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INDUCTION OF OXIDATIVE STRESS RESPONSES BY DIOXIN AND OTHER LIGANDS OF THE ARYL HYDROCARBON RECEPTOR

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□ TCDD and other polyhalogenated aromatic hydrocarbon ligands of the aryl hydrocarbon receptor (AHR) have been classically considered as non-genotoxic compounds because they fail to be directly mutagenic in either bacteria or most in vitro assay systems. They do so in spite of having repeatedly been linked to oxidative stress and to mutagenic and carcinogenic outcomes. Oxidative stress, on the other hand, has been used as a marker for the toxicity of dioxin and its congeners. We have focused this review on the connection between oxidative stress induction and the toxic effects of fetal and adult dioxin exposure, with emphasis on the large species difference in sensitivity to this agent. We examine the roles that the dioxin-inducible cytochromes P450s play in the cellular and toxicological consequences of dioxin exposure with emphasis on oxidative stress involvement. Many components of the health consequences resulting from dioxin exposure may be attributable to epigenetic mechanisms arising from prolonged reactive oxygen generation.

1. INTRODUCTION

Many polynuclear polyhalogenated aromatic hydrocarbons (PHAHs) are known or suspected environmental carcinogens, toxicants and teratogens in animals and humans (Gatmaitan *et al.*, 1977; Talalay *et al.*, 1988; Hebert *et al.*, 1990; Jiang *et al.*, 1991; Butler *et al.*, 1992; Ralston *et al.*, 1994; Hatae *et al.*, 1996). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) is prototypical of PHAH compounds, including polyhalogenated dibenzo-*p*-dioxins, dibenzofurans and coplanar biphenyls, that bind to and activate the cytosolic aryl hydrocarbon receptor (AHR). TCDD is a co-planar polychlorinated biphenyl with among the highest AHR binding affinities and agonistic activities (Poland *et al.*, 1976a). It is this interaction of PHAHs, such as TCDD, with the AHR that mediate most if not all effects of low-concentration TCDD exposures.

The AHR is the only bona fide ligand-activated member of the PAS superfamily of proteins, named for the PER ("period" regulator of circadian rhythm), ARNT ("Ah receptor nuclear translocator") and SIM ("single minded", regulator of midline cell differentiation) members of

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helix-loop-helix transcription factors (Alsharif *et al.*, 1994; Hassoun *et al.*, 2003). Prior to ligand binding the AHR resides in the cytosol, associated with two molecules of HSP90 and HSP90 accessory proteins. Upon TCDD binding, the AHR is released from this cytosolic complex and is translocated into the nucleus where it forms a heterodimeric complex with ARNT (Okey *et al.*, 1989). This complex binds to one or more aryl hydrocarbon response elements (AhRE; also known as xenobiotic response elements, XRE; and dioxin response elements, DRE) that function as *cis*-acting enhancers in the regulatory domains of a growing number of genes collectively known as the *AHR gene battery* (Nebert *et al.*, 1993). Battery members include phase I cytochromes P450 (CYP) *Cyp1a1* and *Cyp1a2*, *Cyp1b1* and NAD(P)H quinone oxidoreductase (*Nqo1*), and phase II antioxidant enzymes such as UDP-glucuronosyltransferase (*Ugt1a1*), glutathione *S*-transferase (*Gst1a1*) and aldehyde dehydrogenase (*Aldh3a1*).

Ligands for the AHR include planar PHAHs and diverse classes of plant-derived chemicals. It has been hypothesized that the AHR/ARNT transcriptional complex evolved for defense against an increasingly diverse array of plant toxins and as a result it is unlikely to serve endogenous physiological functions (Gonzalez *et al.*, 1990). More recently however, the AHR has emerged as an important regulator of physiologic and developmental processes in the absence of an apparent exogenous (xenobiotic) ligand (Fernandez-Salguero *et al.*, 1997; Lahvis *et al.*, 2000). The AHR represents a pivotal upstream event in the apoptosis cascade (Nebert *et al.*, 2000; Slim *et al.*, 2000; Dong *et al.*, 2004), exerts an important level of influence on reproductive success (Abbott *et al.*, 1999) and participates in cell cycle regulation (Puga *et al.*, 2002; Marlowe *et al.*, 2004). Further, acting through the AHR, TCDD has been shown to modulate up- or down-regulation of more than 300 known mRNAs and an equivalent number of expressed sequence tags (Puga *et al.*, 2000b). In part, this effect can be attributed to interactions between the AHR and transcription factors other than ARNT, some of which are involved in the control of complex cellular programs, such as cell division and cell fate (Ge *et al.*, 1998; Kolluri *et al.*, 1999; Tian *et al.*, 1999; Puga *et al.*, 2000a; Elferink *et al.*, 2001; Puga *et al.*, 2002; Marlowe *et al.*, 2004). In light of such studies, it is likely that the AHR has important roles in regulating cellular homeostasis that may be disrupted by environmental chemicals. The diversity of AHR ligand interactions, the complexity of the cellular transcriptome, the persistence of AHR activation, and the nature of agonist exposure determine whether the homeostatic equilibrium is maintained or perturbed.

The toxicologic responses elicited by TCDD differ widely among animal species and strains. These differences are attributable to variations in a number of molecular, tissue specific, biochemical and physiological

characteristics. In making inter-model comparisons, TCDD dose can be expressed as a variety of different metrics such as administered dose, average daily dose, tissue concentration, average body burden and area under the curve (AUC). As a result, clear-cut dose-response assessments of TCDD are made difficult by the complexity of biologic responses to TCDD, the variety of tissues affected by TCDD and gaps in our understanding of the mechanisms relating exposure to toxicity. As a result, body burden rather than daily intake (administration) has been suggested as the best dose metric for interspecies comparisons and extrapolation, although the vast majority of studies describing TCDD toxicity express dose in terms of acute, subchronic and chronic exposures (DeVito *et al.*, 1995).

2. FUNCTIONAL ALTERATIONS OF THE AHR

Like many other transcription factors, the AHR has been amenable to dissection into functional domains. The C-terminal half of the AHR, containing a glutamine-rich domain, is responsible for transactivation; whereas the N-terminal half of the AHR, consisting of a basic-region helix-loop-helix domain and two PAS domains, has overlapping functions responsible for DNA binding, ligand binding and dimerization (Hankinson, 1995). Unfortunately, the AHR peptide sequence is not particularly well conserved across species, especially the C-terminal half of the protein, thus complicating risk assessment. Polymorphisms identified within the coding region of the AHR instill differences in AHR-responsive gene induction and toxicologic responses to numerous PHAHs (Nebert, 1989; Swanson *et al.*, 1993; Poland *et al.*, 1994). Interspecies variation notwithstanding, the AHR has been widely studied in mice and rats, which, relative to the human AHR, have high ligand binding affinity.

In mice, differences in TCDD sensitivity have been related to polymorphisms in the AHR that give rise to the commonly studied "responsive" and "nonresponsive" strains (C57BL/6 and DBA/2, respectively). AHR polymorphisms in DBA/2 mice reduce ligand binding affinity approximately 10-fold and thereby diminish TCDD potency for acute lethality (Chapman *et al.*, 1985; Okey *et al.*, 1989). Several groups have sequenced the AHR alleles from inbred strains of mice. These studies have characterized four distinct alleles in mice, referred to as $Ahr^{b\text{-}1}$, $Ahr^{b\text{-}2}$, $Ahr^{b\text{-}3}$, and Ahr^{d}. Among these, the "responsive" phenotype in C57BL/6 mice is encoded by the autosomal dominant $Ahr^{b\text{-}1}$ allele while the "nonresponsive" DBA/2 phenotype is encoded by the Ahr^{d} allele. The four identified mouse alleles differ by 8 nucleotides in their shared open reading frame. In addition, these AHRs differ by 45 amino acids at their C-terminus as a result of a nucleotide change in the Ahr^{d} allele that replaces the stop codon in the Ahr^{b} allele with an arginine (Chang *et al.*, 1993; Poland *et al.*, 1994). Most of the amino acid changes distinguishing

these strains occur within the transactivation domain and have little or no known functional consequence (Chang *et al.*, 1993). However, a point mutation at position 375 of the DBA/2 AHR results in an ALA to VAL substitution in the second PAS domain of the C57BL/6 strain that is responsible for the difference in ligand binding affinity and transactivation (Poland *et al.*, 1994; Maier *et al.*, 1998). These findings have been further supported in mice with homozygous loss of functional AHR (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996). These $Ahr^{-/-}$ knockout strains were refractory to TCDD-mediated CYP1A1 induction and were highly resistant to TCDD-mediated pathologies up to 2000 μg/kg, a 10-fold higher dose than that which induce severe toxicity in functional AHR expressing mice (Fernandez-Salguero *et al.*, 1996).

Rat strains have also been characterized with respect to their TCDD sensitivity. At the extremes of TCDD responsiveness are the "sensitive" Long-Evans rats (L-E) and the "resistant" Hans/Wistar (H/W) substrain of Wistar rats that differ by at least 1000-fold in the acute lethality of TCDD (LD_{50} between 10 to 20 μg/kg and >9600 μg/kg, respectively) (Pohjanvirta *et al.*, 1994b). Inheritance studies implicate the *AHR* gene locus and a second uncharacterized gene *B* in the TCDD resistance of the H/W rats, with the *AHR* contributing the largest quantitative role (Tuomisto *et al.*, 1999). Unlike the C57BL/6 and DBA/2 mice, TCDD resistance is the dominant trait in rats, segregating with autosomal inheritance (Pohjanvirta *et al.*, 1999). Molecular analysis of the coding region of AHR cDNAs from the H/W and the L-E rat revealed a Val497Ala amino acid change in the transactivation domain and, perhaps more importantly, a single point mutation in the first nucleotide of intron 10, resulting in altered mRNA splicing (Pohjanvirta *et al.*, 1998). The loss of this splice-donor site results in the use of the nearest upstream and two downstream consensus splice sites that yields three different molecular AHR species having either a deletion of 43 amino acids in exon 10, an extra 7-amino acid stretch encoded by intron 10, or no translated contribution from exon 11. The net effect of the exon 10 mutation is a modified AHR transactivation domain that has little or no effect on AHR accumulation, ligand binding affinity, or activation of *CYP1A* gene expression (Pohjanvirta *et al.*, 1988; Unkila *et al.*, 1993; Pohjanvirta *et al.*, 1999), but which effectively converts the Han/Wistar rat into the most resistant naturally occurring mammals to TCDD toxicity (Simanainen *et al.*, 2003).

3. PRINCIPLES OF TCDD SENSITIVITY

Within a single animal, tissues vary in response to TCDD-receptor binding. It is the coupling of TCDD-receptor interaction to a measured response that accounts for varying tissue sensitivities and thereby target organ toxicity. In general, the maximum response elicited by a receptor

agonist for a specific endpoint is defined as the intrinsic efficacy of a ligand. Efficacy is therefore a quantitative measure of the signaling events that couples the formation of a ligand-receptor complex to a biologic response (Hestermann *et al.*, 2000). By measuring nonlethal endpoints, differences in TCDD intrinsic efficacy between L-E and H/W rat strains have been categorized into two classes: Type I endpoints (EROD activities, thymus weight, tooth defect) that showed similar efficacy in both strains; and Type II endpoints (body weight, serum FFA and bilirubin levels, and serum ASAT activity) where the response in H/W rats was less than half that observed in L-E rats (Simanainen *et al.*, 2003). The contribution of the AHR and the product of gene *B* to these endpoints was investigated by segregating the H/W resistant genes into three different rat lines, designated A, B and C, by congenic crossbreeding with inbread L-E rats (Tuomisto *et al.*, 1999). Line A possessed the original "resistant" H/W *AHR* allele but with a wild-type gene *B* allele. Line B possessed a normal *AHR* allele, but was homozygous for the H/W gene *B* allele. Line C possessed neither of the H/W resistance alleles. These studies demonstrated that the AHR is the most important factor decreasing TCDD intrinsic efficacy, and that an uncharacterized mechanistic difference exists between type I and II effects that is linked to the altered AHR transactivation domain. Relative to the large difference in acute LD_{50} values between L-E and H/W rat strains, the potency of TCDD for nonlethal type I endpoints was much less affected by the H/W AHR phenotype (Tuomisto *et al.*, 1999; Simanainen *et al.*, 2003). In rat line B, the mutated *B* allele had only a minor influence on TCDD efficacy and the dose responses did not clearly fit into either Type I or Type II responses, but were clearly different from lines A and C. Thus the B allele is concluded to contribute modestly to TCDD resistance independent of the AHR (Simanainen *et al.*, 2003).

The combination of efficacy and ligand-binding affinity determine the relative potency of TCDD (Hestermann *et al.*, 2000), which is defined as the dose of TCDD required to achieve a specific endpoint. Both parameters contributing to potency can vary between animal species, strains and tissues to produce net sensitivities. For example experiments investigating the relative potency of various AHR ligands have shown that TCDD, PCB126, PCB156 and PCB105 all bind to the AHR with reported affinity constants equivalent to 0.76, 16, 2500 and 4600 nM respectively, relative to [^{3}H]-TCDD binding. However, the stimulus-response relationship demonstrates that while TCDD and PCB126 have high intrinsic efficacy for CYP1A1 induction, PCB126 is much less efficient at eliciting a response *after* binding the AHR. PCB105, which binds the AHR at high concentrations, competes for [^{3}H]TCDD binding while eliciting no response qualifying PCB105 as a competitive antagonist. With the exception of PCB105, each of these agents is a full agonist since each elicits the

same maximal response. Therefore the potency of these AHR ligands can be expressed in terms of the **e**ffective **c**oncentration that elicit 50% maximal response for CYP1A1 induction (EC_{50}) and have been shown to be equivalent to 0.015 nM (TCDD), 0.12 nM (PCB126) and 1900 nM (PCB156). In comparison PCB105 has no EC_{50} since it does not elicit a response (Hestermann *et al.*, 2000).

This same principle has been utilized to describe the biologic responses observed between different animal species and strains. For example, the efficacy of TCDD for lethality ranges among rodent species from the guinea pig (LD_{50} = 1 μg/kg) to H/W rat (LD_{50} >9600 μg/kg) (Henck *et al.*, 1981; Poland *et al.*, 1982; Pohjanvirta *et al.*, 1994a). This range of sensitivity has been attributed to a restructured transactivation domain in the hamster AHR, presumably producing a much less responsive ligand-receptor complex (Korkalainen *et al.*, 2004). In comparison, experiments investigating TCDD resistance in mice have found that sensitivity correlates with binding affinity because the dose-response curve is shifted to the right without a reduction in response magnitude (Poland *et al.*, 1976b). Thus, both intrinsic efficacy and ligand-binding contribute to the manifestation of TCDD toxicity and the tremendous variability in dose response that has been reported between animal species.

In vitro modeling of the TCDD dose-response using CYP1A1 induction as a biomarker has demonstrated that TCDD need only occupy a fraction of AHR receptors to elicit maximal response. In PLHC-1 cells, a hepatocellular carcinoma cell line derived from the teleost *Poeciliopsis lucida*, only 1.9 % of available receptor sites were required to be occupied for 50% maximal response while 28% saturation produced 95% maximal response. These data establish a "spare" receptor relationship for the high-intrinsic efficacy AHR ligands such as TCDD, which contrasts with the low-intrinsic activity of ortho-substituted PCB congeners that fail to elicit maximal response even with AHR saturation (Hestermann *et al.*, 2000).

In addition to the individual contribution of intrinsic efficacy and ligand binding to the wide range of observed species and strain TCDD susceptibilities, receptor density also modulates the response. Regulation of receptor expression levels by its own ligand is a common pharmacologic observation and receptor theory predicts that changes in AHR levels will influence both the potency and the maximal response of TCDD. Up-regulation of a receptor's presence increases the potency of its ligand and is referred to as "sensitization", while down regulation results in "desensitization" and subsequent tolerance. In such a manner, AHR expression is significantly influenced by dose and duration of TCDD exposure (Pollenz, 2002). Following short-term *in vitro* exposure of cultured Hepa-1 cells to 2 nM TCDD, AHR levels are reduced to less than 20% of original levels within 6 hours following treatment, and this desensitization persists for at least 72 hours (Giannone *et al.*, 1998). In vivo studies, however,

have failed to consistently demonstrate a prominent physiologic effect caused AHR by ligand binding. In Sprague Dawley rats, a single oral dose of 10 or 50 μg/kg produced a pronounced initial reduction of liver and cytosol AHR concentrations, though, in the case of the former, depletion persisted for 14 days, while in the latter, depletion was followed by AHR induction (Pollenz *et al.*, 1998; Franc *et al.*, 2001a). A similar effect was also reported for Hans/Wistar rats, though these rats had lower liver AHR concentrations than either SD or L-E rats regardless of TCDD treatment (Franc *et al.*, 2001a). In contrast to high-dose TDCC-mediated loss of the AHR, acute and chronic low-dose TCDD administration produced either an increase, or no change in AHR concentrations (Sloop *et al.*, 1987; Franc *et al.*, 2001a; Franc *et al.*, 2001b). In addition, when increased receptor presence was observed it did not translate into sensitization as determined by CYP1A1 induction (Franc *et al.*, 2001a; Franc *et al.*, 2001b). These reports suggest that low-dose TCDD, such as a typical environmental exposure, is not likely to produce either TCDD tolerance or sensitivity, while higher doses appear to be associated with a transient desensitization. They also demonstrate that receptor density does not contribute to the variation in TCDD responsiveness associated with the L-E and H/W rat strains.

4. EFFECTS OF GENDER AND SEX HORMONES IN THE TCDD DOSE-RESPONSE

In long-term bioassays, TCDD increased the incidence of liver tumors in female, but not male, rats (Kociba *et al.*, 1978; Huff *et al.*, 1991; Sawyer *et al.*, 1999). In general, female rats have been shown to be more susceptible to TCDD-induced oxidative stress (Stohs, 1990), oxidative DNA damage (Wyde *et al.*, 2001b) and hepatocarcinogenesis (Huff *et al.*, 1994). These TCDD-mediated effects are, at least in part, dependent on the presence of estrogen (Jana *et al.*, 2000; Coumoul *et al.*, 2001; Lai *et al.*, 2004), though the role of the estrogen receptor remains equivocal (Wyde *et al.*, 2000; Wyde *et al.*, 2001a). In an initiation-promotion model, ovariectomy inhibited TCDD-induced preneoplastic foci and reduced TCDD-induced liver tumor formation (Lucier *et al.*, 1991) suggesting involvement of estrogen which was later supported by the observation that supplemental 17β-estradiol (E_2) restored tumorogenic sensitivity of ovariectomized females (Wyde *et al.*, 2001b). The presumption that estrogen mediates oxidative stress and carcinogenesis was confirmed in Syrian hamsters, which show 100% kidney tumor incidence following the administration of 17β-estradiol or estrone (Liehr, 1997). Likewise estradiol was also found to produce oxidative DNA damage in hamster tissues and other biological model systems (Han *et al.*, 1995; Tritscher *et al.*, 1996; Wyllie *et al.*, 1997; Hodgson *et al.*, 1998; Cavalieri *et al.*, 2000; Liehr, 2001; Wyde *et al.*, 2001b).

Oxidative estrogen metabolism results in the formation of two estrogen catechols, 2-hydroxylated and 4-hydroxylated estradiol. Under circumstances where these catechols are excessively produced, or where their metabolism is impaired, catalytic oxidation to semiquinones and quinones can occur. Of particular importance is formation of 4-hydroxyestradiol, the oxidized quinone of which has been associated with oxidative DNA damage and increased cancer risk (Bradlow *et al.*, 1985; Bradlow *et al.*, 1986; Telang *et al.*, 1992; Nebert, 1993; Bradlow *et al.*, 1995; Telang *et al.*, 1997; Liehr, 1999; Jefcoate *et al.*, 2000; Cavalieri *et al.*, 2000). In contrast, 2-hydroxyestradiol formation has not been associated with either DNA damage or increased cancer risk (Bradlow *et al.*, 1996; Telang *et al.*, 1997; Cavalieri *et al.*, 2000). Toxicity of 4-hydroxyestradiol results from two types of reactions; a one electron redox cycling reaction that occurs when 4-hydroxyestradiol is oxidized to estrone 3,4-quinone, and a two electron electrophilic addition reaction (Liehr, 2000; Cavalieri *et al.*, 2000). Redox cycling generates superoxide and ultimately the highly reactive genotoxic hydroxyl radical (Roy *et al.*, 1991; Han and Liehr, 1995). Superoxide produced in this way may further enhance redox cycling by mobilizing iron from ferritin, increasing cellular Fenton chemistry (Wyllie and Liehr, 1997; Liehr *et al.*, 2001). Rearrangement of estrone 3,4-quinone produces a strongly electrophilic carbonium cation that may undergo a Michael addition reaction with cellular sulfhydrals such as (i.e. glutathione, protein thiols) or by electrophilic addition to DNA purine bases resulting in depurinating adducts (Cavalieri *et al.*, 2000), and ultimately procarcinogenic mutations (Liehr, 2001; Embrechts *et al.*, 2003). The relative contributions of redox cycling and electrophilic interactions in the oxidative stress response and toxicity have not been firmly established; however, limited evidence suggests that covalent sulfhydral modification by electrophiles is likely to be a greater cytotoxic hazard than transient quinone formation that facilitates disposal from the cell (Buffinton *et al.*, 1989).

Xenobiotics acting through the AHR may alter the metabolic profile of E_2 and therefore its estrogenic and toxicological profile. Metabolism of E_2 to 2-hydroxyestradiol is predominantly catalyzed by cytochrome P450 CYP1A1 (Roy *et al.*, 1992; Spink *et al.*, 1998) with some contribution by members of the CYP3A family (Hammond *et al.*, 1997), while metabolism of E_2 to the 4-hydroxyestradiol is mainly a result of CYP1B1 activity (Spink *et al.*, 1994; Hayes *et al.*, 1996; Jefcoate *et al.*, 2000). In liver, TCDD increases the levels of CYP1A1 and CYP1A2 relative to CYP1B1 (Walker *et al.*, 1999), hence 2-hydroxylation predominates over 4-hydroxylation. Similar results have been reported in several breast epithelial tumor and non-tumor cell lines where TCDD strongly induced CYP1A1 activity with resultant 2-hydroxyestradiol formation as the major E_2 metabolite (Spink *et al.*, 1998). In this regard, increased production of 2-hydroxyestradiol

relative to 4-hydroxyestradiol and 16α-hydroxyestrone has been observed after exposure to indole 3-carbinol, a dietary micronutrient and AHR proligand. This finding is of potential clinical importance for cancer, since indole carbinols, that bind AHR as acid condensation products, are in clinical trials as cancer chemoprotective agents (Gillner *et al.*, 1985; Malloy *et al.*, 1997; Michnovicz *et al.*, 1997; Telang *et al.*, 1997; Rosen *et al.*, 1998; Yuan *et al.*, 1999; Bell *et al.*, 2000) (reviewed by (Shertzer *et al.*, 2000)).

5. EFFECTS OF TCDD EXPOSURE ON DEVELOPMENT

AHR in development

In the mammalian fetus and in fish larvae, the AHR plays prominent roles in both resolving vascular structures and mediating cardiovascular toxicities of TCDD (Lahvis *et al.*, 2000; Bello *et al.*, 2004). In mammals, the importance of functional AHR is demonstrated in AHR-*null* mice by a failure of a fetal vascular structure, the ductus venosus, to close, thus permitting blood from the portal vein to bypass the liver by shunting to the inferior vena cava. Functional AHR is also required for normal vascular "pruning" during fetal development, the absence of which results in the propagation into maturity the highly anastomotic vasculature architecture of the liver, eye and kidney that that are characteristically neonatal (Lahvis *et al.*, 2000).

Because of the involvement of the AHR in resolving fetal vascular structures, it is not surprising that the cardiovascular system has been shown to be an important target of TCDD-mediated toxicity (Jokinen *et al.*, 2003; Karyala *et al.*, 2004). Although the mechanisms underlying cardiovascular risks are undetermined, it has been postulated that TCDD interferes with cardiovascular development by sequestering the AHR or displacing an as yet unidentified ligand, thereby preventing the AHR from carrying out its normal endogenous activity. In support of this hypothesis, knockdown of the TCDD-responsive AHR2 in zebrafish with morpholino-substituted oligonucleotides has specifically demonstrated that TCDD retardation of common cardinal vein (CCV) regression is AHR dependent (Bello *et al.*, 2004). That knockdown of AHR2 expression itself did not inhibit CCV regression in a manner similar to that of the Ahr-null mouse is attributable to the fact that zebrafish possess a second, TCDD refractory a*hr* locus (*ahr*1) that may compensate for the loss of *ahr*2 (Bello *et al.*, 2004). In separate experiments, fish have provided evidence that the vascular endothelium is also a sensitive target for TCDD toxicity, and this may prove important with regards to the human health effects of TCDD. In fish TCDD elicits increased vascular permeability, which in lake trout manifests as yolk sac edema, and in zebrafish as extravascular accumulation of serum proteins in mesencephalic brain tis-

sues (Guiney *et al.*, 2000; Dong *et al.*, 2004). Although the precise nature of this microvascular leakage remains to be determined, decreased cardiac output, increased endothelial vacuolation (Guiney *et al.*, 2000), disruption of peripheral vascular beds (Henry *et al.*, 1997) and/or disruption of angiogenic signaling (Bello *et al.*, 2004) have been suggested. Whether TCDD mediates developmental vascular defects in fish by the same mechanism as those observed in mammals following gestational exposure remains to be determined; however, two separate reports demonstrate that loss of the AHR protects against the teratogenic effects of TCDD (Mimura *et al.*, 1997; Peters *et al.*, 1999b).

In humans there are few reliable studies linking maternal exposure to TCDD and related compounds (e.g., other dioxins, furans, and dioxin-like PCBs) with impaired fetal development. A number of epidemiologic studies have been confounded by the use of indirect estimates of TCDD exposure, such as local soil levels (Stockbauer *et al.*, 1988), estimates of dietary consumption (Svensson *et al.*, 1991; Rylander *et al.*, 2000) and correlation with residential location (Revich *et al.*, 2001). Only a few studies have used biologic measures of exposure, such as dioxin or PCB concentrations in breast milk and serum (Patandin *et al.*, 1998; Eskenazi *et al.*, 2003). In both of these studies birth weight and gestational age did not differ between mothers with higher exposure levels relative to controls, though these findings are somewhat offset by reports that birth weight was negatively correlated with cord plasma PCB and dioxin levels (Patandin *et al.*, 1998; Vartiainen *et al.*, 1998). One mechanistic explanation for the equivocal association between maternal exposure and teratogenesis may relate to the low affinity human AHR, comparable to the nonresponsive DBA/J2 mouse strain (Ramadoss *et al.*, 2004). In experiments with AHR-null mice, oral exposure of pregnant dams (40 μg/kg TCDD) was sufficient to produce cleft palate and hydronephrosis in nearly all wild-type fetuses while no mice with the homologous AHR knockout were sensitive to the teratogenic effects (Mimura *et al.*, 1997; Peters *et al.*, 1999a) . This possibility is further supported by the use of humanized mice expressing human AHR rather than mouse AHR. These studies demonstrated that mice expressing human AHR had a weaker response to TCDD than resistant DAB/2 mice, and that the humanized AHR phenotype protected against cleft palate (Moriguchi *et al.*, 2003).

A variety of human epidemiologic studies have suggested a link between TCDD exposure and cardiovascular morbidity following occupational exposure (Bertazzi *et al.*, 1989; Flesch-Janys *et al.*, 1995; Vena *et al.*, 1998; Pesatori *et al.*, 1998; Pesatori *et al.*, 2003). Retrospective analysis of 1189 chemical plant workers exposed to dioxin and furans reported a highly significant 2.5-fold (95% confidence interval-1.3-4.7) increase in relative-risk of death from heart disease due to dioxin exposure (Flesch-Janys *et al.*, 1995; Pelclova *et al.*, 2002). The body burden at which these

effects were seen ranges from 110 to 4000 ng/kg of TCDD in blood fat, well below the body burden of TCDD shown to induce cancer in rodents (100-140000 ng/kg) (DeVito *et al.*, 1995). These observations were later supported in hyperlipidemic mice subchronicly treated with TCDD (150 ng/kg, 3 times weekly), resulting in increased blood pressure and atherogenic lipids; the two most important clinical risk factors for atherosclerotic plaque formation. Further, TCDD exposed animals had a trend towards earlier onset and increased severity of atherosclerotic plaques compared to vehicle treated mice (Dalton *et al.*, 2001). Similarly, in female Sprague-Dawley rats treated 5 days per week with up to 100 ng/kg/day TCDD for 2 years, cardiomyopathy and chronic active arteritis increased in a dose dependent manner. However the severity of cardiomyopathy did not increase in a dose-responsive manner and only became evident in the later treatment groups (Jokinen *et al.*, 2003)

6. ROLE OF CYTOCHROME P450 ENZYMES IN TCDD TOXICITY

Several cytochrome P450 genes under the control of the AHR, notably those in the CYP1 family (CYP1A1, CYP1A2 and CYP1B1) have been suggested to contribute to TCDD-induced toxicity (Andersen *et al.*, 1998; Nebert *et al.*, 2004). Because TCDD-associated toxicities are slow to develop, requiring days to weeks, it is likely that the transcriptional events elicited by TCDD-mediated AHR activation must be persistent. Therefore, we believe that the persistent changes in gene expression induced by TCDD disrupt signal transduction homeostasis leading to the accumulation of toxicants (i.e reactive oxygen species, lipid peroxidation products) that in turn lead to pathology. In the liver, one such gene circuit involves the *Cyp1a* monooxygenase subfamily. Studies utilizing *Cyp1a1*$^{-/-}$ knockout mice from a C57BL/6J background demonstrate that a single high dose of TCDD (200 μg/kg) is highly lethal to *Cyp1a1*$^{+/+}$ males but not to *Cyp1a1*$^{-/-}$ males or to females of either genotype. This protective effect conveyed by gender, however, is quite limited compared to the protective effect afforded by *Ahr* knockout, that protected against TCDD doses of up to 2 mg/kg (Fernandez-Salguero *et al.*, 1996). Further, *Cyp1a1*$^{-/-}$ mice are resistant to TCDD-induced wasting syndrome, which is manifested by weight loss or poor weight gain in conjunction with marked increases in serum AST levels, reflecting rhabdomyolysis. Glycogen depletion and down regulation of phospho-enol-pyruvate carboxykinase, combined with SRC oncoprotein action have been suggested to play a role in this process (Dunlap *et al.*, 2002). *Cyp1a1*$^{-/-}$ mice, regardless of gender, are more resistant to hepatocyte hypertrophy; likewise, *Cyp1a1*$^{-/-}$ mice experience decreased accumulation of microvesicular and interstitial lipid accumulation for reasons that are not yet clear. It is of interest to note that the H/W rat, which is resistant to TCDD toxicity for reasons already discussed, shows normal acute induction of *CYP1* family

genes and uroporphyria following TCDD exposure (Simanainen *et al.*, 2003; Niittynen *et al.*, 2003; Uno *et al.*, 2004; Korkalainen *et al.*, 2004).

The other member of the cytochrome P4501A family, CYP1A2, is emerging as a monooxygenase that dichotomously contributes to both protective and sensitizing effects to TCDD toxicity. The protective effects result from pharmacokinetic and antioxidant activities associated with CYP1A2 expression. Pharmacokinetically, CYP1A2 is the primary hepatic TCDD-binding protein, capable of sequestering significant quantities of dioxin; this is not true of CYP1A1 (Diliberto *et al.*, 1997; Uno *et al.*, 2004). Further, CYP1A2 is stabilized by TCDD extending its half-life and therefore augmenting its phramacokinetic effect (Andersen *et al.*, 1997; Diliberto *et al.*, 1997). Presumably by sequestering TCDD, CYP1A2 acts to reduce the free fraction of TCDD available to mediate gene induction through AHR interaction. Pharmacokinetic studies have shown that levels of both CYP1A1 and CYP1A2 must be considered in predicting tissue concentrations of TCDD from the administered doses (Wang *et al.*, 1997a; Wang *et al.*, 1997b; Santostefano *et al.*, 1998). In terms of antioxidant protection, CYP1A2 enzyme activity is associated with decreased microsomal H_2O_2 production, possibly by acting as an electron transport pathway or electron sink for uncoupled electron transfer by CYP2E1 or other microsomal enzyme systems.

Contrasting with these apparent beneficial effects, CYP1A2, and to some extent CYP1A1, has been demonstrated in mice to mediate the uroporphyrinogenic effect of TCDD. In brief, uroporphyria results from dysfunction of uroporpheryninogen decarboxylase (UROD) during hepatic heme synthesis, leading to significant hepatocellular uroporphyrin accumulation and possibly liver injury (Pohjanvirta and Tuomisto, 1994a; Smith *et al.*, 1998). In TCDD-mediated uroporphyria, CYP1A2 is necessary and sufficient to inhibit UROD metabolism, resulting in accumulation of uroporphyrin isomers (Smith *et al.*, 1998). Knockout mouse experiments have shown that loss of CYP1A2 completely, and CYP1A1 partially, protects against TCDD-mediated uroporphyrin accumulation (Smith *et al.*, 2001; Uno *et al.*, 2004).

In the course of the reaction catalyzed by monooxygenase P450 enzymes, two electrons are sequentially transferred from NADPH-dependent cytochrome P450 oxidoreductase to each atom of bound oxygen, resulting in the production of oxygenated substrate and water (Guengerich *et al.*, 1985; Poulos *et al.*, 1992). This reaction is reversible, a process that is perhaps toxicologically important, since physiologically-derived peroxides can metabolize various xenobiotics, particularly carcinogenic arylamines, via the peroxidase activity of CYP1A2 (Anari *et al.*, 1997). Although tight coupling normally exists between oxygen reduction and monooxygenation, some reactive oxygen may be released as either superoxide or H_2O_2 in the course of electron transfer. The

monooxygenase-dependent production of ROS in liver microsomes, supported by NADPH, is a well-known phenomenon (Gillette *et al.*, 1957) that clearly contributes to the total cellular production of reactive oxygen in rat liver, without necessitating enzyme induction (Bondy *et al.*, 1994). Even in the absence of exogenous xenobiotic substrates, endogenous substrates, such as any one of the many arachidonic acid metabolites, may stimulate ROS production (Capdevila *et al.*, 1988; Rifkind *et al.*, 1990; Nakai *et al.*, 1992). In this regard, lipoxin A4, a metabolite of arachidonic acid, may act as an inducing ligand for the AHR (Schaldach *et al.*, 1999). Further, substrate independent ROS production, due to inefficient microsomal electron coupling, has been demonstrated for CYP2E (Ekstrom *et al.*, 1986; Dai *et al.*, 1993), CYP2B, and CYP3A (Ahmed *et al.*, 1995).

While xenobiotic AHR ligands, such as TCDD, can induce microsomal CYP1 expression and ROS production, suppression of CYP1A1 activity has been reported with high dose exposure to several PHAH. This phenomenon has been studied in fish and rodent liver microsomes using the AHR inducing compounds 3,3′,4,4′-tetrachlorodiphenyl (TCB) and 3,3′,4,4′,5-pentachlorobiphenyl (PeCB) (Schlezinger *et al.*, 2001). CYP1A1 enzyme activity was strongly inhibited even though treatment with the halogenated biphenyls increased *cyp1a1* mRNA. Since these compounds are poorly metabolized, inhibition by product could not explain the results. The loss of CYP1A1 activity was attributed to the ability of TCB and PeCB to accelerate CYP1A1 electron flow with concomitantly increased ROS production. Although some reactive oxygen species are released by enzyme uncoupling, ROS scavengers were unable to prevent the loss of CYP1A1 activity indicating that the chemistry involved occurs entirely within the enzyme active site. TCB also stimulated ROS production in microsomes from insect cells expressing human CYP1A1, but not in microsomes from cells expressing human CYP1A2 (Schlezinger *et al.*, 1999). These results may explain the previous observation in mice that TCDD produced a sustained elevation of hepatic CYP1A2 activity, while CYP1A1 showed a transient increase, followed by a rapid loss (Shertzer *et al.*, 1998).

7. TCDD-MEDIATED PERTURBATION OF REDOX HOMEOSTASIS

In addition to its involvement in normal physiological processes and signal transduction, the AHR appears to mediate toxicological effects through oxidative stress. As used here, the term *oxidative stress* refers to any condition that produces an oxidative stress response through an increase in the cellular oxidation state. An oxidative shift in cellular redox homeostasis generally results from increased production of reactive oxygen species relative to cellular antioxidant defenses. Although oxidative stress does not necessarily result in toxicity, it is an important

mechanistic component of many toxicologic processes. In this regard, TCDD-mediated activation of the AHR shifts the cellular redox balance to produce an oxidative stress response (Hassoun *et al.*, 1998; Shertzer *et al.*, 1998; Slezak *et al.*, 2000; Senft *et al.*, 2002a; Senft *et al.*, 2002b). For this reason it has become widely hypothesized that the toxicity induced by TCDD involves an oxidative stress component; an observation that has been reported by several laboratories (Stohs, 1990; Alsharif *et al.*, 1994; Shertzer *et al.*, 1998; Slezak *et al.*, 2000).

Several mechanisms have been proposed to explain TCDD-mediated oxidative stress including reduction in expression levels of protective antioxidant enzyme systems (Latchoumycandane *et al.*, 2003) and perturbation of cytochrome P450 levels (Nebert *et al.*, 2000; Lee *et al.*, 2002). The incomplete reduction of O_2 by several enzyme systems, in particular the cytochrome P450 enzymes that are induced by TCDD, is known to result in the generation of superoxide and hydrogen peroxide through poor coupling of electron flow. TCDD has been implicated in the formation of the superoxide anion in rat brain (Hassoun *et al.*, 2003) and hydrogen peroxide in mouse liver (Shertzer *et al.*, 1998; Senft *et al.*, 2002a), with resultant generation of lipid peroxides in rat brain, mouse liver and rat testis (Shertzer *et al.*, 1998; Hassoun *et al.*, 2003; Latchoumycandane *et al.*, 2003). Several lines of evidence support the AHR as a mediator of oxidative stress. It has been observed that peritoneal lavage cells from C57BL/6 mice, which carry the high-affinity *Ahr*^{b1} allele, demonstrated considerably greater production of superoxide anion in response to TCDD relative to cells from low-affinity DBA/2 mice (Alsharif *et al.*, 1994). Likewise, hepatic lipid peroxidation induced by TCDD occurred at low doses (500 ng/kg) in C57BL/6 mice and only at higher doses (5 μg/kg) in DBA/2 mice (Mohammadpour *et al.*, 1988). In addition, inactivation of aconitase activity, a reliable measure of oxidative stress (Pantopoulos *et al.*, 1995), was documented in C57BL/6 but not in DBA/2 mice following TCDD treatment (Smith *et al.*, 1998).

It should be noted that TCDD dose and tissue concentration do not necessarily correlate with ROS production; the pattern of TCDD exposure also has a prominent effect on ROS production. In liver, an acute oral dose of TCDD (10 and 100 μg/kg) administered to C57BL/6 mice produced a sustained increase in liver superoxide anion and thiobarbituric acid reactive substance (TBARS), attaining to hepatic TCDD concentrations of 55 and 321 ng/g respectively at 13 weeks following exposure. In comparison, subchronic TCDD administration (0.15 to 150 ng/kg; 5 days/week for 13 weeks; po) produced increased superoxide and TBARS only with the highest (150 ng/kg/day) exposure level, corresponding to a hepatic TCDD concentration of 12 ng/g of liver. These data suggest that higher tissue TCDD concentrations are required to elicit oxidative stress following acute exposure than with subchronic TCDD

exposure (Slezak *et al.*, 2000). For this reason it is clear that uncharacterized factors remain that can contribute to tissue responses during TCDD exposure.

Enzyme systems catalyzing O_2 reduction must be in balance within the cell because partially reduced oxygen species can be more reactive and deleterious than the parent molecule. Such is the case with hydrogen peroxide, which is generated during superoxide detoxification. Detoxification of superoxide to H_2O requires the sequential action of SOD with catalase or glutathione peroxidase. Three- to six-fold overexpression of Cu/Zn SOD in transgenic mice results in increased production of H_2O_2 and hydroxyl radicals, which accompany enhanced apoptosis of thymocytes and bone marrow cells (Peled-Kamar *et al.*, 1995). This is similar in nature to the enhanced neurotoxicity of kainic acid by SOD overexpression that also occurs through the generation of superoxide (Bar-Peled *et al.*, 1996). Therefore, the consequence of TCDD-induced changes in antioxidant enzyme expression is uncertain, as illustrated by the fact that up-regulation of SOD does not necessarily dictate a decrease in cellular ROS.

Two sites of TCDD-induced reactive oxygen production have been proposed: the microsomes and the mitochondria. Microsomal reactive oxygen production in mouse liver is regulated by at least three forms of cytochrome P450s (Uno *et al.*, 2004; Shertzer *et al.*, 2004b) that clearly contribute to the total cellular production of reactive oxygen in rat liver (Dai *et al.*, 1993; Bondy and Naderi, 1994). CYP1A1 and CYP2E1 generate reactive oxygen in liver microsomes, while CYP1A2 diminishes reactive oxygen production. The stoichiometric ratios of NADPH and O_2 utilized relative to H_2O_2 produced indicate that the pathway of electron flow is short-circuited by TCDD-mediated microsome induction, resulting in increased H_2O_2 production (Shertzer *et al.*, 2004a). CYP1A2 contributes to the time course of the oxidative stress response elicited by AHR ligands by reducing the microsomal oxidative stress response, including lipid peroxidation and decreased membrane fluidity, which is observed following TCDD treatment in mice. CYP1A2 appears to act as an electron sink by accepting electrons generated by CYP1A1 and CYP2E1, preventing the generation of H_2O_2 and the oxidation of microsomal membrane lipids (Shertzer *et al.*, 2004b).

Mitochondria appear to be the major site for reactive oxygen production (Senft *et al.*, 2002b; Latchoumycandane *et al.*, 2003) and as such may represent a target for TCDD-dependent injury. One proposed mechanism by which TCDD may contribute to increased mitochondrially-derived reactive oxygen is inhibition of electron transport at complex III, producing a persistent increase in succinate-dependent superoxide and hydrogen peroxide production (Senft *et al.*, 2002b). Respiratory chain-derived reactive oxygen can result from a decrease in cytochrome *c* oxi-

dase (complex IV) activity, coupled with an increase in succinate-cytochrome *c* reductase (complex II), resulting in an increase in the reduction state of cytochrome bc_1 complex (complex III) to facilitate univalent reduction of oxygen (Senft *et al.*, 2002a; Senft *et al.*, 2002b). Mechanistically, TCDD-dependent electron flow through complex III results in increased electron pressure and increase redox cycling of Coenzyme Q or Fe-S proteins. The physiologic relevance of this mechanism however is not well established, since the concentration of TCDD necessary to act in this manner is far greater than would be expected to be found in naturally-occurring exposures.

Following TCDD treatment, and associated with increases in the production of reactive oxygen, both GSH and GSSG increase in the cytosol and in the mitochondria. However, in the mitochondria, GSH increases to a greater extent relative to the cytosol, while GSSG increases to a lesser extent. These differences resulted in shifts in equilibrium for both type 1 (protein mixed disulfides) and type 2 (protein disulfides) thiol-disulfide switches (Schafer *et al.*, 2001). In the cytosol, TCDD produces an increase in oxidation state, with decreases in type 1 and type 2 switches, as well as an increase (more positive) in the reduction potential (ΔE) of GSSG/2GSH. In sharp contrast, mitochondria display an increase in reduction state, with increases in type 1 and type 2 thiol redox switches, as well as a decrease (more negative) in the ΔE of GSSG/2GSH half reaction (Dalton *et al.*, 2004). These results from the authors' labs are consistent with the hypothesis that TCDD mediates an increase in mitochondrial reactive oxygen result from an overall increase in the reduction state of the mitochondria. As such, the mitochondrial generation of reactive oxygen by TCDD may be considered a form of reductive stress, rather that the clearly defined oxidative stress response that occurs in the cytoplasm.

Although TCDD is not genotoxic in the Ames test, one suggested pathway by which it produces toxic effects involves oxidative DNA damage and increased mutation frequency. A strong relationship has been established between oxidative damage to DNA and chemical carcinogenesis (Cairns *et al.*, 1991). Oxidation of DNA at the 8-position deoxyguanosine produces 8-hydroxydeoxyguanosine (8-OHdG), which represents the major promutagenic lesion produced during oxidative stress. When guanosine base modification is followed by DNA replication G→T and A→C transversions can be produced. In addition, reactive oxygen-induced DNA damage activates error-prone polymerase DNA repair that may in turn produce base mispairing (Cairns *et al.*, 1991). Although exonucleases and glycosylases can repair such oxidative DNA damage, the probability of mutation fixation increases with the duration of exposure to a mutagen and with increases in the mitotic rate (Kasai *et al.*, 1986; Cheng *et al.*, 1992; Aronica *et al.*, 1993; Kamiya *et al.*, 1995), which, given

the biologic persistence of TCDD, increase the total probability of a mutational event to a level comparable with that of stronger mutagens. An increase in 8-OHdG that persisted 8 weeks after treatment with TCDD was observed in the urine of C57BL/6 mice (Shen *et al.*, 1995; Shertzer *et al.*, 1998) and in the tissue culture medium of hepatoma Hepa-1c1c7 cells treated with TCDD (Park *et al.*, 1996).

Though TCDD has been repeatedly linked to oxidative stress and the oxidative stress response to mutagenesis, TCDD has not been shown to be directly mutagenic in either bacterial or most *in vitro* assay systems (Giri, 1986). In this regard, an important negative finding has been that, at a dosing regimen capable of producing an oxidative stress response, TCDD did not alter the mutation frequency or the mutation spectrum of the *lacI* transgene in male or female Big Blue rats (Thornton *et al.*, 2001). Since oxidative stress and some oxidative stress response genes are induced by TCDD *in vivo*, it can be concluded with caution that TCDD-mediated oxidative damage may not be a prominent cause of mutations. For this reason, an alternate pathway for enhancing cell proliferation and malignant conversion appears likely. Despite the ability of TCDD to generate oxidative base products (Park *et al.*, 1996), the health implications of such findings must be questioned.

8. CONCLUDING REMARKS

In all likelihood, there are several interdependent AHR-dependent pathways that lead to the increased generation of ROS and to the decreased ability to defend against their action. For example, AHR activation may lead to an increase in superoxide production through increased expression of xanthine dehydrogenase/xanthine oxidase and monooxygenases; to an increase in capacity for superoxide reduction through increases in CuZnSOD; and to inhibition of glutathione peroxidases through the generation of J series prostaglandins. The net effect of all these changes would be an increased production of H_2O_2 and a decreased capacity to detoxify it. Studies aimed at understanding AHR-mediated toxicity have led to the discovery of AHR variants that appear to maintain physiological function and yet confer greatly diminish toxicity; perhaps this is due to the remarkable structural plasticity of the AHR, as shown by work in inbred mouse strains and in rats. The human AHRs thus far studied demonstrate ligand binding affinity characteristics similar to those of the low-affinity mouse strains. This is likely to be an important factor explaining why PHAHs show relatively low toxicity in humans. It is also intriguing to speculate that AHR variants may exist in the human population that confer sensitivity to PHAH pollutants because they behave more like the high-affinity rodent AHR variants. Indeed the studies in the rat and hamster suggest that poorly understood functions of the

AHR transactivation domain contribute to toxicity.

One of the greater challenges in Ah receptor research is to identify the connection between toxicity and exposure. While the use of various model systems do serve to elucidate mechanism of TCDD toxicity, it remains difficult to draw broad conclusions given the wide variations in TCDD responses associated with species and strain susceptibilities, exposure models and response endpoints. Are changes in the redox state of cells exposed to PHAHs adaptive or toxic? Are the effects of these changes cumulative? The extent to which the AHR ligands elicit oxidative stress may depend on the duration and nature of the exposure, as well as on the properties of the agonist. At times AHR activation may be so transient that it causes modest and largely unnoticed perturbations to the cellular redox status. At other times or with other AHR ligands, the effects might be much more significant and harmful because of the severity and length of the oxidative stress response. We believe that many of the health consequences resulting from TCDD exposure may likely result from epigenetic mechanisms, including those exerted by cytosolic and mitochondrial reactive oxygen production. In this scenario, resultant toxicity would be related to the non-physiological persistent activation of AHR-dependent signaling pathways due to the long biological half-life of the compound.

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