocrine effects, developmental toxicity, lethality, wasting, liver toxicity, teratogenesis, tumour promotion and cancer (Birnbaum, 1994; Pohjanvirta and Tuomisto, 1994; Birnbaum and Tuomisto, 2000). Nevertheless, the toxic responses to TCDD are dependent on many factors, such as exposure dose, age, sex, animal strain and species, target organs and cell types. For example, female rats are twice as sensitive to acute lethality of TCDD as male rats (Pohjanvirta *et al.*, 1993). However, there is strong evidence to suggest that practically all TCDD-induced responses are mediated via the AHR.

TCDD is one of the most anorexigenic compounds known. In rats, even a single sublethal dose of TCDD may lead to a rapid decline in feed intake and bring about a long-lasting or even permanent retardation of body weight gain. At lethal doses, TCDD elicits a precipitous and striking body weight loss. This wasting syndrome mainly results from hypophagia, but the exact mechanisms of wasting are still unknown (Seefeld *et al.*, 1984a, b; Pohjanvirta and Tuomisto, 1994; Tuomisto *et al.*, 2000).

TCDD can also result in adaptive effects, one of which is the induction of xenobiotic-metabolizing enzymes catalysing the metabolic processing of lipophilic chemicals to water-soluble derivatives and thereby facilitating their elimination (Whitlock, 1999; Nebert *et al.*, 2004). TCDD itself is poorly metabolized and thus it can cause chronic, sustained induction of CYP1A1, CYP1A2 and CYP1B1 leading to oxidative stress (Shertzer *et al.*, 1998; Nebert *et al.*, 2000). Oxidative stress following TCDD exposure has been demonstrated to increase the production of reactive oxygen species, lipid peroxidation and DNA damage (Slezak *et al.*, 2000; Hassoun *et al.*, 2001, 2002). Thus, the toxic effects of TCDD may be caused by induction of oxidative stress (Hassoun *et al.*, 2000, 2001, 2002). On the other hand, induction of oxidative metabolic processes by TCDD may cause the production of highly carcinogenic metabolites of polycyclic aromatic hydrocarbons (PAHs) and oestrogens, creating a link between AHR activation and chemical carcinogenesis (Shimizu *et al.*, 2000).

Epidemiological studies in accidentally exposed populations have suggested a possible link between TCDD and certain types of cancer, cardiovascular disease, diabetes, decreased male/female ratio of births, endometriosis and dental defects (Mocarelli *et al.*, 2000; Bertazzi *et al.*, 2001; Eskenazi *et al.*, 2002; Warner *et al.*, 2002; Pesatori *et al.*, 2003; Akhtar *et al.*, 2004; Alaluusua *et al.*, 2004). However, in Seveso the only verified effect of dioxin exposure has been chloracne, but the long-term follow up of this population is still continuing (Caramaschi *et al.*, 1981). In the highly exposed Seveso population, elevated plasma levels of TCDD are still present because of the long biological half-life of TCDD (Landi *et al.*, 2003; Baccarelli *et al.*, 2004).

The most sensitive effects of TCDD observed in experimental animals appear to be developmental, including effects on the developing immune, nervous and reproductive systems (Birnbaum and Tuomisto, 2000; Greene *et al.*, 2003). The endocrine system is also one of the critical targets for dioxins (Birnbaum and Fenton, 2003).

2.2 AH receptor

The AHR mediates most of the biochemical and toxic effects of dioxins and related aromatic hydrocarbons. About 30 years ago it was first discovered in mouse liver, where it was found specifically and with high affinity to bind to the radiolabelled analogue of TCDD (Poland *et al.*, 1976). Since that time, the mechanisms of AHR action and its connections with the toxic responses of TCDD have been studied extensively. As a ligand-activated nuclear receptor, the AHR plays a role in regulation of cytochrome P450 (CYP) genes (Honkakoski and Negishi, 2000), and so far the best-characterized AHR-mediated pathway is the induction of CYP1A1 (Whitlock, 1999).

The structure of AHR required years to resolve due to the instability of the receptor protein and low levels of expression. Biochemical purification of the AHR protein succeeded only after development of a photoaffinity-labelled ligand (Poland *et al.*, 1986; Perdew and Poland, 1988; Okey *et al.*, 1989). The sequence information of purified AHR protein was then used to clone the cDNA (Burbach *et al.*, 1992; Ema *et al.*, 1992). Later on, AHR cDNAs were cloned from several animals, including mammals, birds, fish and some invertebrates (Hahn, 2002).

The AHR is present in a wide variety of tissues. In the adult rat, AHR mRNA was expressed at the highest levels in lung, thymus, kidney and liver, while lower levels were expressed in heart and spleen (Carver *et al.*, 1994). In humans, placenta was reported to exhibit the highest expression, but high expression levels have also been detected in lung, heart, pancreas and liver, with lower levels of expression found in brain, kidney and skeletal muscle (Dolwick *et al.*, 1993a).

2.2.1 Structure of the AHR

The AHR was earlier thought to belong to the steroid receptors because of some functional similarities (Evans, 1988). However, the molecular cloning of AHR cDNA revealed that it belongs to the basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) superfamily, which is a group of structurally related proteins playing roles in detection of and adaptation to environmental change. One of the first members (cloned one year before the AHR) was the ARNT, which acts as a dimerization partner of the AHR (Hoffman *et al.*, 1991). Other founding members were PER (product of the *Drosophila Period* gene) and SIM (product of the *Drosophila Single-minded locus*), which shares a highly conserved PAS domain, named subsequently after these first three members (PER, ARNT, SIM) (Gu *et al.*, 2000; Kewley *et al.*, 2004).

The AHR, similarly to other bHLH/PAS proteins, has a conserved aminoterminus (N-terminus). The bHLH domain located nearest the N-terminus binds DNA and promotes dimerization with the ARNT. The PAS domain affords specificity for dimerization and also

contains most of the ligand-binding domain (LBD) (Fukunaga *et al.*, 1995; Pongratz *et al.*, 1998). In addition, both the bHLH and PAS domains are responsible for interaction with the 90-kDa heat shock protein (hsp90) (Antonsson *et al.*, 1995) (Fig. 2).

The N-terminal end also contains signals for both the nuclear localization (NLS) and the nuclear export signals (NES) (Ikuta *et al.*, 1998). Using these two signals the AHR shuttles between the cytoplasm and the nucleus of the cell (Ikuta *et al.*, 2000). A recent report suggests that the NLS is required for AHR-regulated biology, because mice carrying a mutation in the NLS of the *Ahr* gene were resistant to TCDD-induced toxicity, as are *Ahr* knockout mice (Bunger *et al.*, 2003).

The C-terminal end of the AHR is not conserved among bHLH/PAS proteins. This variable region contains a potent transactivation domain composed of several interacting subdomains (Reen *et al.*, 2002). One of the most important subdomains in this regard is a glutamine-rich (Q-rich) region (Jain *et al.*, 1994; Fukunaga *et al.*, 1995). In some in vitro studies, it exhibited the strongest transcriptional activity of several activation subdomains studied (Sogawa *et al.*, 1995a). In addition, the Q-rich subdomain is necessary and sufficient for *in vitro* interaction with the AHR coactivators receptor interacting protein 140 (RIP140) and steroid receptor coactivator-1 (SRC-1) (Kumar and Perdew, 1999; Kumar *et al.*, 1999). Furthermore, the retinoblastoma tumour suppressor protein (Rb) interacts with the Q-rich subdomain (Ge and Elferink, 1998).

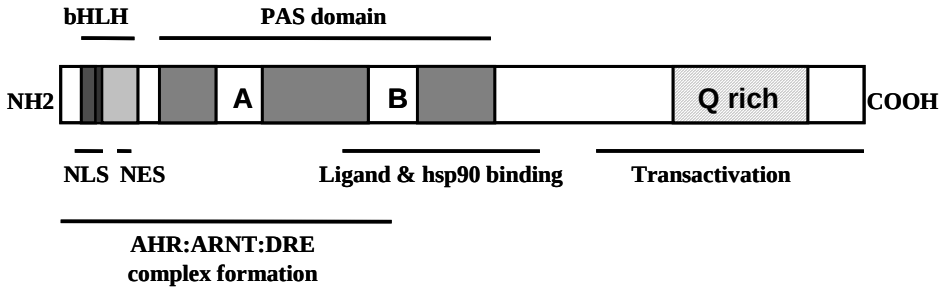

Figure 2. Domain structure of the AHR [modified from Denison *et al.* (2002)].

The 5'-flanking region of the *AHR* gene contains no TATA box, but instead multiple GC boxes near the transcription initiation sites. Comparison of human and murine *AHR* promoters revealed several conserved regions containing binding sites for transcription factors, such as Sp1 (Schmidt *et al.*, 1993; Eguchi *et al.*, 1994; Mimura *et al.*, 1994; Garrison and Denison, 2000; Racky *et al.*, 2004).

2.2.2 Mechanism of action

In the absence of a ligand, the AHR is found in the cytoplasm as a complex with two molecules of hsp90, the immunophilin like X-associated protein 2 (XAP2, known also as ARA9 or AIP1) and some other proteins such as p23 (Fig. 3) (Kazlauskas *et al.*, 1999, 2000; Meyer and Perdew, 1999; Petrulis and Perdew, 2002). In this complex, hsp90 is important for maintaining the receptor in a conformation exhibiting high affinity for ligand binding and repressed DNA-binding ability (Pongratz *et al.*, 1992; Whitelaw *et al.*, 1995). XAP2 binds to both hsp90 and the AHR and appears to stabilize the complex. It was also implicated in regulation of the intracellular localization of the AHR, in protection of the receptor against degradation and in repression of its transcriptional activity (Ma and Whitlock, 1997; Meyer and Perdew, 1999; Kazlauskas *et al.*, 2000, 2002; Petrulis *et al.*, 2003; Hollingshead *et al.*, 2004). p23 appears to stabilize the AHR-hsp90 complex in a ligand-inducible form (Kazlauskas *et al.*, 1999). There are also suggestions that some auxiliary cochaperones, such as p60 and hsp70, may associate with this complex, but neither their presence nor functional role is yet clear (Nair *et al.*, 1996; Petrulis and Perdew, 2002).

Ligands for the AHR are thought to enter cells by simple diffusion and to bind to the PAS B domain. Ligand binding induces a conformational change in the receptor exposing the NLS (Lees and Whitelaw, 1999; Henry and Gasiewicz, 2003; Ikuta *et al.*, 2004). The AHR complex then translocates into the nucleus, dissociates from its partner molecules and heterodimerizes with the ARNT (Probst *et al.*, 1993; McGuire *et al.*, 1994; Denison and Nagy, 2003). Formation of the AHR-ARNT heterodimer converts the complex to its high-affinity DNA-binding form (Kronenberg *et al.*, 2000). This complex binds to its specific DNA recognition site (DRE), located upstream of the target genes, such as CYP1A1 (Denison *et al.*, 1988, 1989; Whitlock, 1999). The DRE is composed of two half-sites, TNG and GTG, recognized by the AHR and ARNT, respectively (Denison *et al.*, 1988; Swanson *et al.*, 1995). The gene activation involves DNA bending, nucleosomal disruption and interaction with some transcription factors and coactivators/corepressors (Rowlands *et al.*, 1996; Nguyen *et al.*, 1999; Garrison *et al.*, 2000; Beischlag *et al.*, 2002; Wang *et al.*, 2004).

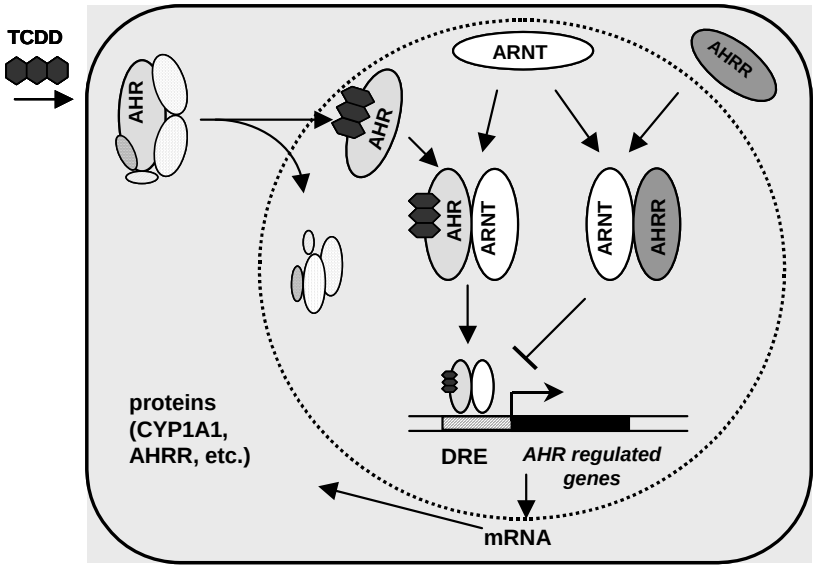

Figure 3. Mechanism of transcriptional activation by AHR and negative feedback regulation of AHR by AHRR.

The aryl hydrocarbon receptor repressor (AHRR), also a member of the bHLH/PAS family, is a negative regulator of the AHR, competing with it for formation of a heterodimer with the ARNT (Mimura *et al.*, 1999; Karchner *et al.*, 2002). The ARNT-AHRR complex is capable of binding to DRE, but not of transactivating genes. The AHRR itself is induced by AHR ligands, and thus the AHR and AHRR form a regulatory feedback loop in the AHR signal transduction pathway (Mimura and Fujii-Kuriyama, 2003).

Another mechanism by which the AHR can be down-regulated is degradation. The TCDD-induced degradation is ubiquitin-mediated and occurs via the 26S proteasome pathway following nuclear export of AHR (Roberts and Whitelaw, 1999; Ma and Baldwin, 2000; Swanson, 2002). This agonist-dependent degradation may protect cells from the consequences of prolonged exposure to high concentrations of agonists (Gu *et al.*, 2000). Recently, the existence of novel labile proteins was suggested both in the negative regulation and in the TCDD-induced degradation of AHR (Ma and Baldwin, 2002; Monk *et al.*, 2003).

2.2.3 Interactions with other proteins

The AHR interacts with a number of proteins, including transcription factors and coactivator or corepressor proteins. For example, the transcriptional coactivators SRC-1, nuclear activator (NcoA), and p160 directly interact with the AHR and coactivate transcription of TCDD-responsive genes (Beischlag *et al.*, 2002; Hankinson, 2005). In contrast, interactions with the silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) inhibit AHR-dependent gene expression (Nguyen *et al.*, 1999; Rushing and Denison, 2002; Fallone *et al.*, 2004). There are distinct motif(s) for the recruitment of coregulators to AHR; e.g. the coactivators SRC-1 and RIP140 interact with the Q-rich subdomain of the AHR transactivation domain (Kumar and Perdew, 1999; Kumar *et al.*, 1999).

The AHR is known to interact directly with the Rb, which controls cell cycle progression (Ge and Elferink, 1998). It was reported that this interaction potentiates repression of E2F-dependent transcription and cell cycle arrest (Puga *et al.*, 2000a). Very recent studies showed that TCDD treatment causes recruitment of the AHR to E2F-dependent promoters and the concurrent displacement of p300, which leads to repression of S-phase-specific genes and thus inhibition of the cell cycle (Marlowe *et al.*, 2004). Rb may also act as a coactivator of the AHR, since interactions of the AHR with Rb have appeared to be necessary for maximal AHR activity (Elferink *et al.*, 2001). Another mechanism by which the AHR is suggested to affect the cell cycle as well as apoptosis is interaction with transforming growth factor-beta (TGF-β) (Zaher *et al.*, 1998).

There is also evidence for cross-talk between the AHR and steroid hormone receptor signal transduction pathways, including the oestrogen (ER), androgen, progesterone and thyroid hormone receptors (Porterfield, 1994; Kharat and Saatcioglu, 1996; Jana *et al.*, 1999; Selmin *et al.*, 2005). For example, the TCDD-activated AHR directly interacts with oestrogen receptor alpha (ERα) and beta (ERβ) and orphan receptors COUP-TFI and ERRα1 to mediate the antioestrogenic effect of TCDD (Klinge *et al.*, 2000; Ohtake *et al.*, 2003). In addition, the AHR may interact with the hypoxia-inducible factor (HIF) signalling pathways independently of competition for heterodimerization with ARNT (Chan *et al.*, 1999). Furthermore, the AHR interacts with the nuclear factor kappaB (NF-κB) signalling pathway, which plays an important role e.g. in immune responses, inflammatory reactions and apoptosis (Tian *et al.*, 2002). It has been reported that activation of NF-κB is responsible for tumour necrosis factor-α (TNF-α) and lipopolysaccharide-induced suppression of CYP1A1 by AHR (Ke *et al.*, 2001).

The protein kinase C (PKC) pathway has been linked to TCDD-mediated AHR-dependent processes in several controversial reports (Berghard *et al.*, 1993; Chen and Tukey, 1996; Long *et al.*, 1998), but evidence on direct interactions between PKC and AHR has not been found until recently (Minsavage *et al.*, 2004). The AHR tyrosine 9 appears to be a critical residue required for phosphorylation of the AHR and AHR-mediated gene transcription

(Bacsi and Hankinson, 1996; Park *et al.*, 2000; Minsavage *et al.*, 2003, 2004). Furthermore, direct interactions of the AHR with $pp60^{src}$, a tyrosine kinase, were reported (Enan and Matsumura, 1996; Blankenship and Matsumura, 1997). In addition to PKC, mitogen-activated protein (MAP) kinases may also play a role in TCDD-induced AHR phosphorylation (Tan *et al.*, 2004a, 2004b). However, despite the evidence of direct phosphorylation of AHR, its functional significance is still unclear.

2.2.4 Ligands

The AHR can be bound and activated by structurally diverse ligands. The best-characterized, synthetic AHR ligands are planar and hydrophobic HAHs and PAHs. HAHs include, in addition to dioxins, compounds such as dibenzofurans and biphenyls, and PAHs include 3-methylcholanthrene, benzo(a)pyrene, benzanthracenes and benzoflavones (Poland and Knutson, 1982; Denison and Nagy, 2003). HAHs are the most potent class of AHR ligands, e.g. TCDD is 30000 times more potent than benzo(a)pyrene in causing enzyme induction response in rats (Nebert *et al.*, 2000). Therefore, TCDD is used as the prototype and model substance in cell and animal experiments.

There is a wide variety of naturally occurring AHR ligands, most of which are dietary plant-derived chemicals such as flavonoids, carotenoids and phenolics. They can activate the AHR signalling pathway, although the majority are relatively weak ligands (Denison *et al.*, 2002). One exception is indolo[3,2-*b*]carbazole (ICZ), which is an indole derivative present in some cruciferous vegetables and which has a high affinity for the AHR (Gillner *et al.*, 1993). However, in rats, it failed to cause toxicity and CYP1A1 induction (Pohjanvirta *et al.*, 2002).

To date, a true endogenous ligand for the AHR has not been found, although a number of reports have suggested potential canditates. Most of these suggested compounds are tryptophan derivatives (Rannug *et al.*, 1987). For example, the tryptophan photoproduct, 6-formylindolo[3,2-*b*]carbazole (FICZ), possesses a very high AHR-binding affinity and transiently induces *CYP1A1* gene expression in cultured cells (Wei *et al.*, 1999, 2000). Other potent AHR ligands are indigo and indirubin, which were isolated from human urine (Adachi *et al.*, 2001) and induce AHR-mediated microsomal drug-metabolizing enzyme activity in mice (Sugihara *et al.*, 2004). Another possible canditate for endogenous ligand was isolated from porcine lung tissue (Song *et al.*, 2002). In addition, many other endogenous chemicals have been identified that can bind to the AHR and activate AHR-mediated gene expression. These chemicals include bilirubin and biliverdin (Sinal and Bend, 1997; Phelan *et al.*, 1998), arachidonic acid metabolites such as lipoxin A4 (Schaldach *et al.*, 1999) and several prostaglandins (Seidel *et al.*, 2001), 7-ketocholesterol (Savouret *et al.*, 2001) and retinoids (Soprano *et al.*, 2001; Gambone *et al.*, 2002).

2.2.5 Target genes

Although numerous genes are regulated by the AHR, the best-studied target genes are those encoding xenobiotic-metabolizing enzymes. In mice, the conventional [Ah] gene battery comprises six members, two phase I cytochrome P450 genes and four phase II detoxification enzyme-encoding genes (Fig. 4) (Nebert *et al.*, 1990, 1993, 2000). In addition, *CYP1B1* gene is also regulated by the AHR (Sutter *et al.*, 1994). Moreover, even within the CYP superfamily, new genes continue to be identified that are under AHR control (Rivera *et al.*, 2002).

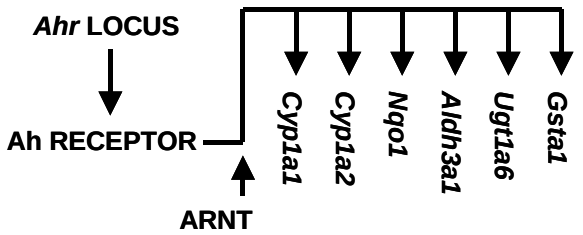

Figure 4. The six members of the mouse [Ah] gene battery [modified from Nebert *et al.* (2000)]. Abbreviations: *Cyp1a1*, cytochrome P4501A1; *Cyp1a2*, cytochrome P4501A2; *Nqo1*, NAD(P)H:quinone oxidoreductase; *Alhd3a1*, cytosolic aldehyde dehydrogenase 3; *Ugt1a6*, UDP glucuronosyltransferase 1A6; *Gsta1*, glutathione transferase.

Recent studies using DNA microarray techniques suggested that approximately 300 genes are potentially altered by AHR activation in the human hepatoma HepG2 cell line (Puga *et al.*, 2000b; Frueh *et al.*, 2001). However, only one third of these genes were directly regulated by TCDD while the remaining genes required protein synthesis to show their regulated expression by TCDD. When the genes were clustered in groups with related functions, several groups could be identified, e.g. genes involved in calcium regulation; receptor-associated kinases, phosphatases and their effectors; coding transcription factors; cardiovascular and pulmonary function; cell cycle regulation, differentiation and apoptosis; development, cell adhesion, cancer and metastasis; protein traffic and membrane integrity and drug metabolism and DNA stability (Puga *et al.*, 2000b). In another study using microarray and reverse transcriptase-polymerase chain reaction (RT-PCR) techniques, 114 genes (64 human and 50 rat genes) changed their expression on AHR activation in hepatocytes. Some of these genes were well-known targets of the AHR such as genes in the [Ah] gene battery, but also recently reported AHR targets, such as *Bcl-2*, *Bcl-xl*, *BAD* and *cyclooxygenase 1* (Kel *et al.*, 2004).

The development of new computational methods and availability of the human, mouse and rat genomic sequences have enabled the identification and characterization of promoters and other regulatory elements. In a recent study, promoters of AHR-regulated genes were analysed to search for new targets within the human genome (Kel *et al.*, 2004). At least one potential AHR site was found in 864 genes (and 71 of these potential genes with the highest prediction scores were also verified by RT-PCR and microarray studies). Likewise, a comparative computational scanning approach was used to identify putative DREs in the genomic sequences of human, mouse and rat target genes (Sun *et al.*, 2004). This screening observed a few thousand DRE-containing genes, but only 48 of these were common in human, mouse and rat. Among the 48 genes, 7 were classified as being involved in oxidative stress, hypoxia and detoxification, 5 were associated with calcium homeostasi, and 5 were localized in the endoplasmic reticulum (Sun *et al.*, 2004).

In addition to its traditional role as a ligand-activated transcription factor, the AHR also functions as a coactivator (Boutros *et al.*, 2004; Sogawa *et al.*, 2004). Upstream of the rat *CYP1A2* gene, a novel response element (called XRE-II, DRE-II or AHRE-II) was characterized, to which the AHR-ARNT heterodimer can bind while associated with an unidentified factor (protein X) (Sogawa *et al.*, 2004) (Fig. 5). Binding of this complex leads to an activation of a set of genes called the AHRE-II gene battery (Boutros *et al.*, 2004). So far, a total of 36 genes have been found that contain AHRE-II motifs conserved across human, mouse and rat genomes and over one third of these genes responded to TCDD. In addition to CYP1A2, the AHRE-II gene battery encodes a large number of transporters and ion channels (Boutros *et al.*, 2004). In oestrogen signalling, activated AHR-ARNT heterodimer also functions as a coactivator. This heterodimer directly recruits ER in the absence of ER ligands and activates the transcription of ER-mediated genes (Ohtake *et al.*, 2003).

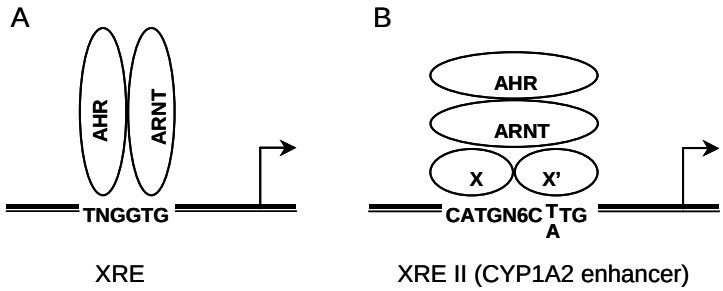

Figure 5. Schematic representation of two types of transcription mechanism mediated by AHR-ARNT. A. The classical model of induction of genes mediated by the AHR-ARNT heterodimer. B. The novel induction mechanism in which the AHR-ARNT heterodimer functions as a coactivator in the rat *CYP1A2* gene [modified from Sogawa *et al.* (2004)].

New AHR-regulated genes have also been identified by studying individual genes. For example, *HES-1*, which plays a role in neuronal differentiation and also in the cell cycle, as well as *Socs2*, which suppresses cytokine signalling, are new targets for AHR regulation (Boverhof *et al.*, 2004; Thomsen *et al.*, 2004). In addition, several hypothalamic neuropeptide genes contain DREs, which suggests that TCDD can directly regulate their expression in hypothalamic neurons (Fetissov *et al.*, 2004). Interestingly, some genes of transcription factors are also regulated by the AHR (Puga *et al.*, 2000b; Borlak *et al.*, 2002; Kel *et al.*, 2004), which could explain the AHR-mediated regulation of several genes that are not direct targets of the AHR (Kel *et al.*, 2004).

2.2.6 Diversity, evolution and physiological significance

Characterization of AHR in diverse species is one approach to understanding the physiological significance of the AHR. So far, AHR cDNAs have been cloned and sequenced from a variety of mammalian species as well as birds, fishes and some invertebrate species (Table 1). The conservation of functional AHRs among species suggests that the AHR plays an important physiological role in addition to its role in xenobiotic metabolism. Evidence from AHR-deficient mice has revealed that the AHR can affect reproduction, survival and growth (Gonzalez and Fernandez-Salguero, 1998). Interestingly, recent studies provided evidence that AHR activation and developmentally induced heterodimerization with the ARNT are essential for normal vascular development (Lahvis *et al.*, 2000; Walisser *et al.*, 2004a, 2004b). Furthermore, the AHR regulates neuronal differentiation in *C.elegans*, suggesting that the AHR has an evolutionarily conserved role in neuronal development (Qin and Powell-Coffman, 2004).

Table 1. Full-length AHR cDNAs cloned to date [modified from Hahn (2002)].

Species	Accession number	Length (aa)	Reference
Mammals			
Human (*Homo sapiens*)	NM-001621	848	(Dolwick *et al.*, 1993a)
Mouse (*Mus musculus*)	NM-013464	805	(Ema *et al.*, 1992)
Rat (Sprague Dawley) (*Rattus norvegicus*)	NM-013149	853	(Carver *et al.*, 1994)
Hamster (*Mesocricetus auratus*)	AF275721	920	(Korkalainen *et al.*, 2000)
Guinea pig (*Cavia porcellus*)	AY028947	846	(Korkalainen *et al.*, 2001)
Rabbit (*Oryctolagus cuniculus*)	D38226	847	(Takahashi *et al.*, 1996)
Beluga Whale (*Delphinapterus leucas*)	AF332999	845	(Jensen and Hahn, 2001)
Harbor seal (*Phoca vitulina*)	AB056700	843	(Kim and Hahn, 2002)
Baikal seal (*Phoca sibirica*)	AB072432	843	(Kim *et al.*, 2002)
Birds			
Chicken (*Gallus gallus*)	AF260832, AF192502	858	(Walker *et al.*, 2000)
Common tern (*Sterna hirundo*)	AF192503	859	(Karchner *et al.*, 2000)
Black-footed albatross (*Diomeda nigripes*)	AB106109 (AHR1)	861	(Yasui *et al.*, 2004)
Black-footed albatross (*Diomeda nigripes*)	AB106110 (AHR2)	861	(Yasui *et al.*, 2004)
Cormorant (*Phalacrocorax carbo*)	AB109545 (AHR1)	860	(Yasui *et al.*, 2004)
Amphibians			
Frog (*Xenopus laevis*)	AB109555	834	(Ohi *et al.*, 2003)
Bony fish			
Killifish (*Fundulus heteroclitus*)	AF024591 (AHR1)	944	(Karchner *et al.*, 1999)
Killifish (*Fundulus heteroclitus*)	U29679 (AHR2)	952	(Karchner *et al.*, 1999)
Rainbow trout (*Oncorhynchus mykiss*)	AF065137 (AHR2α)	1058	(Abnet *et al.*, 1999)
Rainbow trout (*Oncorhynchus mykiss*)	AF065138 (AHR2β)	1059	(Abnet *et al.*, 1999)
Zebrafish (*Danio rerio*)	AF258854 (AHR1)	805	(Andreasen *et al.*, 2002)
Zebrafish (*Danio rerio*)	AF063446 (AHR2)	1027	(Tanguay *et al.*, 1999)
Medaka (*Oryzias latipes*)	AB065092 (AHR1)	879	(Kawamura and Yamashita, 2002)
Atlantic tomcod (*Microgradus tomcod*)	AF050489 (AHR2)	823	(Roy and Wirgin, 1997)
Jawless fish			
Lamprey (*Petromyzon marinus*)	AF024595	1076	(Hahn *et al.*, 1997)
Invertebrates			
Soft-shell clam (*Mya arenaria*)	AF261769	843	(Butler *et al.*, 2001)
Nematode (*Caenohabditis elegans*)	AF039570	602	(Powell-Coffman *et al.*, 1998)
Fruit fly (*Drosophila melanogaster*)	AF050630 (spineless)	884	(Duncan *et al.*, 1998)

Two AHR genes were identified in fish and very recently in aquatic birds (Karchner *et al.*, 1999; Andreasen *et al.*, 2002; Yasui *et al.*, 2004). Whether amphibians also have AHR2 is not yet clear (Ohi *et al.*, 2003). AHR1 shows a high level of identity with the mammalian AHR, whereas AHR2 does not have a mammalian counterpart (Hahn, 2001). It has been suggested that AHR1 and AHR2 arose from gene duplication, which may have occurred after the diversion of fish from other vertebrate species. Mammalian AHRs belong to the AHR1 clade (Fig. 6). The third clade is the AHRR group, which due to another gene duplication diverged from the AHR clades before AHR1 and AHR2 diverged from each other (Hahn, 2002). AHRR has been identified both in mammalian and fish species (Mimura *et al.*, 1999; Karchner *et al.*, 2002). The fourth clade consists of invertebrate AHRs. Both AHR1 and AHR2 are capable of specific binding of TCDD, whereas neither AHRR nor the invertebrate AHR homologues possess this property (Hahn, 2002). Instead of its transactivation function, the AHRR group has acquired activity as a repressor.

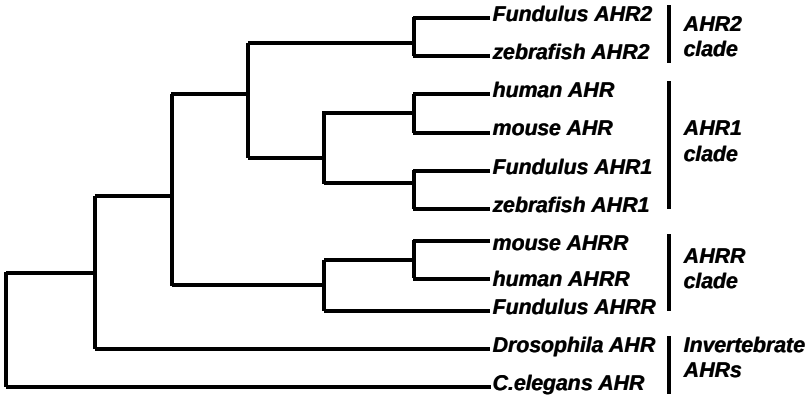

Figure 6. Phylogenetic tree showing relationship among vertebrate *AHR1*, *AHR2*, *AHRR* and invertebate *AHR* genes [modified from Hahn (2002)].

2.2.7 Induction mechanisms independent of the AHR

TCDD has been reported to activate MAP kinases, such as Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), independently of AHR (Tan *et al.*, 2002). However, further elucidation of the mechanism revealed that TCDD-activated MAP kinases enhance the transcriptional activity of ARNT and therefore the AHR-ARNT-dependent gene expression (Tan *et al.*, 2004a). MAP kinases may regulate the transcriptional activity of cofactors (p300, p160, Rb and SMRT), which are associated with the AHR/ARNT complex (Tan *et al.*, 2004a).

Moreover, other signal transductions may also be modulated independently of the DRE-binding and transcriptional activation roles of AHR (Enan and Matsumura, 1996; Chan *et al.*, 1999; Reiners and Clift, 1999; Puga *et al.*, 2000a; Dunlap *et al.*, 2002; Guo *et al.*, 2004). However, it remains to be determined whether there is cross-talk with AHR through the common coregulator proteins shared by different pathways (Carlson and Perdew, 2002). Very recently, it was reported that nuclear localization of the AHR was an essential step in most TCDD-mediated responses, such as xenobiotic metabolism, TCDD toxicity as well as regulation of normal liver development (Bunger *et al.*, 2003).

2.3 Other bHLH/PAS proteins in dioxin signalling

The AHR, ARNT and AHRR are all members of the bHLH/PAS protein family, which is a rapidly growing family of signal transduction molecules playing roles in development and environmental sensing, including xenobiotic metabolism, hypoxic response, circadian rhythm, development of the central nervous system (CNS) and tracheal formation (Gu *et al.*, 2000; Kewley *et al.*, 2004). The family was originally identified and named according to three founder members: human ARNT, *Drosophila* PER and *Drosophila* SIM. All these proteins possess the essential bHLH/PAS homology domain, which mediates the interaction between two different family members.

The bHLH/PAS proteins can be divided into two phylogenetic groups, based on sequence similarity. The class I factors include AHR, single-minded proteins (SIM1 and SIM2), hypoxia inducible factors (HIF-1α, HIF-2α and HIF-3α) and inhibitory PAS protein (IPAS) (Ema *et al.*, 1996; Makino *et al.*, 2002; Bracken *et al.*, 2003). To form active transcription complexes they must dimerize with class II factors, such as ARNT, ARNT2 or circadian rhythm proteins PER, BMAL1 (= ARNT3) and BMAL2 (Hirose *et al.*, 1996; Gekakis *et al.*, 1998; Takahata *et al.*, 1998).

2.3.1 ARNT and ARNT2

ARNT is a dimerization partner not only for the AHR but also for several other members of the bHLH/PAS family and thus it plays a central role in regulating divergent signalling pathways (Sogawa *et al.*, 1995b; Swanson, 2002). While ARNT can form heterodimers with many partner proteins and also homodimers with itself, ordinarily there exists a sufficient ARNT pool to prevent its levels from limiting AHR signalling (Pollenz *et al.*, 1999; Tomita *et al.*, 2000). However, the simultaneous inductions of the AHR and hypoxia signalling pathways were recently reported to reduce the AHR mediated responses but not the hypoxia response (Hofer *et al.*, 2004; Prasch *et al.*, 2004).

ARNT was originally identified as a factor required for the nuclear translocation of the AHR from the cytoplasm in response to dioxins (Hoffman *et al.*, 1991). ARNT has since

been shown to be a protein localized in the nucleus (Eguchi *et al.*, 1997) and to contain NLS in its N-terminus in addition to bHLH and the PAS A and B domains (Whitelaw *et al.*, 1994). ARNT also contains a potent C-terminal transactivation domain (Jain *et al.*, 1994) (Fig. 7). As in AHR, the promoter region of *ARNT* has no TATA box, but on the other hand, it has several potential regulatory sequences, such as two GC boxes, a cAMP-responsive element, AP-1 site, CAAT box and E box, which may contribute to a high level of expression of the ARNT (Wang *et al.*, 1998). One splice variant of ARNT, the deletion of exon 5, was reported but does not impair the protein function (Hoffman *et al.*, 1991; Drutel *et al.*, 1996; Jana *et al.*, 1998; Wang *et al.*, 1998).

ARNT has been characterized in various mammalian and fish species (Powell and Hahn, 2000). For invertebrate species, ARNT homologues were found in both *Drosophila* (called Tango) and *C.elegans* (called AHA-1) (Duncan *et al.*, 1998; Powell-Coffman *et al.*, 1998). Their heterodimers with AHR homologues recognize the same DRE sequence as does mammalian AHR/ARNT heterodimer, which suggests that the basic mechanism is conserved between vertebrates and invertebrates, although the functions of these proteins are different (Duncan *et al.*, 1998; Karchner *et al.*, 2000). Studies with ARNT knockout mice show that the ARNT protein serves an indispensable function in development, probably via its role in hypoxic induction of angiogenesis (Kozak *et al.*, 1997; Maltepe *et al.*, 1997). Recently, it was suggested that ARNT may be involved in the development of nonsyndromic oral cleft (Kayano *et al.*, 2004).

ARNT2 is a close structural homologue of ARNT (Hirose *et al.*, 1996) and is expressed primarily in brain and kidney, while ARNT is expressed ubiquitously (Drutel *et al.*, 1996; Hirose *et al.*, 1996; Jain *et al.*, 1998; Petersen *et al.*, 2000). The ARNT2 protein may play a role in dioxin signal transduction by acting as an alternative dimerization partner for AHR (Gu *et al.*, 2000). ARNT2 also forms functional HIF complexes in neurons and plays an integral role in hypoxic responses in the CNS (Maltepe *et al.*, 2000). In addition, ARNT2 has other important biological roles, such as controlling the development of neuroendocrine lineages in hypothalamic nuclei together with SIM1 (Michaud *et al.*, 2000).

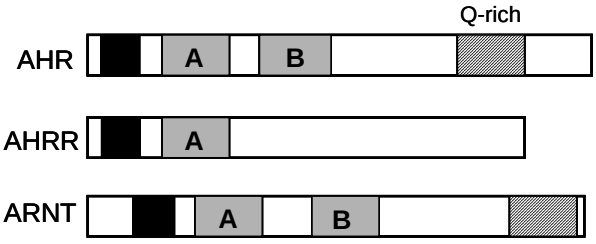

Figure 7. Schematic representation of the structures of AHR, AHRR and ARNT. The bHLH and PAS domains and Q-rich region are marked [modified from Kewley *et al.* (2004)].

2.3.2 AHRR

AHR repressor was first identified as a protein closely related to AHR in mice (Mimura *et al.*, 1999), in which it inhibits AHR function by competing with AHR for dimerization with ARNT and for binding to the XRE sequence. In addition, AHRR is inducible by AHR ligands and thus regulates AHR function by a negative feedback mechanism affecting the expression of genes that are induced by dioxins (Mimura *et al.*, 1999).

AHRR is not capable of binding ligands, probably due to the presence of a truncated PAS B domain (Mimura *et al.*, 1999; Karchner *et al.*, 2002). Despite this deviation, AHRR can still form a functional heterodimer with ARNT (Kikuchi *et al.*, 2003). Another domain, which is not functional in AHRR, is the Q-rich region (Fig. 7). This probably explains the inability of the AHRR-ARNT complex to transactivate DRE-regulated genes (Mimura *et al.*, 1999; Karchner *et al.*, 2002). The characterization of *AHRR* gene promoter sequences revealed no TATA box, but instead, several GC box and DRE sequences as well as a single NF-κB-binding site were detected (Baba *et al.*, 2001). It was suggested that the low constitutive activity of the *AHRR* gene is dependent on these GC boxes (Baba *et al.*, 2001).

To date, the *AHRR* gene has been identified in mouse, human, killifish and zebrafish (Mimura *et al.*, 1999; Watanabe *et al.*, 2001; Fujita *et al.*, 2002; Karchner *et al.*, 2002) (Evans *et al.*, 2004). The basal expression of AHRR mRNA is very low in untreated tissues of mice, but after 3-methylcholanthrene treatment AHRR mRNA levels were induced in several tissues (Mimura *et al.*, 1999). The high constitutive expression of AHRR has been reported to repress the induction of CYP1A1 in human fibroblasts (Gradin *et al.*, 1999). In humans, AHRR is constitutively expressed in various normal tissues, especially in testis (Tsuchiya *et al.*, 2003; Yamamoto *et al.*, 2004). In a population in Japan, two polymorphism of *AHRR* were detected, one of which may be involved in the susceptibility to dioxin-related male infertility (Watanabe *et al.*, 2004) and the incidence of micropenis (Fujita *et al.*, 2002). In contrast, no associations were detected between the *AHRR* polymorphism and uterine endometriosis (Watanabe *et al.*, 2001).

2.3.3 Other bHLH/PAS proteins

Recent data suggest that other bHLH/PAS proteins may also have connections with AHR signalling. First, Yang et al. (2004) reported that TCDD induces the expression of SIM1 via interaction with the AHR-ARNT2 complex and that this interaction is involved in the control of food intake. Moreover, SIM1 requires ARNT2 as an obligatory heterodimerization partner in the developmental process of neuroendocrinological cell lineages (Michaud *et al.*, 2000; Hosoya *et al.*, 2001). Second, TCDD alters the expression of PER in the suprachiasmatic nuclei, which is the master circadian clock (Li *et al.*, 2004). In the same mouse model, persistent activation of the AHR pathway by TCDD led to a failure in circadian homeostasis (Frame *et al.*, 2004). Third, AHR and HIF-1α have been shown to

inhibit each other independently of competition for ARNT (Chan *et al.*, 1999; Pollenz *et al.*, 1999).

2.4 Role of AHR genetic variability in dioxin sensitivity

A characteristic feature of TCDD toxicity is wide variation in sensitivity among species and even strains of the same species (Table 2). The largest interspecies difference in TCDD toxicity exists between guinea pig and hamster, while L-E and H/W rats show the largest intraspecies difference. Both these animal pairs, exhibiting over 1000-fold difference in susceptibility to the acute lethality of TCDD, have been used as animal models in dioxin sensitivity studies. These animal models are particularly interesting because only some endpoints of TCDD toxicity are different in contrast to the mouse model employing the C57BL/6 and DBA/2 strains, in which all endpoints are similarly altered by TCDD treatment. The molecular basis for the rat strain difference appears to reside mainly in AHR polymorphism (see 2.4.2), but that for species difference is unknown.

Table 2. Species differences in acute TCDD toxicity.

Species/strain	LD_{50}(μg/kg)	Reference
Mouse		
C57BL/6	180	(Chapman and Schiller, 1985)
DBA/2	2600	(Chapman and Schiller, 1985)
Rat		
Sprague-Dawley	60	(Beatty *et al.*, 1978)
L-E	18	(Pohjanvirta *et al.*, 1993)
H/W	> 9600	(Unkila *et al.*, 1994)
line A	> 10 000	(Tuomisto *et al.*, 1999)
line B	830	(Tuomisto *et al.*, 1999)
line C	40	(Tuomisto *et al.*, 1999)
Guinea pig	1-2	(Schwetz *et al.*, 1973) (McConnell *et al.*, 1978b)
Hamster	3000-5000	(Olson *et al.*, 1980b) (Henck *et al.*, 1981)
Rabbit	115	(Schwetz *et al.*, 1973)
Chicken	< 25	(Greig *et al.*, 1973)
Monkey	< 70	(McConnell *et al.*, 1978a)
Fish		
Lake trout (sack fry)	0.074	(Walker *et al.*, 1996)
Rainbow trout (embryo)	0.20	(Walker *et al.*, 1996)
Killifish (embryo)	0.25	(Toomey *et al.*, 2001)
Zebra fish (embryo)	2.50	(Henry *et al.*, 1997)

2.4.1 Mouse model

C57BL/6 and DBA/2 mice

One of the best-characterized strain differences in TCDD toxicity exists between the sensitive C57BL/6 and resistant DBA/2 mouse strains. This approximately 10-fold difference in sensitivity exists in a wide variety of TCDD-induced biochemical and toxic effects including CYP1A1 induction, acute lethality, teratogenicity, hepatic porphyria and thymic atrophy (Poland and Knutson, 1982; Nebert, 1989). The response to TCDD is dependent on the *Ahr* alleles; C57BL/6 mice carry a wild-type Ahr^{b1} allele that encodes a high-affinity AHR, while the DBA/2 strain carries a low-affinity-type Ahr^{d} allele (Okey *et al.*, 1989; Poland *et al.*, 1994). cDNA cloning of the Ahr^{d} allele revealed an Ala-to-Val substitution at codon 375 in the LBD that resulted in markedly reduced binding affinity for TCDD (Ema *et al.*, 1994). Many other genetic variations were found in the mouse *Ahr* gene, but none of these polymorphisms has yet been associated with any change in receptor function (Thomas *et al.*, 2002).

Genetically engineered mice

AHR knock out mice have been developed by three independent laboratories (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996; Mimura *et al.*, 1997). These mice do not exhibit induction of typical AHR target genes, such as of *Cyp1a1* or *Cyp1a2*, when treated with TCDD (Fernandez-Salguero *et al.*, 1995). They are also highly resistant to the toxic effects of TCDD including acute lethality, thymus atrophy, liver toxicity, teratogenesis, hydronephrosis and reproductive effects (Fernandez-Salguero *et al.*, 1995; Fernandez-Salguero *et al.*, 1996; Mimura *et al.*, 1997; Thurmond *et al.*, 1999; Lin *et al.*, 2001). In addition, these mice are resistant to the carcinogenity of benzo[a]pyrene (Shimizu *et al.*, 2000).

Although AHR knock out mice appear normal at birth, they have several phenotypic abnormalities, such as immune system impairment, reduced fecundity and hepatic defects including smaller liver size, fibrosis and liver retinoic accumulation (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996; Andreola *et al.*, 1997; Abbott *et al.*, 1999). Lesions are found in many tissues, e.g. in skin, liver, heart, eye and kidney (Fernandez-Salguero *et al.*, 1997; Lahvis *et al.*, 2000). However, the most reproducible phenotype across laboratories is a reduction in relative liver weight, which may be the result of the persistence of a fetal vascular structure, the *ductus venosus*, leading to massive portosystemic shunting (Lahvis *et al.*, 2000). Identical patent *ductus venosus* was recently observed in mice harbouring a hypomorphic *Ahr* or *Arnt* allele, but AHR activation by TCDD during late development enables rescue from this vascular defect (Walisser *et al.*, 2004a, 2004b).

Recently, mice carrying a mutation in the nuclear localization sequence of the *Ahr* were developed (Bunger *et al.*, 2003). These Ahr^{nls} mice are resistant to TCDD toxicity and

display the same developmental defects as were previously observed in AHR knock out mice, which suggests that nuclear localization is required for most, if not all, of AHR-regulated biology (Bunger *et al.*, 2003).

Furthermore, a transgenic mouse model that expresses a constitutively active AHR was developed (McGuire *et al.*, 2001; Andersson *et al.*, 2002). These mice have reduced life span, show increased expression of AHR-dependent genes such as *Cyp1a1*, develop spontaneous stomach tumours, show decreased thymus weight and diminished population of peritoneal B1 cells and have a higher prevalence of liver tumours than wild-type mice (Andersson *et al.*, 2002, 2003; Moennikes *et al.*, 2004).

A 'humanized' mouse was developed by introducing human AHR cDNA in place of mouse *Ahr* (Moriguchi *et al.*, 2003). The human AHR expressed in these mice is functionally less responsive to TCDD than the AHR of DBA/2 mice. Possibly, this humanized model mouse could be used to predict the biological effects of bioaccumulative environmental toxicants such as TCDD in humans (Moriguchi *et al.*, 2003).

2.4.2 Rat model

H/W and L-E rats

The TCDD-sensitive L-E and TCDD-resistant H/W rats show the largest intraspecies difference, about 1000-fold, in acute lethality of TCDD. The lethal dose 50 (LD50) values for these rat strains are about 10 and > 9600 µg/kg, respectively (Pohjanvirta *et al.*, 1993; Unkila *et al.*, 1994). Thus, the H/W rat represents the most TCDD-resistant mammal known. However, despite their resistance to the acute lethality of TCDD, H/W rats are sensitive to certain biochemical and toxic responses of TCDD, with the magnitude of responses being similar to those in L-E rats. These responses, called type I endpoints, include induction of CYP1A1, thymic atrophy, fetotoxicity and tooth defect (Pohjanvirta *et al.*, 1989; Alaluusua *et al.*, 1993; Huuskonen *et al.*, 1994; Simanainen *et al.*, 2002; 2003). Type II endpoints with a clear sensitivity difference include, in addition to TCDD lethality (Pohjanvirta *et al.*, 1993; Unkila *et al.*, 1994), e.g. wasting syndrome (Pohjanvirta *et al.*, 1987), hepatotoxicity (Pohjanvirta *et al.*, 1989), liver tumor promotion (Viluksela *et al.*, 2000), teratogenesis (Huuskonen *et al.*, 1994), hyperbilirubinaemia and accumulation of biliverdin in the liver (Niittynen *et al.*, 2003) and increased serum tryptophan and free fatty acids (Pohjanvirta *et al.*, 1989; Unkila *et al.*, 1994; Simanainen *et al.*, 2002, 2003).

In vitro studies show that the binding affinity of AHR for TCDD is similar in both strains, although the number of binding sites was 2-3-fold higher in L-E rats (Pohjanvirta *et al.*, 1999). Moreover, both AHRs are able to dimerize with ARNT and bind to DRE in DNA (Pohjanvirta *et al.*, 1999). In addition, there are no major differences in tissue distribution, metabolism and excretion of ^{14}C-TCDD between L-E and H/W rats (Pohjanvirta *et al.*,

1990). The TCDD-induced upregulation of AHR is also similar in both rat strains (Franc *et al.*, 2001a, b). Nevertheless, Western blot analysis revealed a notable size difference in the AHR proteins (Pohjanvirta *et al.*, 1999). While the L-E rat AHR was identical to that previously reported for other rat strains (106 kDa), the AHR in H/W rats was significantly smaller (98 kDa).

Molecular cloning and sequencing of the H/W rat AHR revealed two point mutations (Pohjanvirta *et al.*, 1998). The first mutation found in exon 10 results in change in a single amino acid, but it probably has no functional consequences because this mutation occurs in a hypervariable region. Instead, the other point mutation at the first nucleotide of intron 10 is critical, because it destroys the normal exon/intron junction and leads to use of cryptic splice sites (Fig. 8). Three different mRNA products have been detected: a deletion of 129 bp and two insertions 29 bp and 134 bp long. Since the shorter insertion variant contains a stop codon and the same insertion is included at the 5' end of the longer insertion variant, they translate into an identical protein, which has a total deletion of 38 amino acids with 7 novel amino acids at its C-terminus. Another type of H/W AHR protein, due to a deletion, is 43 amino acids shorter than the wild-type AHR (Pohjanvirta *et al.*, 1998). Consequently, both types of H/W AHRs have shorter transactivation domains and therefore a restructured C-terminus. Genetic studies imply that the reconstructed AHR is the principal reason for TCDD resistance in H/W rats (Pohjanvirta *et al.*, 1999; Tuomisto *et al.*, 1999).

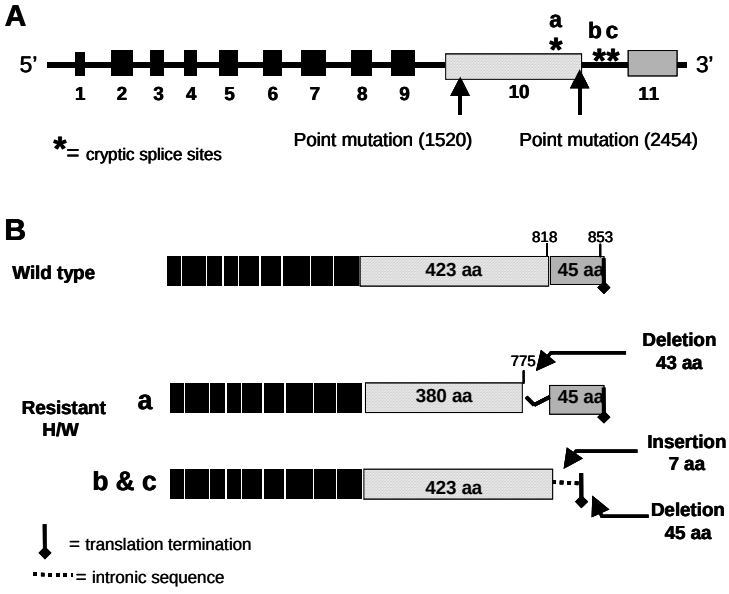

Figure 8. Structure of H/W AHR at the DNA level (A) and the protein level (B). a, b, and c act as cryptic splice sites resulting in two protein products with total deletions of 43 and 38 amino acids [modified from Okey *et al.* (2005)].

Interestingly, both H/W and L-E rats exhibit equal susceptibility to the acute lethality of perfluorodecanoic acid, which also causes a wasting syndrome similar to that induced by TCDD but does not act via the AHR (Brewster and Birnbaum, 1989; Unkila *et al.*, 1992). Thus, the sensitivity difference between H/W and L-E rats appears to be specific for TCDD. In addition, it decreases gradually along with increasing chlorination of the dioxin molecule (Pohjanvirta *et al.*, 1995; Simanainen *et al.*, 2002).

Line A, B and C rats

The genes affecting dioxin sensitivity in H/W rats were segregated into new rat lines, using conventional crossbreeding studies (Tuomisto *et al.*, 1999). Line A has the mutated H/W-type AHR and is as resistant to TCDD as the H/W. Line B has another resistance gene, the still unknown gene B, and is intermediately resistant. Line C has wild alleles of both genes and is almost as sensitive as L-E rats. These experiments showed that resistance to TCDD is associated with these mutated genes (Tuomisto *et al.*, 1999).

2.4.3 Hamster and guinea pig

The largest interspecies difference in acute TCDD toxicity exists between guinea pig and hamster. Guinea pigs are the most TCDD-sensitive mammals known with an LD50 value of about 1 µg/kg, while hamsters are extremely resistant with LD50 values of about 3000-5000 µg/kg (McConnel *et al.*, 1978; Olson *et al.*, 1980a; Henck *et al.*, 1981; Poland and Knutson, 1982). However, these species exhibit no apparent difference in the binding affinity of AHR to TCDD or in the binding of transformed AHR to the DRE (Gasiewicz and Rucci, 1984; Denison and Wilkinson, 1985; Bank *et al.*, 1992). As also shown in the rat model, some responses, such as enzyme induction and fetotoxicity, are fairly similar between these animal species, despite the wide divergence in acute lethality of TCDD (Pohjanvirta and Tuomisto, 1994).

Hamsters and guinea pigs have large physiological differences that complicate their use in dioxin sensitivity studies. For example, guinea pigs are strict herbivores whereas hamsters are omnivores and also hibernators (Pohjanvirta and Tuomisto, 1994). In addition, the half-life of TCDD elimination differs, being 11-15 days in hamsters and up to 94 days in guinea pigs (Olson *et al.*, 1980a; Olson, 1986).

2.4.4 Other animal models

Fish

Fish have been used to study the molecular basis of differences in susceptibility to drugs and environmental chemicals, because they are among the vertebrate animals most sensitive to dioxin toxicity, especially during their early life stages. In contrast to mammals, many fish species possess at least two AHRs (Hahn, 2001). AHR1 is the orthologue of the mammalian AHR, whereas AHR2 appears to be present only in early vertebrates (Karchner *et al.*, 1999). While most fish species are highly sensitive to dioxin toxicity, some populations of Atlantic killifish *(Fundulus heteroclitus)* have developed a heritable resistance to TCDD and PAH toxicity following long-term exposure (Bello *et al.*, 2001). Hahn *et al.* (2004) showed that the AHR1 locus in *F. heteroclitus* is highly polymorphic and that allele frequencies differ between some dioxin-sensitive and dioxin-resistant populations.

Others

Several frog species are resistant to TCDD toxicity, especially during development. However, cloning of *Xenopus laevis* AHR revealed that its AHR was similar to those of other vertebrate species, which suggests that the low responsiveness of frogs to TCDD is not accounted for by the structural characteristics of AHR (Ohi *et al.*, 2003). Invertebrate animals are also relatively insensitive to the toxicity of dioxin-like compounds (Hahn, 1998), but are not useful in dioxin sensitivity studies because their AHRs lack TCDD binding (Butler *et al.*, 2001).

2.4.5 Polymorphism of human AHR

In humans, several genetic variations in the *AHR* gene have been detected. Most of these polymorphisms are present in exon 10, which is the region responsible for transactivation of the AHR (Harper *et al.*, 2002). Kawajiri *et al.* (1995) were the first to report the replacement of the amino acid arginine by lysine at codon 554 in a population in Japan. Very recently, this polymorphism was shown to be significantly associated with survival in soft tissue sarcoma (Berwick *et al.*, 2004). The second *AHR* polymorphism was found at codon 570, which results in replacement of valine by isoleucine (Smart and Daly, 2000; Wong *et al.*, 2001). The third genetic variation at codon 517 replaces the amino acid proline with serine (Wong *et al.*, 2001). The fourth variant in exon 10 is localized at codon 786 and leads to replacement of methionine with valine (Cauchi *et al.*, 2001). Exept for Arg554Lys polymorphism, none of these other polymorphisms alone appears to alter receptor function, but Wong *et al.* (2001) reported that the combination of mutations at codons 554 and 570 abrogates CYP1A1 induction.

In addition to variations in exon 10, a single polymorphism has been reported at codon 44 (Kawajiri *et al.*, 1995) and several in the 5' flanking sequence of AHR (Cauchi *et al.*, 2001; Racky *et al.*, 2004) (Fig. 9).

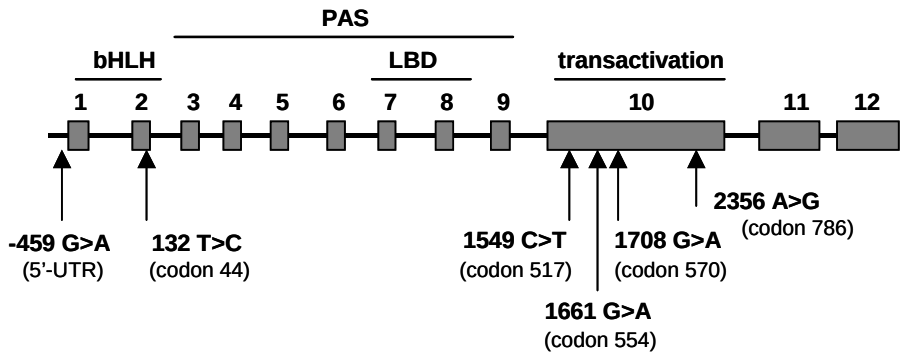

Figure 9. Sites of known polymorphisms or genetic variants in human AHR. Exons 1-12 are marked. Sequence variations are numbered according to recommended nucleotide nomenclature and previously used nomenclature is indicated in parentheses. Abbreviations are basic helix–loop–helix domain (bHLH), ligand-binding domain (LBD); Per/Arnt/Sim (PAS) domain [modified from Harper *et al.* (2002)].

2.5 Risk assessment of dioxins

The risk assessment of dioxins suffers from lack of knowledge regarding the molecular mechanism of dioxin toxicity. Only one mechanism of dioxin action, induction of CYP1A1, has been elucidated in some detail. However, CYP1A1 induction is not predictive of toxicity, although both effects are mediated via the AHR. A useful tool for studying key mechanisms in dioxin toxicity is the large strain-specific sensitivity difference between H/W and L-E rats. While there is a very large sensitivity difference in some endpoints of TCDD toxicity, some responses such as induction of CYP1A1 are similar between these strains.

Another fact that complicates dioxin risk assessment is the exceptionally wide species differences in dioxin sensitivity. In the mouse and rat models, the primary structure of the AHR was regarded as one of the most critical factors determining dioxin sensitivity (Ema *et al.*, 1994; Pohjanvirta *et al.*, 1998; Tuomisto *et al.*, 1999). The 10-fold difference between the dioxin-sensitive C57BL/6 and the dioxin-resistant DBA/2 mice is explained by polymorphic variations in the LBD of AHR (Ema *et al.*, 1994). The over 1000-fold difference between the dioxin-resistant H/W and the dioxin-sensitive L-E rats is due to a mutation in the AHR transactivation domain of the H/W rat (Pohjanvirta *et al.*, 1998). In

human AHR, several polymorphisms have been detected, but none has yet been associated with altered function of AHR or impact on human health (Harper *et al.*, 2002).

One important question in dioxin risk assessment is whether animal models are appropriate for assessing risk in humans. In addition to species differences in TCDD toxicity, the use of animal data for extrapolation to humans also suffers from interspecies differences in the range of observed effects and elimination half-life. While the structure and mode of action of AHR appear to be highly conserved across species, a recent study comparing DREs in human, mouse and rat sequences showed that only 39% of human orthologues with a positionally conserved DREs had a rodent counterpart with a positionally conserved DRE (Sun *et al.*, 2004). These results suggest that AHR-mediated gene expression may not be well conserved across species. In addition, many target genes of TCDD do not have DREs, suggesting that some TCDD-induced responses are due to secondary effects (Frueh *et al.*, 2001; Sun *et al.*, 2004). These facts may challenge the simple cross-species extrapolation from animal data to humans and the suitability of rodent models for assessing the potential human health risks associated with TCDD exposure.

A critical question in risk assessment is whether humans are sensitive or resistant to dioxin toxicity. Data from industrial and occupational exposures reveal no cases of acute mortality among highly exposed persons (Bertazzi *et al.*, 2001). However, the highest TCDD exposure ever encountered among humans corresponded to an acute (single) dose of approximately 25 μg/kg (Landi *et al.*, 1998; Abraham *et al.*, 2002; Geusau *et al.*, 2002; Eskenazi *et al.*, 2004), which is already lethal to the most TCDD-sensitive animal, the guinea pig, but not to the most common laboratory animals (Table 2). Furthermore, in vitro studies with human cell lines and tissue cultures have suggested that humans are susceptible to certain biochemical and toxic impacts of dioxins to the same degree as rats (Lucier, 1991). Despite these facts, the general view is that humans are rather resistant to the acute toxicity of dioxins. Moreover, there are large differences in susceptibility among individuals: again in Seveso, some people did not develop chloracne even though they had very high levels of TCDD (Mocarelli, 2001). In addition, women had higher TCDD concentrations and also appeared to show metabolic behaviour for TCDD different from that of men, which suggest a gender difference in TCDD toxicity (Landi *et al.*, 1997, 1998).

In 1997, the IARC changed the classification of TCDD from “a possible human carcinogen” (Group 2B) to “a human carcinogen” (Group I) (IARC, 1997). However, this classification has been criticized because it was mainly based on strong evidence in experimental animals but only limited epidemiological evidence in humans and generalizations from animals to humans are limited by the wide interspecies variation in TCDD responses (Cole *et al.*, 2003; Greene *et al.*, 2003). For example, it was estimated that humans are less sensitive than rats to the carcinogenic effects of TCDD (Hays *et al.*, 1997). In addition, the elimination half-life of TCDD reported for background and moderate exposure levels varies between humans

and rodents, being about 7 years in adult humans and about 2-4 weeks in mice and rats (Allen *et al.*, 1975; Gasiewicz *et al.*, 1983; Flesch-Janys *et al.*, 1996) and therefore the cumulative effects of TCDD may be different between humans and experimental animals. Notably, the elimination half-live depends on the dose being shorter at high doses; for example, in the most severely TCDD-contamined woman it was reported to be as short as 1.5 years (Geusau *et al.*, 2002).

Although human intake levels of TCDD have decreased notably over the past two decades, a significant baseline exposure to dioxins appears to be inevitable (Aylward and Hays, 2002). It is believed that the current background exposure should not pose a significant risk to the general population (Greene *et al.*, 2003). However, there are some subgroups in the general population that are exposed to higher intakes of dioxins, such as breast-fed infants and families of fishermen who frequently consume large amounts of Baltic herring and salmon (Kiviranta *et al.*, 2000, 2003; Lorber and Phillips, 2002).

The WHO as well as several other authoritative agencies have recommended a tolerable daily intake (TDI) of 1-4 pg WHO-TEQ/kg body weight per day (van Leeuwen *et al.*, 2000; Hays and Aylward, 2003). This recommendation was based on the most sensitive effects of dioxins that were considered adverse (hormonal, reproductive and developmental effects) seen at low doses in animal studies. It also includes an overall uncertainty factor of 10 to account for possible differences in susceptibility between humans and experimental animals. However, the USEPA concluded that safe doses of dioxins are as much as a thousand-fold lower (USEPA, 2000). Their latest risk assessment was also based on noncancer endpoints, but they used a linear model for exploring the exposure-response relationship instead of the threshold models used by other agencies.

3 AIMS OF THE STUDY

Sensitivity to the toxic effects of TCDD varies widely among animal species and even within strains. There are 1000-fold sensitivity differences involved in acute lethality of TCDD between L-E and H/W rats as well as between hamsters and guinea pigs. The AHR is the major reason for the sensitivity difference between H/W and L-E rats, but the reason for the sensitivity difference between hamsters and guinea pigs is unknown. Since the AHRs of hamsters and guinea pigs were not cloned earlier, the first aim in this study was to clone them to determine whether the structure of the AHR also plays a critical role in this dioxin sensitivity difference. In addition to the AHR, there is another, currently unknown factor involved in dioxin sensitivity differences. Therefore, other aims in this study were to compare the structures of ARNT, ARNT2 and AHRR between H/W and L-E rats to find out whether these important proteins in the AHR signalling pathway could contribute to strain-specific sensitivity differences in TCDD toxicity. Since the *AHRR* gene has not been identified earlier in rats, its expression in addition to cloning also needed to be characterized. The final aim was to examine the hypothalamic effects of TCDD on expression of genes encoding the AHR-regulated bHLH/PAS proteins, which are potentially involved in molecular pathogenesis of the wasting syndrome, utilizing our differentially sensitive rat strains.

The specific aims in each study were as follows:

1. To clone and sequence the AHR from TCDD-resistant hamsters,
2. To clone and sequence the AHR from the most TCDD-sensitive mammal known, the guinea pig,
3. To clone and sequence the ARNT and the ARNT2 from H/W and L-E rats,
4. To clone and sequence the AHRR from H/W and L-E rats and to study its time-, dose-, and tissue-dependent expression and
5. To study the effects of TCDD on the hypothalamic expression of several bHLH/PAS proteins participating in dioxin signal transduction and possibly the wasting syndrome.

4 MATERIALS AND METHODS

4.1 Animal husbandry and sample collection (I-V)

Two golden Syrian hamsters, a male and a female, were purchased from Harlan Nederland (Horst, the Netherlands). After arriving, the hamsters were killed by decapitation at the age of 4 weeks and various tissues were rapidly removed. The tissues were flash-frozen in liquid nitrogen, and stored at –80 ºC until analysis (I). Liver samples of guinea pigs were provided by Dr. Niku Oksala (Kuopio University Hospital). Two domestic guinea pigs were killed by decapitation at the age of 4 weeks and the livers were collected (II).

H/W and L-E rats were obtained from the breeding colony of the National Public Health Institute, Department of Environmental Health, Kuopio, Finland. Before the experiments, young adult male L-E and H/W rats were transferred from the barrier unit to an artificially illuminated animal room with a constant temperature of 21.5 ± 1 °C, humidity 55 ± 10% and a 12-h/12-h light/dark rhythm (lights on at 7 a.m.). The rats were housed in single-rat stainless-steel wiremesh cages on aspen wood chips (Tapvei, Kaavi, Finland) (III-V). The rats were killed by decapitation and various tissues were rapidly removed, flash-frozen in liquid nitrogen, and stored at –80 ºC for subsequent analysis. The doses used, exposure times and tissues analysed in each study (I-V) are presented in Table 3.

Table 3. Experimental design

Study	**Animals**	**Doses** (µg/kg TCDD)	**Exposure times** (hours)	**Analysed tissues**
I	hamsters	-	-	liver, lung, heart, kidney, spleen, thymus, hypothalamus, testis, ovary
II	guinea pigs	-	-	liver
III	H/W and L-E rats	50 or 100	24 or 96	liver, hypothalamus, kidney, lung, adipose tissue
IV	H/W and L-E rats	0.001-100	3, 6, 19, 24 or 96	liver, heart, kidney, spleen, testis
V	H/W and L-E rats	50 or 100	6, 96 or 120	hypothalamus

4.2 Chemicals

TCDD was purchased from the Ufa Institute (Ufa, Russia) and was over 98% pure, as assessed with gas chromatography-mass spectrometry (III-V).

4.3 RT-PCR cloning

The cloning procedure was essentially similar in each cloning study (I-IV). First, frozen liver samples were homogenized, using an Ultra-Turrax homogenizer (T-25 basic, IKA-WERKE GMBH & Co, Germany). Total RNA was isolated, using Trizol reagent (Life Technologies, Eggenstein, Germany) or the GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO) and cDNA was synthesized with Omniscript reverse transcriptase (Qiagen, Hilden, Germany). The PCR was performed with the DyNAzyme EXT DNA polymerase blend (Finnzymes, Espoo, Finland) or with FastStart DNA polymerase (Roche, Mannheim, Germany) on an Uno II, TPersonal or TGradient thermocycler (Biometra, Göttingen, Germany). The touchdown method was applied throughout all reactions: the annealing temperature was set at about 5 °C above the calculated melting temperature of the primers for the first cycle and then decreased by 1 °C per cycle down to the desired final temperature. Modified RACE techniques were used to obtain the 5’ and 3’ ends. Detailed descriptions of the primers and cloning strategies are presented in the original publications (I-IV).

The PCR products were cloned into a cloning vector, using the blunt-end technique (I-IV). The PCR products were purified from 1% agarose gels with the QIAquick Gel Extraction Kit (Qiagen), Wizard SV Gel and PCR Clean-Up Systems (Promega, Madison, WI) or GenElute Gel Purification Kit (Sigma-Aldrich). The purified PCR products were concentrated using Pellet Paint Co-precipitant (Novagen, Madison, WI). Before ligation, T4 DNA polymerase (MBI Fermentas, Vilnius, Lithuania) was used to blunt the possible 3’ or 5’ protruding termini of the DNA and T4 polynucleotide kinase (MBI Fermentas) was used to transfer the phosphate groups from the ATP to the 5’ termini of the DNA. The DNA inserts were ligated into pCR-Script SK(+) Amp plasmid (Stratagene, La Jolla, CA), which was digested beforehand using SmaI restriction endonuclease to create the blunt ends and dephosphorylated with Calf Intestine Alkaline Phosphatase (CIAP) to prevent self-ligation of the vector. In addition to an insert and a vector, a typical ligation reaction contained T4 DNA ligase (1-3 units) (MBI Fermentas), 5% polyethylene glycol 4000 and ligation buffer consisting of 40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DDT and 0.5 mM ATP, pH 7.8. The ligation reactions were usually carried out at 22 °C 1-3 h or at 16 °C overnight.

The ligation mixture was transformed into *E. coli* XL1-Blue supercompetent bacterial cells (Stratagene). Ampicillin selection and blue/white screening were used to separate colonies with recombinant plasmids. The colonies were further screened with PCR to reveal the size of the inserts. The primers were designed to amplify the entire polylinker area in the

plasmid. The desired colonies were picked and cultured overnight in LB medium containing 50 μg/ml ampicillin. The plasmid DNA was isolated from bacterial DNA using the Wizard Plus SV Minipreps DNA Purification System (Promega). Sequencing was performed in the AIV institute, University of Kuopio, Finland, with an A.L.F. or A.L.Fexpress DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden) using either Thermo Sequenase Fluorescent Labelled Primer Cycle Sequencing Kit or Thermo Sequenase CY5 Dye Terminator Kit (Amersham Pharmacia Biotech). All ambiguities were resolved by auxiliary clonings (I-IV).

4.4 Northern blot

For Northern blot analysis, poly(A)+ RNA was purified from total RNA. The denatured mRNA samples were fractionated by electrophoresis in an agarose-formaldehyde gel, transferred onto a nylon membrane (Hybond N^+, Amersham Pharmacia Biotech), hybridized with a digoxigenin-labelled probe (Roche) and finally detected with the colorimetric method using NBT/BCIP (Roche) (I).

4.5 Western blot

Western blot analysis was performed using protein fractions isolated from liver (I-II) and lung (I) as well as *in vitro*-translated protein (I) generated from hamster AHR cDNA. *In vitro* transcription and translation were carried out using Single Tube Protein System3 (Novagen). The proteins were analysed with immunoblotting after sodium dodecylsulphate – polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA) and then subjected to Western blot analysis using rabbit polyclonal anti-AHR antibody (1:50 000) as the primary antibody (BioMol Research Laboratories, Plymouth Meeting, PA) and an alkaline phosphatase-labelled anti-rabbit IgG as the secondary antibody (Roche). The bands were visualized with NBT/BCIP (Roche).

4.6 Expression analysis

RT-PCR was used to study gene expression. The total RNA was isolated and cDNA generated as earlier described in the cloning procedure. To study the expression of different splice variants of ARNT and ARNT2 (III), semiquantitative PCR was used and the band intensities were assessed using the Pharmacia Gel Documentation System and the Imagemaster 1D software (Amersham Pharmacia Biotech). In subsequent studies (IV-V), quantitative RT-PCR analyses were performed employing the QuantiTect SYBR Green PCR Kit (Qiagen) and Rotor-Gene 2000 Real-Time Amplification System (Corbett Research, Mortlake, NSW, Australia). The LightCycler instrument and LightCycler

FastStart DNA Master SYBR Green I Kit (Roche) were also used in quantification in some expression analyses (V). The expression levels were related to mRNA concentrations of the housekeeping gene β-actin. Detailed description of primers and PCR conditions are presented in the original publications (III-V).

4.7 Statistics

In the time-course and dose-response analysis (IV) with three or more groups, statistical comparisons were performed by one-way analysis of variance (ANOVA) if the variances were homogenous. Duncan's multiple range test was employed for multiple comparisons post hoc if ANOVA showed a statistically significant difference ($p < 0.05$) among the groups. In the case of nonhomogenous variances, the Kruskal-Wallis nonparametric ANOVA followed by the Mann-Whitney U test were used. The time-course data from days 1 and 4 were broken down strainwise and assessed statistically with Student's t-test. In study V, the data were statistically analysed for the presence of differences between the control and TCDD-exposed groups (separate comparisons strainwise at each time point) or between corresponding groups of the strains with two-tailed t-test for independent samples.

5 RESULTS

5.1 Structure of the AHR in hamster and guinea pig (I-II)

The coding region of hamster AHR proved to be longer than in guinea pig, because of the incorporation of short satellitelike DNA repeats in the Q-rich subunit of the C-terminal transactivation domain (I: Fig. 2). This repetitive sequence contained codons for glutamine and thus it increased the number of glutamine residues in the Q-rich subdomain (Table 4). The size of the receptor protein was also larger in hamster (I: Fig. 6; II: Fig. 4).

Table 4. Comparison between hamster and guinea pig.

	Hamster	**Guinea pig**
Coding region of AHR (bp)	2763	2541
Number of amino acids in AHR protein	920	846
Estimated size of AHR protein (kDa)	120	103
Number of glutamines in Q-rich subdomain	49	23

When the amino acid sequences of hamster and guinea pig AHR were compared with the corresponding human, rat and mouse sequences, the greatest similarity was found in the aminoterminus within the bHLH and PAS domains (II: Fig. 3). In the variable C-terminus, guinea pig AHR also resembled that of the human, rat and mouse receptors. Only the exceptionally large Q-rich subdomain in hamster AHR differed strikingly from other sequences. The overall homology between hamster and guinea pig was only 56%. Surprisingly, guinea pig showed the highest degree of homology to human AHR (II: Table 1).

5.2 Novel splice variants of ARNT and ARNT2 in the rat (III)

ARNT and ARNT2 were cloned from both L-E and H/W rats via RT-PCR, using primers based on published rat sequences (Drutel *et al.*, 1996). The sequences of both ARNT and ARNT2 proved to be identical between these strains.

Several different products of alternative splicing were detected (Table 5; III: Fig. 1). All ARNT variants were in-frame changes, while one insertion detected in the structure of ARNT2 contained a premature stop codon resulting in a truncated protein in C-terminus.

The relative expression levels of the splice variants were examined with semiquantitative RT-PCR (III: Figs. 2, 3). The pattern of expression was similar in untreated and TCDD-treated rats. Furthermore, there were no differences in the expression of splice variants between H/W and L-E rats.

Table 5. Splice variants detected in the sequences of ARNT and ARNT2 in the rat.

Gene	Location	Mutation	Size (bp)	Relative expression level vs. wt (%)
ARNT	exon 5	deletion	45	60-70
ARNT	3' end of exon 6	deletion	141	very low
ARNT	5' end of exon 11	deletion	15	not detected
ARNT	5' end of exon 16	interindividual variation in the number of trinucleotide repeats (CAG)	variable (39-63)	not determined
ARNT	5' end of exon 20	insertion	3	not determined
ARNT2	5' end of exon 19	insertion	31	40

5.3 AHRR cloning from H/W and L-E rats (IV)

Molecular cloning of rat AHRR revealed that it is highly identical to mouse AHRR, which was the first AHRR to be characterized (Mimura *et al.*, 1999). The N-terminal end of rat AHRR was highly conserved, but the PAS B and Q-rich domains typical of AHR structure were severely truncated or lacking (IV: Figs. 2, 3). The structures were identical in both the H/W and L-E strains.

The time-, dose- and tissue-dependent expression of AHRR was determined, using quantitative real-time RT-PCR. There was wide variation among individual rats in the expression of liver AHRR mRNA. When the expression of CYP1A1 was measured using the same cDNA samples, the variation was strikingly less. The lowest dose of TCDD tended to increase AHRR mRNA levels slightly, while the CYP1A1 levels remained unaffected (IV: Fig. 6). The constitutive expression levels of AHRR were very low in untreated rats but increased rapidly after TCDD exposure (IV: Fig. 5). Testis exhibited the highest constitutive expression of AHRR but very low expression of CYP1A1. The TCDD-induced levels of AHRR were highest in kidney, spleen and heart, in which CYP1A1 induction was relatively

low. Liver displayed the lowest AHRR levels in response to TCDD but the highest induction of CYP1A1 (IV: Fig. 7). Again, no marked differences were found between H/W and L-E rats.

5.4 Expression of bHLH/PAS proteins in rat hypothalamus (V)

The effect of TCDD on the hypothalamic mRNA expression of the bHLH/PAS proteins AHR, ARNT, ARNT2, AHRR, SIM1 and PER2, as well as that of CYP1A1 and CYP1A2, was analysed using quantitative real-time RT-PCR. The expression levels were measured in both the H/W and L-E strains, which show an over 1000-fold sensitivity difference also in the wasting syndrome.

In both strains, high constitutive levels of ARNT2 and AHR, moderate levels of ARNT and CYP1A1 and very low levels of AHRR, PER2, CYP1A2 and SIM1 mRNAs were recorded. The only genes whose expressions were modified by TCDD were *AHRR*, *CYP1A1* and *CYP1A2*. Importantly, we could not reproduce the previously reported changes in *SIM1* or *PER2* expression after TCDD exposure. Differences between the H/W and L-E rats appeared in the constitutive levels of AHR and ARNT and in the TCDD-induced levels of CYP1A2, AHRR, AHR and ARNT, all of which were about 2-4-fold lower in H/W rats.

5.5 Novel sequence data (I-IV)

The sequences of cloned cDNAs as well as sequence data of novel splice variants were submitted to GenBank (National Center for Biotechnology Information, National Library of Medicine, Bethesda, MD) (Table 6).

Table 6. Complete coding sequences submitted to GenBank.

Accession number	Species	Description
AF275721	hamster (*Mesocricetus auratus*)	AHR mRNA
AY028947	guinea pig (*Cavia porcellus*)	AHR mRNA
AY264361	rat (*Rattus norvegicus*)	ARNT mRNA
AY264362	rat (*Rattus norvegicus*)	ARNT, exon 5 deletion variant
AY264363	rat (*Rattus norvegicus*)	ARNT, exon 6 deletion variant
AY264364	rat (*Rattus norvegicus*)	ARNT, exon 11 deletion variant
AY264365	rat (*Rattus norvegicus*)	ARNT, exon 20 insertion variant
AY264366	rat (*Rattus norvegicus*)	ARNT2, exon 19 insertion variant
AY367561	rat (*Rattus norvegicus*)	AHRR mRNA

6 DISCUSSION

6.1 AHR structure as a determinant of dioxin sensitivity

Hamsters and guinea pigs are used as animal models in dioxin sensitivity studies. Guinea pigs are the most TCDD-sensitive mammals, while hamsters can tolerate over 1000-fold higher doses of TCDD. A similar difference in acute TCDD toxicity exists between two rat strains, the sensitive L-E and the resistant H/W. Recent cloning of H/W rat AHR revealed changes in the architecture of the transactivation domain, which appears to be the principal reason for TCDD resistance in H/W rats (Pohjanvirta *et al.*, 1998; Tuomisto *et al.*, 1999). Therefore, the primary structure of hamster AHR was determined to ascertain whether TCDD resistance in hamsters has a similar basis. Moreover, the cloning and sequencing of guinea pig AHR was performed to compare its transactivation domain structure especially with that in hamster and also with other AHR sequences. Our goal was to better understand the role played by AHR structure as a determinant of dioxin sensitivity.

The N-terminal ends of the hamster and guinea pig AHRs containing the bHLH and the PAS domains are highly conserved. This high degree of homology is not surprising, since these domains have important functions such as DNA binding, ligand binding and heterodimerization with the ARNT (Dolwick *et al.*, 1993b). The most conspicuous difference among AHR sequences appears in the C-terminal transactivation domain, which is essential for transactivation function of the AHR *in vitro* (Jain *et al.*, 1994; Fukunaga *et al.*, 1995; Kumar *et al.*, 2001). In the TCDD-resistant hamster, the Q-rich subdomain is larger and also contains twice as many glutamine residues as the AHR of the TCDD-sensitive guinea pig. Across published mammalian species, there is a distinct correlation between the number of glutamine residues and sensitivity to the acute lethality of TCDD. This suggests that the Q-rich subdomain in the C-terminal transactivation domain may be causally involved in the differences in sensitivity to TCDD toxicity.

6.1.1 Restructured transactivation domain selectively affects TCDD responses

Studies in our laboratory have revealed that both of the most TCDD-resistant animals known, hamsters and H/W rats, have remodelled transactivation domains in their AHRs. An interesting feature is that despite the large deletion or enlargement in the transactivation domain the induction of CYP1A1 remains normal in both animals (Pohjanvirta and Tuomisto, 1994). In addition, hamsters and outbread Long-Evans rats display similar reproductive alterations after perinatal TCDD exposure to nearly identical dosage levels (Gray *et al.*, 1995). The dosage levels associated with fetotoxicity or monooxygenase induction in hamsters and H/W rats are also almost similar to those in sensitive species (Henry and Gasiewicz, 1987; Pohjanvirta *et al.*, 1988; Pohjanvirta and Tuomisto, 1994; Birnbaum and Tuomisto, 2000). Thus, in TCDD-resistant hamsters and H/W rats, the

change in the transactivation domain may not influence the expression of some genes (type I responses), while it has a dramatic effect on many other responses such as lethality, toxicity and wasting (type II responses) (Fig. 10). In other words, the alteration appears to be endpoint-dependent: it selectively affects the transcription of only some genes, notably those that are central to dioxin toxicity. Therefore, the existence of type I and II endpoints in H/W rats suggests that different genes may have different requirements for the structure of the transactivation domain.

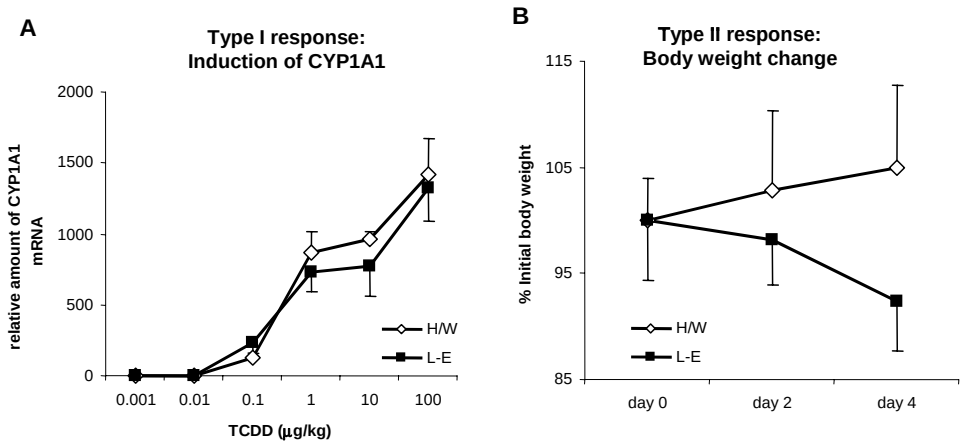

Figure 10. Examples of type I and II responses. **A.** Hepatic induction of CYP1A1 mRNA. H/W and L-E rats were treated with 0.001-100 µg/kg TCDD 19 h before liver removal. Expression levels are given relative to β-actin concentrations. Each data point represents mean ± SD of four individual rats (IV). **B**. Relative body weight change in H/W and L-E rats after a single dose of 50 µg/kg TCDD. Points represent means ± SD of 4 individual rats (V).

6.1.2 Possible mechanisms by which the restructured transactivation domain affects transcription machinery

Protein-protein interactions

The AHR/ARNT complex can activate target gene expression through direct protein-protein interactions with transcription factors and coactivators or corepressors, some of which are known to bind to the transactivation domain of AHR (Rowlands *et al.*, 1996; Kobayashi *et al.*, 1997; Nguyen *et al.*, 1999; Beischlag *et al.*, 2002; Hankinson, 2005). For example, the coactivators SRC-1 and RIP140 bind to the Q-rich region *in vitro* (Kumar and Perdew,

1999; Kumar *et al.*, 1999). Furthermore, the Q-rich subdomain is one of the two binding sites for Rb (Ge and Elferink, 1998). The AHR/ARNT heterodimer may also interact with other signal transduction pathways, which may occur indirectly via shared coactivators, e.g. via p300/CBP coactivators (Kobayashi *et al.*, 1997; Kumar and Perdew, 1999; Carlson and Perdew, 2002). In addition, the transactivation domain of human AHR is able to recruit cofactors similar to those of the LBD of ERα (Reen *et al.*, 2002). Since there is little information on the protein-protein interactions underlying transcriptional regulation of AHR, it is difficult to assess to what degree the restructured transactivation domains in dioxin-resistant animals affect these interactions and thereby the transactivation potential of AHR.

Conformation

Activation of genes by the AHR/ARNT complex is a multistep process that includes several conformational changes in the AHR protein (Kronenberg *et al.*, 2000; Henry and Gasiewicz, 2003). The remodelled transactivation domain of AHR may have some effect on the three-dimensional structure, but probably not on the first structural alterations arising from ligand binding and heterodimerization with ARNT, because it has been established that the domains for ligand binding, heterodimerization and DNA binding can function even when the transactivation domain is completely deleted (Dolwick *et al.*, 1993b; Ko *et al.*, 1997). In later steps, the transactivation domain of the AHR is needed for facilitating the alteration of the promoter chromatin structure to a form that is capable of binding transcription factors and other cofactors (Ko *et al.*, 1996; Whitlock *et al.*, 1996). In human AHR, the amino acids 663-688 in the Q-rich region probably form an α-helical secondary structure, in which a single amino acid Leu-678 may play a critical role in making contacts with coregulators (Kumar *et al.*, 2001). This same leucine residue was conserved in the AHR sequences of both hamster and guinea pig; thus, it may not play a role in dioxin sensitivity differences between these animals.

Enhancer-promoter communications

Gene activation by the AHR requires a number of sequential steps, beginning from ligand binding and ending at interactions of the AHR/ARNT heterodimer with proteins that facilitate changes in chromatin structure (Whitlock, 1999; Swanson, 2002). In our rat model, the first events in AHR signalling are identical between TCDD-resistant and TCDD-sensitive rats (Pohjanvirta *et al.*, 1999) and probably also between TCDD-resistant hamster and TCDD-sensitive guinea pig (Pohjanvirta and Tuomisto, 1994). The differences arise after binding of the AHR/ARNT complex to enhancer sequences (DREs) upstream of the target genes. This binding promotes the alteration in chromatin structure and disruption of nucleosomes (Okino and Whitlock, 1995). The transactivation domain of AHR, but not that

of ARNT, mediates the TCDD-inducible enhancer-promoter communication, which thereby increases promoter accessibility and facilitates promoter occupancy by the transcription factors (Ko *et al.*, 1996; Ko *et al.*, 1997). In this regard, the restructured transactivation domains in the AHRs of hamsters and H/W rats may impair mediation of the induction signal from enhancer to promoter and thus affect the expression of some specific genes.

Subdomains in the transactivation domain

The AHR has a complex transactivation domain that is composed of several segments which are often classified with respect to their amino acid composition (Ko *et al.*, 1997; Kumar *et al.*, 2001). In mice, the Q-rich subdomain harbours most of the transactivation potential, but other subdomains were also potent transcriptional activators of the mouse *Cyp1a1* gene (Ko *et al.*, 1997). In the transactivation domain of the human AHR, potentially distinct acidic, Q-rich and proline/serine/threonine-rich subdomains have been identified (Rowlands *et al.*, 1996; Reen *et al.*, 2002). These subdomains are able to function independently as well as to cooperate and thus result in a synergistic activation of transcription (Rowlands *et al.*, 1996; Kumar *et al.*, 2001). In addition to the Q-rich region, other subdomains are also capable of sequestering cofactors involved in transcription (Reen *et al.*, 2002) and thus this flexibility via multiple activation subdomains could make the AHR a versatile transcription factor. However, in TCDD-resistant hamsters, the other subdomains in the transactivation domain may not compensate for the deviant Q-rich subdomain; instead, synergistic transactivation of some genes may be impaired.

AHR as a coactivator

In oestrogen signalling, the activated AHR/ARNT complex functions as a coactivator by directly recruiting the ER and thereby activating the transcription of ER-mediated genes (Ohtake *et al.*, 2003). Very recently, the AHR/ARNT heterodimer was shown to bind via an unidentified X-protein to a novel response element called XRE-II (called also DRE-II or AHRE-II) upstream of the rat *CYP1A2* gene (Sogawa *et al.*, 2004). Binding of this complex leads to an activation of a set of genes, called the AHRE-II battery that encodes a large number of transporters and ion channels (Boutros *et al.*, 2004). It remains to be determined whether the reconstructed transactivation domain of the AHR in TCDD-resistant animals plays a role in this coactivation function of AHR. Furthermore, it would be interesting to find out if some of the species differences in dioxin toxicity could be explained through this aspect of AHR function.

Quantitative difference

L-E rats express two- to three-fold higher levels of the AHR and ARNT than H/W rats both before and after TCDD treatment (Pohjanvirta *et al.*, 1999) (V). However, these differences in the amounts of AHR and ARNT may not play a role in strain-specific sensitivity differences, because DRE binding of the AHR/ARNT heterodimers occur similarly in these rats (Pohjanvirta *et al.*, 1999). Recently, studies using mice harbouring a hypomorphic *Arnt* allele showed that the AHR is dependent on the ARNT for adaptive, toxic and developmental pathways. Interestingly, those mice whose ARNT expression is reduced to 10% of wild-type levels are resistant to dioxin toxicity, but retain TCDD-induced CYP1A activity (Walisser *et al.*, 2004b). Moreover, Tomita *et al.* (2000) reported that despite more than 80% loss in ARNT expression in lung, maximal induction of CYP1A1 can still be found. These results suggest that adaptive CYP1A1 response to TCDD does not require as many AHR/ARNT heterodimers as toxic or developmental pathways. This may provide a simplified explanation for the existence of type I and type II responses: due to a restructured transactivation domain of AHR, the transactivation ability of the AHR/ARNT complex may be reduced, but is still sufficient for maximal activation of the *CYP1A1* gene.

6.2 ARNT, ARNT2 and AHRR may not contribute to dioxin sensitivity in the rat model

Although the AHR appears to be the major reason for TCDD resistance in H/W rats, some other factors are also involved (Pohjanvirta, 1990). When the resistance genes of H/W rats were segregated into new rat lines, it was observed that in addition to the AHR the resistance was also associated with an unknown gene B. Line B rats bearing the B^{hw} allele are intermediately resistant to TCDD (Tuomisto *et al.*, 1999; Simanainen *et al.*, 2003) and possess their own characteristic feature of TCDD toxicity, namely predisposition to accumulation of biliverdin in the liver (Niittynen *et al.*, 2003).

Gene B is still unidentified, but it may encode a protein closely involved in the AHR signalling pathway. Therefore, we cloned and sequenced the cDNAs of ARNT, ARNT2 and AHRR from H/W and L-E rats to determine if these proteins could be auxiliary factors accounting for the strain-specific differences in TCDD toxicity. However, the cDNA sequences proved to be identical, suggesting that the structures of these proteins do not contribute to dioxin sensitivity. Surprisingly, we found several splice variants in the structures of ARNT and ARNT2, but none of these variants appeared to be related to TCDD resistance. Instead, they could have other functions suggesting an intricate regulation of ARNT and ARNT2 activities.

The cDNA sequence of rat AHRR showed high levels of sequence identity to mouse AHRR and also to rat AHR, except that the PAS B and Q-rich subdomains were lacking. We found

no marked differences in the expression of AHRR between H/W and L-E rats before or after TCDD treatment in any of the tissues examined. Thus, the rat strain differences in TCDD toxicity cannot be explained by the differential expression of AHRR. However, simultaneous determination of CYP1A1 mRNA suggested that AHRR may play a modulatory part in CYP1A1 regulation.

6.3 Differences in the hypothalamic expression of bHLH/PAS proteins may not account for the wasting syndrome

The TCDD-induced wasting syndrome is a type II endpoint that shows a clear sensitivity difference between differentially sensitive rat strains. This suggests the involvement of the AHR signalling pathway in regulation of genes contributing to the wasting syndrome. The exact mechanism by which TCDD affects feeding is unknown, but due to the central role of the hypothalamus in the control of appetite and body weight, it may involve hypothalamic activation of some currently unidentified genes. Therefore, H/W and L-E rats were used to examine the effects of TCDD on hypothalamic expression of several bHLH/PAS genes that are involved in, or related to, AHR signalling. If the wasting syndrome seen in L-E rats were due to changes in expression levels of these studied genes, a difference should be seen in comparison to H/W rats, which maintained their body weight four days after TCDD treatment.

No dramatic differences between differentially sensitive rat strains appeared in the expression levels of eight studied genes. However, the L-E rats expressed two- to three-fold higher constitutive or TCDD-induced mRNA levels of AHR, ARNT, ARNT2 and AHRR. This sensitive rat strain has been reported earlier to possess higher hepatic concentrations of AHR and ARNT (Pohjanvirta *et al.*, 1999). Despite these differences, DRE binding of the AHR/ARNT heterodimers occurs similarly in both rat strains, suggesting that differences in the amounts of AHR and ARNT do not play a noticeable role in this interstrain difference.

The largest difference between H/W and L-E rats was seen in the TCDD-induced expression of CYP1A2, which was about four-fold higher in L-E rats. However, no major differences in hypothalamic kinetics of ^{14}C-TCDD between these strains have been detected (Pohjanvirta *et al.*, 1990); induction of CYP1A1 also occurs similarly. Interestingly, it was reported that the absence of CYP1A2 may afford partial protection against TCDD-induced liver toxicity (Smith *et al.*, 2001). In addition, recent studies with male *Cyp1a1* knockout mice showed that these mice are somewhat resistant to high-dose TCDD-induced toxicity and the wasting syndrome (Uno *et al.*, 2004). However, the protection due to loss of the *Cyp1a1* gene is very small compared with that seen in *Ahr* knockout mice (Fernandez-Salguero *et al.*, 1996). Besides, studies using our rat model suggest that induction of CYP1A activity is independent of TCDD-induced toxic endpoints, such as the wasting

syndrome. Concordantly, CYP1A1 induction could be dissociated from the toxic effects of TCDD in ARNT hypomorphic mice (Walisser *et al.*, 2004b).

Although the changes found do not account for the wasting syndrome, the presence of all principal genes of the AHR signalling pathway in rat hypothalamus makes it a candidate target for TCDD and shows that the basic mechanisms for AHR signalling are functional. The AHR appears to be an essential mediator of both the adaptive and toxic effects of TCDD, but only the mechanism of CYP1A1 induction has been well elucidated. In contrast, the exact mechanism as well as the genes mediating TCDD-induced toxicity and the wasting syndrome are still poorly known.

6.4 Implications for risk assessment

Risk assessment of dioxins is complicated by the exceptionally wide species differences in dioxin sensitivity. In the mouse and rat models, the primary structure of the AHR appears to be the most critical factor determining dioxin sensitivity. Both the 10-fold sensitivity difference between C57BL/6 and DBA/2 mice and the over 1000-fold difference between L-E and H/W rats are explained by the presence of polymorphic variation in the AHR (Ema *et al.*, 1994; Pohjanvirta *et al.*, 1998). Accordingly, the structure of the AHR also appears to be an important determinant of dioxin sensitivity differences in hamster and guinea pig, which also show over 1000-fold differences in sensitivity to the acute lethality of TCDD. Moreover, the data from the present study emphasize the importance of the transactivation domain and especially the Q-rich subdomain in dioxin sensitivity.

Would it then be possible to make future predictions of species-specific dioxin sensitivity differences by characterizing the structure of the AHR? This appears to hold in many species, but probably not in all species. In the dioxin literature, humans are considered to be highly resistant to the acute toxicity of TCDD, but the structure of the human AHR does not support the resistance. On the contrary, the human AHR turned out to be highly homologous to that of the most dioxin-sensitive species known, the guinea pig. The assumption of human resistance is mainly based on the fact that the human AHR appears to have a lower relative affinity for TCDD than the receptors of most susceptible laboratory species (Ema *et al.*, 1994; Harper *et al.*, 2002; Ramadoss and Perdew, 2004). In an AHR-humanized mouse model, the expressed human AHR was also less responsive to TCDD than the AHR of resistant DBA/2 mice (Moriguchi *et al.*, 2003). Another reason is that no cases of acute mortality have been detected among persons who have received high doses of dioxins in industrial or occupational exposures (Bertazzi *et al.*, 2001). In Seveso and later in an individual intoxication case in Vienna, the highest TCDD concentrations in blood lipid were 56 000 ng/kg and 144 000 ng/kg, respectively. The highest concentration in the most exposed woman in Vienna roughly corresponded to a single dose of 25 μg TCDD per kilogram of body weight, and this dose caused a severe intoxication syndrome (Geusau *et*

al., 2001, 2002). This dose indicates that humans are more resistant to the acute toxicity of TCDD than guinea pigs and L-E rats, but it may not be reasonable to assume that humans are less sensitive than most laboratory animals. Evidence from *in vitro* studies suggested that humans appear to be at least as sensitive as rats to some biochemical effects of dioxins (Lucier, 1991). However, in that study, one of the measured parameters was induction of CYP1A1, which is a classical adaptive response to TCDD, but not apparently in relation to toxic responses caused by TCDD. Studies using our rat model support the present view that the adaptive pathway participating in metabolism of PAHs is unrelated to the toxic pathway mediating the deleterious effects of dioxins (Walisser *et al.*, 2004b).

In addition to the AHR, also ARNT and AHRR are important in mediating the biological effects of dioxins. Thus, it could be expected that these genes may in part contribute to dioxin sensitivity differences. However, the present study showed that neither the structural variation nor the expression of these genes play a role in the strain-specific differences in TCDD toxicity in our rat model. This fact may somewhat facilitate dioxin risk assessment, because it further emphasizes the importance of the AHR structure as a determinant of dioxin sensitivity differences.

The fact that the molecular mechanisms of dioxin toxicity are still poorly known complicates dioxin risk assessment. One mechanism that we tried to elucidate in this study was the TCDD-induced wasting syndrome, which shows clear sensitivity difference between H/W and L-E rats, indicating that this effect is also mediated via AHR signalling. Although no clear relationship between the TCDD-induced expression of the genes studied and the wasting syndrome was found, the present study showed the usability of our rat model in elucidating mechanisms in dioxin toxicity. Since the endpoints, which differ most between H/W and L-E rats, include acute lethality, wasting and hepatotoxicity, our rat model would be useful in the search for genes that are critical to development of those TCDD toxicities.

7 CONCLUSIONS

1. The C-terminal transactivation domain of the AHR appears to be an important determinant of the species- and strain-specific sensitivity differences in TCDD toxicity. A Q-rich subregion of this domain is aberrant in hamsters and this may account for the exceptional resistance of this species to the acute lethality of TCDD.
2. Among the most common laboratory animals used in dioxin sensitivity studies, namely rat strains with wild-type AHR, mouse, hamster and guinea pig, there is an inverse correlation between the number of glutamine residues in the Q-rich subdomain and TCDD sensitivity.
3. The human AHR shows the highest homology to the AHR of the most TCDD-sensitive species known, the guinea pig.
4. L-E and H/W rats express several splice variants of ARNT and at least one splice variant of ARNT2. At least the largest deletions of ARNT as well as the insertion in ARNT2 leading to a truncated protein are likely to have functional consequences.
5. The large strain-specific differences in susceptibility to TCDD lethality between H/W and L-E rats are not explained by the structural variations of ARNT and ARNT2.
6. Rat AHRR lacks the PAS B and Q-rich domains. In mice, this explains the inability of AHRR both to bind ligand and transactivate DRE-regulated genes.
7. The constitutive expression of the AHRR is very low, but after TCDD exposure the mRNA levels of the AHRR increase rapidly. The induction of AHRR is as sensitive a response to TCDD as the induction of CYP1A1, although the maximal induction levels are considerably lower.
8. The AHRR shows tissue-dependent expression, so that testis exhibits the highest constitutive expression of AHRR, whereas kidney, spleen and heart show the highest induction of AHRR in response to TCDD treatment.
9. The structures of AHRR cDNA as well as the expression patterns by TCDD are similar between H/W and L-E rats; therefore the AHRR may not contribute to strain differences in dioxin sensitivity in this rat model.
10. The presence of all principal proteins in the AHR signalling pathway in rat hypothalamus makes it a good candidate for TCDD regarding its influence on development of the wasting syndrome.
11. TCDD does not modulate the expression of *SIM1* or *PER2* in rat hypothalamus; therefore these genes may not be involved in the effects of TCDD on feeding.

12. The constitutive expression levels of AHR and ARNT and the TCDD-induced levels of CYP1A2, AHRR, AHR and ARNT are the only differences among the parameters measured that can be detected between H/W and L-E rats in rat hypothalamus. However, the changes found probably do not account for development of the wasting syndrome.

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