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Commentary

Ah receptor: Dioxin-mediated toxic responses as hints to deregulated physiologic functions

Karl Walter Bock *, *Christoph Köhle*

Department of Toxicology, Institute of Pharmacology and Toxicology, University of Tübingen, Tübingen, Germany

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AhR, aryl hydrocarbon receptor
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XRE, xenobiotic response element
TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

ABSTRACT

The aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor and member of the bHLH/PAS (basic Helix-Loop-Helix/Per-Arnt-Sim) family of chemosensors and developmental regulators. It represents a multifunctional molecular switch regulating endo- and xenobiotic metabolism as well as cell proliferation and differentiation. Physiologic functions of the AhR are beginning to be understood, including functions in vascular development, and in detoxification of endo- and xenobiotics. The AhR is also recognized as the culprit for most toxic responses observed after exposure to dioxins and related compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The non-metabolizable AhR agonist TCDD has to be distinguished from the myriad of metabolizable agonists present as dietary contaminants and plant constituents as well as endogenous toxins. The hypothesis is emerging that the diverse tissue-specific, TCDD-mediated toxicities are due to sustained and inappropriate AhR activation leading to deregulated physiologic functions. In support of this hypothesis recent observations in the context of some TCDD-mediated toxic responses are discussed, such as chloracne, cleft palate, thymus involution and in particular carcinogenesis. Major open questions are addressed, such as ligand-independent AhR activation by phosphorylation and the large differences in species-dependent susceptibility to toxic responses. Though important issues remain unresolved, the commentary is intended to stimulate efforts to understand dioxin-mediated toxic responses with emphasis on carcinogenesis in comparison with AhR-mediated physiologic functions.

1. Introduction

The aryl hydrocarbon receptor (AhR; also termed dioxin receptor) is a ligand-activated transcription factor and member of the bHLH/PAS (basic Helix-Loop-Helix/Per-Arnt-Sim) family of chemosensors and developmental regulators [1]. The AhR is involved in organogenesis, in detoxification of endo- and xenobiotics and in mediating diverse organ-specific toxic responses of dioxins. A number of reviews discussed various aspects of the AhR and its role in mediating the toxicity of dioxins and related compounds [1–9]. However, physiologic mechanisms remain largely unknown, and molecular mechanisms responsible for the diverse dioxin-mediated toxic responses are poorly understood. In the present commentary an attempt was made to facilitate understanding of dioxin-mediated toxicities as deregulated physiologic functions due to sustained and inappropriate AhR activation.

* *Corresponding author*. Tel.: +49 7071 2972274; fax: +49 7071 292273.
E-mail address: bock@uni-tuebingen.de (K.W. Bock).
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2. Molecular mechanisms of AhR signaling

The AhR is present in the cytosol of most cells in complex with the chaperone Hsp90 and co-chaperones. Ligand binding leads to nuclear translocation, to the release of chaperones and to interaction with its partner protein Arnt, a closely related bHLH/PAS family member [1]. Typical ligands are aryl hydrocarbons (responsible for the name of the receptor), halogenated aryl hydrocarbons such as TCDD, and numerous dietary plant constituents such as indole 3-carbinol generating the potent agonist indolo-(3,2-*b*)-carbazole [8]. Interestingly, tryptophan has been shown to be converted by UV light to potent AhR agonists [8] or to indole 3-pyruvate via aspartate aminotransferase, the latter generating a number of potent AhR agonists predominantly in heart tissue [10]. Tryptophan metabolites may be interesting candidates of potential endogenous AhR ligands. Multiple mechanisms of AhR signaling have been identified: (1) the AhR/Arnt complex interacts with XREs (xenobiotic response elements, also termed AhREs) in the regulatory region of target genes, including genes coding for Phases I and II biotransformation enzymes and genes involved in regulation of development, proliferation and differentiation, (2) recently, a large number of coregulators have been recognized, which are involved in the interaction between enhancers and promoters. In addition, direct interaction of the AhR with the retinoblastoma protein (Rb) and NF-κB has been identified, (3) AhR signaling also includes cross-talk with a number of protein kinases, and (4) evidence for ligand-independent activation of the AhR by phosphorylation was obtained.

2.1. *AhR/Arnt complex as ligand-activated transcription factor*

2.1.1. *Biotransformation of endo- and xenobiotics*

Originally, genes encoding Phases I and II enzymes were identified and termed 'AhR gene battery' using studies with mouse strains expressing high and low affinity AhR receptors [7]. In Table 1, two clusters of AhR target genes are listed for which functional XREs have been characterized. Coordinate induction of Phases I and II enzymes (cluster a) together with conjugate transporters by the AhR may facilitate efficient detoxification of xenobiotics and homeostasis of endobiotics [9,11]. The best studied among these proteins is CYP1A1 which was used as prototype to investigate mammalian gene expression [6]. Interestingly, evidence was obtained that CYP1A1 may be involved in the G1 phase of cell cycle progression [12]. In addition to coordinate induction of Phases I and II induction by the AhR/Arnt complex by so-called bifunctional inducers, a second gene battery has been identified which selectively induces Phase II enzymes together with a large number of other enzymes involved in antioxidant defense (activated by monofunctional inducers) [13]. The key transcription factor inducing this antioxidant gene battery is Nrf2 (NF-E2-related factor-2, a leucine zipper protein) which binds to AREs (antioxidant response elements) [14]. The Nrf2-ARE and AhR-XRE pathways directly cross-talk since XREs were identified in the regulatory region of the Nrf2 gene [15,17]. Cross-talk between these biotransformation systems greatly facilitates detoxification and protection against oxidative/electrophile stress (see Section 3.2), and is intensely investigated in efforts for chemoprotection of cancer and degenerative diseases [14]. It is also interesting that function of the AhR itself is tightly controlled by the AhR repressor, which competes with AhR for Arnt, thereby interfering with the ability of the AhR to bind the XRE and stimulate transcription [26].

Table 1 – XRE-mediated AhR target genes

Target gene/protein	References
Biotransformation	
CYP1A1	[18]
CYP1A2	[18]
CYP1B1	[19]
UGT1A1	[20]
UGT1A6	[21,22]
GSTA2	[23]
NQO1	[24]
ALDH3A1	[25]
Nrf2	[17]
AhR repressor	[26]
Cell proliferation and differentiation	
$p21^{CIP1}$	[27]
$p27^{KIP1}$	[28]
c-jun	[29]
junD	[29]
Hes-1	[30]
IL-2	[31]
Bax	[32]
IGFBP-1	[33]
Filaggrin	[34]
ecto-ATPase (CD39)[a]	[35]

[a] XRE not identified.

2.1.2. *Proliferation and differentiation*

Recently, evidence has been obtained for a heterogeneous group of XRE-modulated proteins involved in cell cycle control and differentiation (Table 1, cluster b): Binding of AhR to XREs in the first intron of the cyclin-dependent kinase inhibitor $p21^{CIP1}$ may lead to inhibition of cyclin E-cdk-2 kinase activity and inhibit retinoblastoma protein (Rb) phosphorylation and cell cycle progression [27]. In addition, AhR-mediated induction of cyclin-dependent kinase inhibitor $p27^{KIP1}$ was observed [28]. The AhR also controls expression of immediate-early protooncogenes c-jun and junD (but not c-fos and junB) in mouse hepatoma Hepa-1 cells [29]. Jun/fos proteins are known fundamental transcription factors controlling a large gene cluster. One recently discovered mechanism of c-Jun activation is discussed under Section 2.3. Novel gene targets of AhR include HES-1 (a bHLH transcriptional repressor) [30], interleukin 2, a mandatory cytokine for homeostasis of T cells (discussed under Section 4.3) [31], the proapoptotic Bax gene [32], IGFBP-1(insulin-like growth factor binding protein-1), discussed under Section 4 [33], filaggrin [34] (a key protein involved in skin differentiation, discussed under Section 4.1), and an interesting ecto-ATPase (CD39) [35], possibly involved in cell recognition. With the use of gene array technology, increasing numbers of AhR target genes are being identified. Recently, the complete spectrum of genes upregulated in wild-type versus AhR-null mice (dioxin-independent gene battery) has been compared with

TCDD-induced genes [36]. XRE-I and XRE-II regulated genes as well as up and downregulated genes were identified (XRE-II and downregulated genes are not discussed here). In addition to identification of new target genes, cross-talk opportunities of the AhR were extended, including the observation that p53 binding sites were found to be overexpressed in genes downregulated by TCDD (see Section 4.4).

2.2. AhR interaction with coactivators/corepressors, NF-κB and the retinoblastoma protein (Rb)

Connection of the AhR/Arnt enhancer complex with promoters of target genes need complex interaction with coactivators/corepressors and the multisubunit mediator complex (Fig. 1) [6,37]. In the case of CYP1A1 it has been demonstrated that in the absence of AhR/Arnt binding, the promoter exists in a tightly-packed nucleosomal configuration [6]. Binding of AhR/Arnt complexes to XREs recruits coregulators such as histone acetyltransferases (HATs). Acetylation of core histones releases the tightly packed DNA strands and allows bending of the AhR/Arnt enhancer, interaction with other coregulators such as mediator complex and with general transcription factors. Evidence has also been obtained that the AhR directly interacts with NF-κB (a transcription factor mediating oxidative stress and inflammatory reactions when NF-κB is released from protection by IκB) [40] and with hypophosphorylated Rb [38,39]. Interaction between AhR and Rb has been shown to affect a major G1 checkpoint of the cell cycle. Both mitogenic signaling by protein kinases and cell contact-mediated mitoinhibition by protein phosphatases may affect phosphorylation of Rb. It has been proposed that two mechanisms may be operative in Rb-mediated growth arrest: coactivation leading to induction of $p27^{Kip1}$ and corepression of cyclin E by a quaternary complex between AhR/Rb and E2F/DP (the latter representing the E2F binding partner in transactivation) [39].

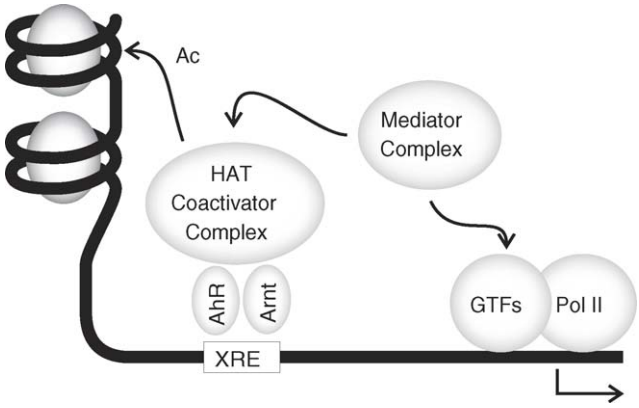

Fig. 1 – Simplified scheme of the interaction of the AhR/Arnt complex with the promoter of target genes. The AhR/Arnt complex often binds to one or more XREs of target genes several kb distant from the promoter. To be able to interact with the promoter the AhR/Arnt complex recruits coregulators such as histone acetyltransferases (HATs). In the case of CYP1A1 the promoter exists in a nucleosomal configuration in the absence of TCDD. Acetylation (Ac) of core histones by HATs releases DNA strands from the tightly packed nucleosome structure. This process allows looping of the DNA strand to interact with other coregulators such as multisubunit mediator complex and general transcription factors (GTFs) of the promoter. Pol II, RNA polymerase II.

2.3. AhR-mediated cross-talk with protein kinase networks

TCDD-mediated activation of multiple protein kinases is known to depend upon the cellular context [41,42]. With regard to phosphorylation of key regulators of cell cycle control and apoptosis such as p53 (see Section 4.4), it is important to note that their regulation is complex and their function may be activated or inhibited by phosphorylation of serine/threonine or tyrosine residues. Recently, evidence was obtained that TCDD induces c-jun expression by an AhR-stimulated p38-MAPK pathway [43]. Increased p38-MAPK phosphorylation in response to dioxin does not require ongoing protein synthesis. Therefore a novel 'cross-talk' between AhR and MAPK signaling was proposed utilizing adaptor proteins similar to TAK-1. In the case of the c-Src tyrosine kinase it was shown that c-Src kinase was released from the cytosolic AhR/Hsp90 complex upon ligand binding [44–46], leading – in a cell context-dependent manner – to membrane translocation and activation of c-Src in studies with WB-F344 cells [47], and to induction of Cox-2 [48].

2.4. Modulation of AhR function by phosphorylation

Observations from a number of laboratories have led to the suggestion that AhR phosphorylation is an important step in AhR signaling. PKA and PKC sites have been identified in the murine and human AhR [49]. Recently it has been suggested that AhR activation by ligand binding or cAMP-mediated phosphorylation may stimulate divergent signaling pathways [50]. Evidence was obtained that cAMP-activated AhR does not interact with Arnt but may allow new protein–protein interactions. The complex responses resulting from AhR phosphorylation have been discussed [42]. In addition, loss of cell contact by growing cells in suspension was shown to promote nuclear translocation of the AhR [19]. This observation may be an example for ligand-independent AhR signaling, which may represent an old evolutionary-conserved process discussed in the following paragraph. It has to be emphasized, however, that the distinction between ligand-dependent and -independent AhR activation needs further scrutiny since in the case of weak ligands the two possibilities are difficult to distinguish [8].

3. Physiologic AhR functions

The AhR and its partner Arnt are expressed in all studied vertebrates, and homologous proteins have been identified in invertebrates. The Drosophila homologues Spineless and Tango are involved in development of antennae and legs [51], and the corresponding homologues in *C. elegans*, Ahr-1 and Aha-1, are involved in development of the nervous system, such as the fate of GABAergic neurons [52,53]. Ligand activation was not detected in invertebrates, and evidence

for ligand-independent AhR signaling has also been obtained in vertebrates [50]. Expression of the AhR in the mouse embryo suggests an important role in embryonic development [54]. Teratogenic effects of dioxins such as cleft palate may be due to deregulation of developmental functions of the AhR, discussed under Section 4.2.

3.1. *Organogenesis*

A robust phenotype arising from AhR-null mice is reduced liver size at birth. The cause of this atrophy was identified as a congenital vascular defect, failure of ductus venosus closure [55]. Patent ductus venosus leads to reduced portal blood supply. Ductus venosus is a bypass between the umbilical vein and the inferior vena cava in fetal circulation. Normally this bypass closes a few days after birth, and blood flow is shifted to the liver via the portal vein. Elegant studies with AhR hypomorphic transgenic mice demonstrated that dioxin-mediated activation of the AhR led to closure of the ductus venosus [55]. The mechanism how the activated AhR leads to atrophy of the ductus venosus is unknown.

3.2. *Detoxification of endo- and xenobiotics*

A major AhR function (leading to its discovery and its name) is the upregulation of detoxification enzymes involved in metabolism of aryl hydrocarbons such as benzo(a)pyrene [2]. It was therefore puzzling that CYP1A1, the major enzyme involved in benzo(a)pyrene metabolism, actually was involved in bioactivation of benzo(a)pyrene to its ultimate carcinogen [56]. However, investigations with CYP1A1-null mice recently demonstrated that after oral exposure the defective mice died, whereas the upregulated CYP1A1 protected wild-type mice from bone marrow toxicity, due to efficient first pass metabolism in the intestinal mucosa and liver [9]. Cooperative regulation of Phases I and II enzymes and conjugate transporters facilitate efficient detoxification [9,11] together with the AhR-linked Nrf2-controlled gene battery which preferentially upregulates Phase II enzymes [14,15]. This link may have far reaching implications since recent studies with Nrf1- and Nrf2-null mice have shown that these proteins are protecting against oxidative stress during embryonic development [16]. The AhR and Nrf2 are known to be modulated by a variety of dietary plant constituents present in vegetables and fruits [8]. Since these compounds are metabolizable agonists (or antagonists) they may be regarded as potential therapeutic ligands of the AhR.

Biotransformation enzymes are not only involved in metabolism of xenobiotics but also in homeostasis of signaling molecules, such as steroid hormones, thyroxin, neurotransmitters and retinoids [57–59]. However, their role in the complex homeostatic mechanisms evolved for signaling molecules is poorly understood.

4. Organ-specific toxic reactions

A plethora of TCDD-mediated toxic reactions has been observed in experimental animals and humans, including endocrine and reproductive toxicity, cardiovascular diseases and multisite cancer [1–3,60]. Dioxin-activated AhR leads to endocrine disruption, which may result in a number of toxicities in target organs, for example in endometriosis and breast cancer [61]. Developmental neurotoxicity may produce neurobehavioral abnormalities associated with cognitive and locomotor systems: AhR and Arnt have been shown to be present in mouse cerebellar granule cell layer and TCDD may disrupt granule cell neurogenesis [62]. Exposure of human populations to TCDD and related compounds was found to be associated with hyperinsulinemia, and links have been proposed to type 2 diabetes [63,64].

A distinction has to be made between metabolizable AhR agonists and practically non-metabolizable agonists such as TCDD (biologic half-life in humans: 7–10 years) which may disrupt physiologic AhR responses (in the case of TCDD 'the button is pushed too long and too hard'). To understand the plethora of toxic responses a detailed knowledge of the tissue-specific physiologic and pathophysiologic processes is mandatory. Therefore, in the present commentary only a few examples of toxic responses are discussed in detail with the aim (i) to substantiate the hypothesis that dioxin-mediated toxicities are possible hints to deregulated physiological functions (Fig. 2), and (ii) to emphasize the large species-dependent differences in sensitivity to toxic responses, discussed under Section 5.2.

4.1. *Skin toxicity: chloracne*

Chloracne represents a hyperkeratotic skin disorder, which affects the hair follicles, sebaceous glands and interfollicular epidermis. It is the most consistently observed pathology in exposed humans since 1957 when dioxins were first identified as culprits for chloracne [2] till to the recent criminal exposure of Viktor Jushchenko 2004 (president of the Ukraine). It is currently assumed that the AhR fulfills an important but

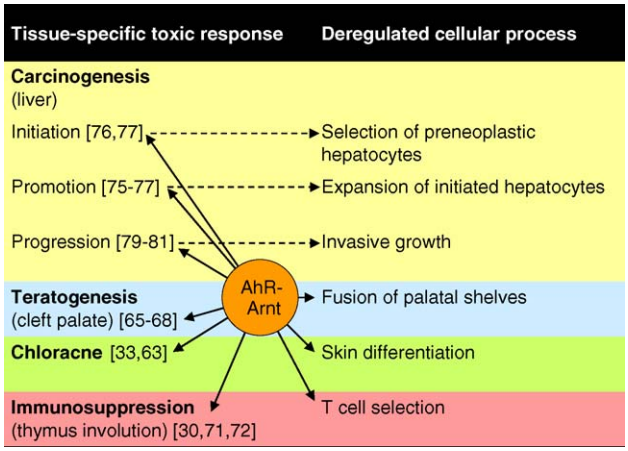

Fig. 2 – Overview on hypothesized dioxin-mediated toxic responses due to deregulated AhR-mediated cellular processes. Solid lines, toxic responses due to sustained AhR activation; broken lines, presumed AhR-modulated cellular processes. Note that in the case of carcinogenesis affected cellular processes are due to the interaction between genotoxically-injured initiated cells and surrounding tissue [92].

unknown role in the complex late stages of keratinocyte differentiation; filaggrin, one of the precursors of cornified epithelium, has been described as XRE-controlled gene product (Table 1) [34,65]. Sustained and inappropriate AhR activation by dioxin exposure is assumed to be the cause of chloracne. The endogenous function of the AhR in skin differentiation is supported by observations with AhR-deficient mice: They developed (53% incidence) severe, localized, interfollicular and follicular epidermal hyperplasia with hyperkeratosis and acanthosis, dermal fibrosis and anagenic hair follicles [66].

4.2. *Teratogenic responses, murine cleft palate as example*

Exposure to dioxins leads to a number of reprotoxic and teratogenic responses [60]. Cleft palate in mice has been well studied [67–70]. In order to form a barrier between the oral and nasal cavities two opposing palatal shelves have to meet and to fuse. The opposing medial edges consist of an outer layer of continuously shed periderm that overlays a layer of basal cells resting on basal lamina. Before fusion, the lamina disappears, basal cells of the apposing medial seam lose epithelial characteristics, extend filopodia into the adjacent connective tissue and gain fibroblast-like features (epithelial to mesenchyme transformation). In this way a single fused tissue is formed. TCDD-exposed murine palatal shelves (at 10^{-10} M) grow and make contact, but the subsequent process of epithelial-to-mesenchyme transformation does not occur. Therefore, a cleft is formed as the palatal shelves continue to grow without fusing. Human embryonic palatal shelves are similarly affected, but at a much higher TCDD concentration (10^{-8} M) [68]. The observation that humans are less sensitive than mice is supported by studies with AhR-transgenic 'humanized' mice [69]. It has also been found that levels of cytokines such as EGF, TGFα and TGFβ1–3 are generated at an inappropriate time [67]. Interestingly, addition of TGFβ3 to dioxin-exposed organ cultures restored fusion of palatal shelves [70], suggesting that stimulators of similar signaling pathways may be candidates of potential antidotes.

TGFβ1 and TGFβ3 are markedly increased in AhR-null mice [71], suggesting that the AhR is a negative regulator of TGFβ levels. However, physiologic functions of the AhR in fusion of palatal shelves are still unknown.

4.3. *Immunosuppression*

The immune system has long been recognized as a target of dioxin toxicity. B and T cell responses as well as host resistance to bacterial and viral infection appear to be affected [72–75]. Involution of the thymus was an early finding of TCDD-mediated toxicity in rodents [2]. Recently, evidence was obtained in experiments with transgenic mice expressing CA-AhR that these mice had a decreased number of thymocytes and an increased percentage of CD8-single-positive thymocytes, showing that AhR activation in T-lineage cells is directly involved in thymocyte loss and skewed differentiation [74]. TCDD may alter the dynamics of T cell selection through dysregulation of coregulators and costimulatory molecules, such as CD30 [75]. It enhances negative selection of T cells in the thymus with functional consequences for peripheral immune functions. Signaling through the T cell receptor primarily controls transcription of the XRE-mediated IL-2 gene, which is mandatory for the homeostasis of T cells (Table 1) [31]. An endogenous role of the AhR in differentiation of the immune system is supported by observations with AhR-deficient mice, which exhibited T-cell deficiency in their spleens [66].

4.4. *Carcinogenicity of dioxins*

4.4.1. *Stages of chemical carcinogenesis*

In experimental hepatocarcinogenesis stages of initiation, promotion and progression can be distinguished. Genotoxic chemicals may lead to oncogene and tumor suppressor gene mutations, a process termed initiation. Tumor promoters are non-genotoxic compounds that increase the probability of cancer development by stimulating clonal expansion of preneoplastic 'initiated' cells. In mouse studies using the prototypical tumor promoter phenobarbital evidence was obtained that the promoter may select for certain preneoplastic genotypes. For example, preneoplastic nodules generated by diethylnitrosamine (DEN) are mainly Ha-ras-mutated whereas in mice treated with phenobarbital the Ha-ras-genotype is suppressed whereas mutated β-catenin was observed in preneoplastic nodules [76]. Finally, at the stage of progression cancer cells gain the ability of invasive and metastasizing growth.

A working group of the WHO evaluated TCDD as a human carcinogen based on (i) its action as a multisite carcinogen in experimental animals and its mode of action via the AhR, (ii) conservation of the receptor in animals and humans, (iii) similar tissue concentrations in heavily exposed human populations and in rats exposed to carcinogenic dosage regimens in bioassays [77]. TCDD has been mostly studied as non-genotoxic tumor promoter, which epigenetically expands preneoplastic lesions [78]. However, TCDD may also affect other stages of carcinogenesis, for example, the antiapoptotic action of TCDD may influence the stage of initiation by facilitating survival of genotoxically injured cells [79–81]. It may also affect the stage of tumor progression by affecting cell surface plasticity of cells and thereby possibly affecting invasive growth (Fig. 2). Human mammary carcinoma MCF-7 cells stably expressing a constitutively active AhR lose their dense growth characteristics and form filopodia and lamellipodia, in contrast to non-transfected controls [82]. Moreover, TCDD has been shown to increase matrix metalloproteinase-1 expression in keratinocytes, and matrix remodeling is a key process in cell migration and tumor invasion [83]. In addition, fibroblasts lacking AhR have impaired tumorigenicity in a subcutaneous mouse xenograft model [84]. Nevertheless, TCDD has been mainly established as a potent tumor promoter.

Due to the fact that AhR signaling strongly depends upon the cell context, we focused on rat liver tumor promotion in the two-stage model of hepatocarcinogenesis [79–81]. It is noteworthy that in this model TCDD acts on at least two different cell populations: normal hepatocytes and enzyme altered foci (EAF). We found that TCDD inhibits apoptosis in EAF while cell division is only moderately enhanced [79–81]. In contrast, studies using rodent liver and various cell lines revealed that TCDD leads to cell cycle arrest and to stimulation

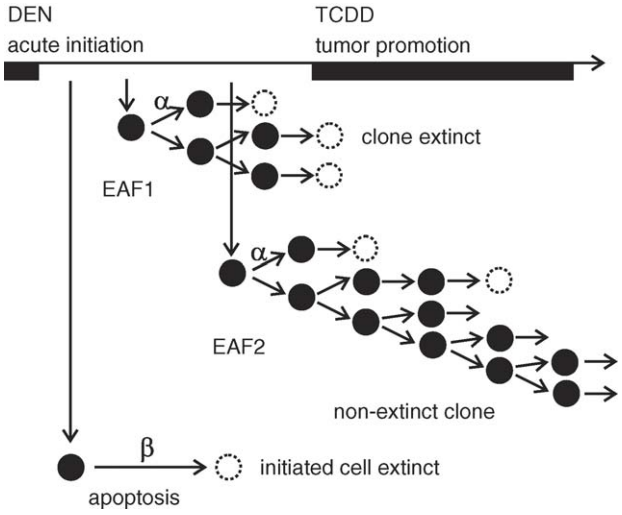

Fig. 3 – Selection and expansion of enzyme-altered focal hepatocytes (EAF) by the tumor promoter TCDD. Most DEN-initiated EAF are extinct (EAF1). After TCDD treatment more initiated hepatocyte clones may survive (EAF2). DEN, diethylnitrosamine.

of apoptosis (Fig. 3 and Table 2). Although differential effects of TCDD in different zones of the liver are not the focus of the present communication, it should be noted that TCDD-mediated growth arrest was not observed in periportal hepatocytes [88]. To reconcile the discrepant TCDD actions on EAF hepatocytes and surrounding liver tissue it has been proposed that TCDD selects and expands cell populations, which gained the ability to evade growth arrest and apoptosis [92]. Despite our focus on liver it should be kept in mind that TCDD is also known as a potent tumor promoter in skin [2]. Multiple mechanisms may facilitate evasion of EAF hepatocytes from TCDD-mediated mitoinhibition and apoptosis. Three processes will be subsequently discussed: (i) release from cell contact inhibition, (ii) protection against hepatotoxic oxidative stress, and (iii) suppression of p53-mediated apoptotic responses.

Table 2 – Differential effects of TCDD on preneoplastic EAF and normal hepatocytes

Cell model	Growth arrest/ apoptosis (+) or inhibition of apoptosis (–)	References
Liver EAF	– (inhibition of apoptosis)	[79–81]
WB-F344 cells	– (release from cell contact inhibition)	[47,85–87]
Liver (centrilobular)	+ (inhibition of DNA synthesis)	[88]
Primary hepatocytes	+ (inhibition of EGF-stimulated DNA synthesis)	[85,89]
5L hepatoma cells	+ (inhibition of proliferation)	[28]
	+ (stimulation of apoptosis)	[90]
Jurkat T cells (CA-AhR)*	+ (cell cycle arrest, stimulation of apoptosis)	[91]

* Constitutively active AhR mutant.

4.4.2. *Release from cell contact inhibition*

Cell–cell contact represents a fundamental regulator of differentiation and of carcinogenesis. Disruption of this process transiently activates AhR-mediated transcription [19,93–95]. Cell–cell contact is already loosened in EAF following initiation by DEN [79–81], and this feature may be synergistically augmented by TCDD. Evidence has been obtained in liver stem cell-like WB-F344 cells that TCDD leads to release from contact inhibition [47,85]. Involvement of cyclin A [86] and downregulation of γ-catenin [87] has been demonstrated under these conditions. Signaling pathways responsible for cell contact inhibition are poorly understood. It is possible that the AhR generates opposing responses at different cellular targets. For example, it is conceivable that TCDD-mediated release from contact inhibition overrules its mitoinhibitory actions, as observed in comparative studies of cell density-dependent growth using primary cultures of normal and nodular hepatocytes [96].

4.4.3. *Protection from hepatotoxic oxidative stress*

In many studies a correlation between hepatotoxicity and hepatic neoplasms has been observed. TCDD is known to induce liver hypertrophy and hyperplasia [2] as well as oxidative stress in this tissue [97]: TCDD may increase reactive oxygen production in mitochondria [98]. In female liver estrogens may generate additional oxidative stress (a possible explanation for the sensitivity of female rats in liver tumor promotion experiments) [99]. Oxidative stress has also been associated with the induction of CYP1A1 and CYP1A2 [100,101]. Based on the CYP1A2-mediated side pathway of heme synthesis leading to the formation of uroporphyrin III, it has been suggested that hepatic uroporphyrin may be a sensitive endogenous marker of oxidative stress. In livers of our tumor promotion experiments uroporphyrin levels were found to be moderately but significantly increased at 13 and 17 weeks of TCDD treatment in the absence of morphologic signs of hepatotoxicity [102], suggesting chronic oxidative stress, which may stimulate growth arrest in liver tissue. In contrast, EAF may be protected from oxidative stress because of their high antioxidant defense capacity, due to low Phase I and high Phase II enzyme levels, termed toxin-resistance phenotype [103].

4.4.4. *Suppression of p53-mediated apoptosis*

p53 is a key tumor suppressor acting as a transcription factor for a number of genes, including Mdm2, encoding a p53-degrading ubiquitin ligase, which acts in a negative regulatory loop [104]. Normally p53 is present in minute amounts. After genotoxic stress, generated by DEN treatment, p53 is stabilized and carries out its functions in cell cycle control, DNA repair and apoptosis (Fig. 4). TCDD has been shown to attenuate DEN-mediated p53 accumulation by hyperphosphorylation of p53 via c-Src protein kinase [105]. However, in the absence of genotoxic stress, TCDD-mediated oxidative stress may be responsible for the moderately enhanced p53 level, which may

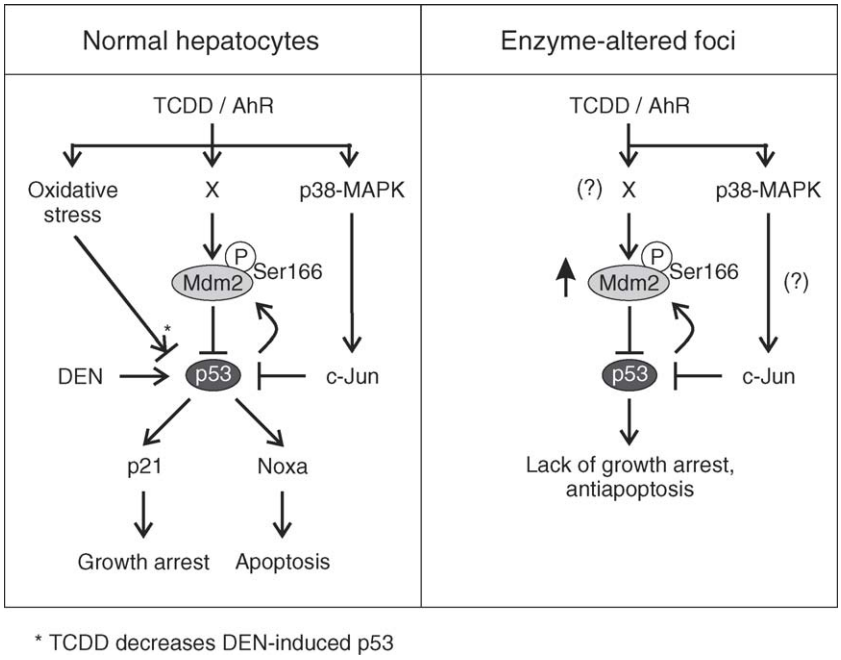

Fig. 4 – TCDD suppresses p53-mediated apoptosis in EAF. Left panel: in normal hepatocytes p53 is kept in minute amounts by rapid degradation. In the presence of genotoxic stress p53 is stabilized and functions in cell cycle control, DNA repair and apoptosis. p53 is tightly regulated by Mdm2 which controls degradation of p53. Mdm2 is transcriptionally upregulated by p53, and thus functions in an autoregulatory loop. TCDD has been shown to decrease DEN-induced p53 accumulation [105]. However, in the absence of genotoxic stress TCDD-mediated oxidative stress moderately enhances p53. TCDD activates Mdm2 by phosphorylating serine 166 via an unidentified protein kinase [106], leading to loss of p53-mediated growth arrest and apoptosis. In addition, p53 may be suppressed by AhR-mediated activation of c-Jun [29,43]. Levels and activity of p53 may subsequently stimulate either growth arrest via $p21^{CIP1}$ or apoptosis via Bcl-2 family members such as Noxa [107,108]. Right panel: in EAF Mdm2 is constitutively upregulated, and p53-mediated genotoxic stress response is attenuated [109]. X, unidentified protein kinase(s).

contribute to growth arrest and apoptosis in TCDD-treated livers. Interestingly, TCDD activates Mdm2 via unidentified protein kinases (X) leading to phosphorylation of serine 166 and attenuates genotoxic stress responses [106]. In addition, p53-mediated apoptosis may be inhibited by AhR-mediated activation of c-Jun [29,43] or other mechanisms [36]. Subsequently, p53-mediated apoptosis by Bcl-2 family members such as Noxa may be suppressed [107,108]. In EAF Mdm2 is constitutively upregulated and p53-mediated stress response is attenuated [109]. Whether TCDD-mediated activation of Mdm2 and c-Jun leads to suppression of p53-mediated apoptosis in EAF remains to be tested.

In conclusion, TCDD-mediated liver tumor promotion may be due to multiple mechanisms favoring growth advantage of cell clones, which are able to overcome major barriers of carcinogenesis such as apoptosis. To evaluate the relevance of the proposed mechanisms, investigations using AhR-mutated rodent models may be useful, for example, using TCDD-sensitive and -resistant rat strains [110,111] or mice expressing a constitutively active AhR [112].

As indicated in Fig. 2, the AhR affects multiple stages of carcinogenesis. (i) Its adaptive detoxification function may inhibit generation of genotoxic carcinogens but may also reduce production of cytotoxic metabolites affecting promotion and progression. (ii) As discussed in chapter 4, AhR functions in regulation of cell growth and differentiation are major factors of dioxin-mediated tumor promotion. To what extent these observations provide hints for physiologic AhR functions in adult life remains unknown. Control of cell proliferation is particularly critical in embryonic/fetal development, and some of these functions may be retained in adult life and may become visible when deregulated by sustained activation of the AhR.

5. Open questions

Many issues remain unresolved. Physiologic AhR functions are just beginning to be understood. Understanding the diverse range of tissue-specific TCDD-mediated toxic responses remains a challenging issue since AhR signaling may differ in different tissues, and may require detailed knowledge of the particular physiologic and pathologic tissue responses. Questions regarding ligand-dependent and -independent AhR activation responses need to be answered, and species differences in sensitivity to toxic responses remain a central issue of human risk assessment.

5.1. *Ligand-dependent and -independent AhR activation*

With regard to ligand-dependent AhR activation a distinction has to be made between persistent and transient ligands. Most transient ligands such as benzo(a)pyrene or dietary plant

constituents are rapidly metabolized by AhR-controlled biotransformation enzymes. In contrast, the AhR is activated by TCDD in a sustained and inappropriate way, and may thus disrupt physiologic AhR signaling. Tissue-specific endogenous ligands and toxins generated during intermediary metabolism need to be identified [8]. In addition, evidence for ligand-independent AhR signaling has been obtained. For example, AhR phosphorylation by cAMP may modulate AhR signaling [50]. It has been speculated that this kind of activation may be related to an ancient way of AhR activation. No AhR ligands could so far be identified for invertebrate AhR homologues [51,52].

5.2. *Species differences in susceptibility to dioxin-mediated toxic responses*

Exceptionally large differences in species-dependent susceptibility to dioxin-mediated toxic responses have been observed, even in strains of the same species. A known example is the more than 1000-fold difference in sensibility to the acute lethal effects of TCDD between the sensitive guinea pigs and resistant hamsters, but also between the sensitive Long–Evans and the resistant Han/Wistar rat strains; the latter two rat strains were similar in sensitivity to CYP1A1 induction, thymus involution but differed 100-fold in sensitivity to tumor promotion in the two-stage model of hepatocarcinogenicity [110]. The resistance of the Han/Wistar rats is due to a mutated transactivation domain of the AhR [111]. In contrast to rodents, primary liver cancer has been rarely observed in highly TCDD-exposed human populations; instead there was a modest increase for all cancers combined (relative risk 1.4), in particular for lung cancer, non-Hodgkin lymphoma [77] and soft tissue sarcoma [113].

A well studied example for a species difference in sensitivity to TCDD was observed in the case of cleft palate. Whereas the mouse is very sensitive, in humans TCDD-mediated cleft palate was not observed in dioxin-exposed human populations. The low risk to develop cleft palate in humans is supported by observations in human fetal organ cultures of palatal shelves [68] and by studies with 'humanized' AhR-mutated mice [69].

Humans are known to be sensitive to develop chloracne when exposed to TCDD, in contrast to rodents [2]. The reason for the sensitivity of humans is unknown. However, it was recognized that mice harboring the 'hairless' gene mutation develop chloracne. The sensitizing hairless gene was identified as a corepressor interacting with the vitamin D receptor, a transcription factor required for hair cycling [114]. Further work on the complex cross-talk between the AhR and receptors for steroids, retinoids and vitamin D may elucidate the sensitivity of humans to chloracne. Elucidating the mechanisms for species differences in dioxin toxicities may greatly improve human risk assessment.

6. Conclusions

The AhR, a ligand-activated bHLH/PAS family member and multifunctional molecular switch, remains a fascinating and rewarding research object, in regard to its role in detoxification, in regulation of vascular development, cell proliferation, differentiation and apoptosis. The present commentary focuses on the hypothesis that disruption of the AhR's tissue-specific physiologic functions by exposure to non-metabolizable, persistent dioxins and related compounds may explain the diverse range of toxicities observed in animals and humans, such as chloracne, teratogenicity, immunosuppression and carcinogenicity.

The discussion is based on brief summaries of molecular mechanisms of AhR signaling, on the limited knowledge about physiologic functions in detoxification and in organ development. Selected organ-specific toxic reactions are described, and proposals are given to understand underlying deregulated processes. However, to what extent these observations provide hints for physiologic AhR functions in adult life remains challenging. Control of cell proliferation by the AhR may be particularly critical in embryonic/fetal development. In adult life detoxification of xenobiotics represents an obvious AhR function. This function becomes evident by its direct coupling to a second transcription factor, Nrf2, a basic leucine zipper protein, mainly involved in protection against oxidative stress. The Nrf2 gene battery is known to selectively induce Phase II reactions of xenobiotic metabolism. Hence, co-regulation of the AhR and Nrf2 gene batteries may lead to a better coupling of Phases I and II enzymes thereby avoiding effective bioactivation of low concentrations of carcinogens in Phase I. Numerous publications have been devoted to transient activation of these two coupled transcription factors by a variety of dietary plant constituents, mainly from Brassica species, offering possibilities for chemoprevention of cancer and degenerative diseases.

It is understood that the range of toxic reactions has to be considerably expanded to prove the hypothesis that dioxin-mediated toxicities may be due to deregulated physiologic AhR functions. Even in the case of the selected toxic reactions, current knowledge of the molecular mechanisms responsible for dioxin-mediated toxicities is unsatisfactory. Difficulties arise from the fact that AhR signaling depends upon the cellular context. Hence, detailed knowledge is required of physiologic and pathophysiologic tissue responses in a given species. Knowledge about molecular mechanisms may improve treatment strategies, as demonstrated by TGFβ3-mediated restored fusion of palatal shelves in TCDD-exposed mice [70].

Two major open questions remain challenging and have been emphasized: (i) ligand-independent AhR activation, presumably via phosphorylation, and (ii) large species differences in susceptibility to toxic reactions. Elucidation of these species differences may considerably improve risk assesment. Obviously, much more knowledge is required to answer the question to what extent dioxin-mediated toxic responses are due to deregulated physiologic functions of a given tissue. However, this knowledge may open new insights into physiologic functions of this exciting orphan receptor in detoxification and development.

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