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Beyond the Aryl Hydrocarbon Receptor: Pathway Interactions in the Hepatotoxicity of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin and Related Compounds

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Abstract

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototypical ligand for a group of environmental halogenated aromatic hydrocarbon contaminants which elicit hepatotoxicity and other toxic responses through activation of the aryl hydrocarbon receptor (AhR). Despite the conservation of the AhR and its signaling pathway, TCDD-elicited differential gene expression networks are species-specific, consistent with differences in sensitivity and toxic responses between species. This review integrates gene expression studies with complementary phenotypic analyses (e.g., metabolomics, clinical biochemistry, and histopathology) to elucidate the pathways through which TCDD and related compounds cause hepatotoxicity beyond AhR activation. We propose that AhR-mediated toxicity is a collective response to the cumulative burden of metabolic reprogramming across multiple pathways. Consequently, nutrition, health status, and genetic background establish the basis for differences in sensitivity and predisposition to adverse outcomes between species, sub-populations, tissues, and cells.

Keywords

Aryl hydrocarbon receptor; Hepatotoxicity; Toxicogenomics; Metabolic Reprogramming; Species-Specific Sensitivity

Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototypical member of a class of persistent environmental halogenated aromatic hydrocarbon (HAH) contaminants that elicit a broad spectrum of biochemical and toxic effects in a species-, sex-, age-, tissue-, and cell-specific manner. These effects include immuno-, hepato-, cardio-, and dermal toxicity, tumor

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promotion, teratogenicity, modulation of cell proliferation and differentiation, alterations in endocrine homeostasis, wasting, and lethality [1,2]. In addition to TCDD, there are 6 other polychlorinated dibenzo-*p*-dioxins (PCDDs), 10 polychlorinated dibenzofurans (PCDFs), and 12 co-planar polychlorinated biphenyl (PCBs) congeners, as well as chloro-substituted dioxin-like naphthalenes and diphenyl ethers that may also elicit dioxin-like toxicity [3]. Brominated analogues of PCDDs, PCDFs, and PCBs elicit comparable dioxin-like activity with relative effect potencies within one order of magnitude [4]. Environmental exposure typically involves a complex mixture of these compounds, which are assumed to share a common mechanism of action. Structure-activity relationships, antagonist studies, and knock-out models provide compelling evidence that most, if not all, of the toxic effects elicited by TCDD and dioxin-like compounds are mediated by the aryl hydrocarbon receptor (AhR). The AhR is a basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) domain-containing ligand activated transcription factor that is functionally similar but structurally different from members of the nuclear receptor superfamily [5,6].

In the canonical pathway, ligand binding to cytosolic AhR causes dissociation of heat shock protein 90 (HSP90), AhR-interacting protein (AIP; also known as ARA9 or XAP2), and p23, followed by nuclear translocation and dimerization with the AhR nuclear translocator (ARNT) [2,7,8]. The heterodimer complex then binds to dioxin response elements (DREs; core sequence 5'-GCGTG-3') and recruits transcriptional co-regulators, leading to the differential expression of target genes [2,7-9]. However, an increasing number of reports describe AhR-mediated changes in gene expression independent of DREs [2,5,10-12]. Moreover, the structure of the ligand can influence which co-regulators are recruited to the promoter, similar to the selective modulation of nuclear receptors [13].

As we approach ~50 years of dioxin research, it is well established that the toxicity of TCDD and related compounds is due to changes in gene expression, yet the pathways affected and mechanisms of toxicity beyond AhR activation remain poorly understood. More specifically, which primary AhR-mediated gene expression responses trigger toxicity? Which secondary responses are required and/or contribute to the development of toxicity? Which metabolic pathways are most affected, and how does AhR-mediated metabolic reprogramming lead to adverse outcomes? Extending our mechanistic understanding of AhR-mediated toxicity is crucial not only to assess the relevance of rodent studies in predicting human risk, but also to identify vulnerable targets that are relevant to human diseases associated with metabolic disruption (e.g., diabetes, cardiovascular disease, metabolic syndrome) and potentially amenable to therapeutic intervention.

Comparative Gene Expression Studies

Despite the conservation of the amino acid sequence, structural organization, and mode of action of the AhR, accumulating evidence suggests that AhR-mediated gene expression networks are not conserved across species, consistent with reported differences in species-specific adverse responses (Figure 1). Moreover, there is a 5,000-fold difference in species-specific sensitivity to TCDD, with LD₅₀ values ranging from 1 µg/kg for highly sensitive guinea pigs to 5000 µg/kg for resistant hamsters. Mice and rats are moderately sensitive with LD₅₀ values of 114 and 22 µg/kg, respectively [14]. Using EC₅₀ values for *Cyp1a1*

induction as an indication of sensitivity, human primary hepatocytes are 2–50 times less sensitive to TCDD compared to rodent hepatocytes [15,16].

Computational analysis using a position weight matrix based on bona fide functional DREs revealed that DRE locations and distributions differ markedly between the human, mouse, and rat genomes [9]. Accordingly, transcriptomic analysis of TCDD-treated human, mouse, and rat primary hepatocytes identified only 16 orthologous genes that were differentially expressed in all three species, while the majority of orthologs exhibited species-specific expression [15]. Likewise, comparisons between human HepG2, mouse Hepa1c1c7, and rat H4IIE hepatoma cells report <8% of differentially expressed orthologs were conserved across all three species, with examples of divergent regulation (e.g., ortholog induced in one species but repressed in another) in the presence of conserved “AhR battery” gene responses (e.g., *Cyp1a1*, *Tiparp*, *Nqo1*, *Ugt1a6*) [17–19]. However, divergent gene expression between hepatoma cell lines and their relevance to human toxicity is rightly scrutinized due to model differences from inherent mutations, genetic instability, and clonal selection under differing culture conditions. Yet, *in vivo* studies also report minimal overlap (15%) between TCDD-responsive orthologs when comparing C57BL/6 mouse and Sprague Dawley rat liver gene expression datasets [20,21]. Similarly, studies which integrate comparative gene expression analysis with complementary phenotypic measurements report species-specific changes in metabolites, serum biochemistry, and histopathology [16,22–25]. Although the AhR and its signaling pathway are highly conserved, these studies suggest AhR-mediated gene expression patterns are species-specific, which may account for differences in sensitivity between species.

Beyond Gene Expression – An Integrated Systems Approach

A) Dysregulation of Iron Homeostasis and Heme Metabolism

In addition to facilitating comparative analyses, toxicogenomic studies can be used to further elucidate mechanisms of toxicity, which is particularly effective when integrated with complementary clinical biochemistry, metabolomics analysis, AhR enrichment (i.e., ChIP-chip, ChIP-Seq), and histopathology. This approach has been used to provide mechanistic insight into previously reported yet poorly understood adverse responses elicited by TCDD and related compounds. For example, Goldstein *et al* (1973) identified a 60% increase in the hepatic iron levels of TCDD-treated mice [26]. Later, al-Turk *et al* (1988) reported iron-supplementation in the diet increased TCDD-elicited lipid peroxidation while induced iron deficiency was protective [27]. More recently, duodenal epithelial and hepatic RNA-Seq analyses identified TCDD-elicited changes in gene expression consistent with iron overloading. Specifically, TCDD caused the dose-dependent repression of hepcidin, the master regulator of systemic iron homeostasis, resulting in a 2.6-fold increase in serum iron with accumulating levels spilling into urine [28]. Iron lies at the cross-section of multiple pathways associated with TCDD-elicited toxicity. For example, iron not only catalyzes the production of reactive oxygen species (ROS) via the Haber-Weiss reaction, but is also required for the biosynthesis of heme, a potentially toxic porphyrin-based cofactor required for hemoprotein assembly and function.

TCDD increases hepatic levels of free heme (oxidized version of heme), consistent with the repression of several highly-expressed cytochrome P450 subfamilies (2c, 2d, 2e, 2j, and 3a) and the induction of aminolevulinic synthase 1 (*Alas1*), the rate limiting step in heme biosynthesis. Free heme not only oxidizes biological macromolecules including proteins, DNA, and lipids, but also contributes to inflammation. Concomitantly, TCDD represses downstream enzymes involved in heme biosynthesis including uroporphyrinogen decarboxylase (*Urod*), which catalyzes the conversion of uroporphyrinogen III to coproporphyrinogen III [28]. Interestingly, human porphyria cutanea tarda (PCT), a photosensitive skin disease involving the overproduction of hepatic porphyrins, is the result of insufficient UROD activity. In the presence of iron and limited UROD activity, CYP1A2 oxidizes uroporphyrinogen III to uroporphomethene, a potent UROD inhibitor, and finally, uroporphyrin III, a urinary metabolite identified in rodent models of porphyria [29,30]. Uroporphyrin III is not a substrate for heme synthesis and thus accumulates in the liver, skin, and urine [31]. Accordingly, TCDD increases total urinary porphyrin levels with collected urine exhibiting a dark tea color indicative of PCT [28]. Fe deficiency has been shown to completely protect against TCDD-elicited porphyria in mice [32,33] while Fe supplementation exacerbates toxicity [34]. In humans, exposure to the weak AhR agonist hexachlorobenzene (HCB) induces PCT [35], while evidence of an association between TCDD and porphyria is inconclusive [36,37].

Beyond its role as a cofactor for hemoproteins, heme also serves as a prosthetic group for heme regulatory motif (HRM)-containing proteins such as BACH1. TCDD-elicited heme accumulation promotes the induction of heme oxygenase 1 (*Hmox1*) through the release of BACH1-mediated repression [28]. Although *Hmox1*-catalyzed catabolism of heme to bilirubin, carbon monoxide (CO), and iron reduces free heme-elicited toxicity, this reaction contributes to the free iron pool and thus promotes TCDD-elicited oxidative stress. Furthermore, the binding of heme to the REV-ERB α/β transcriptional repressors leads to dysregulation of genes associated with the circadian regulation of hepatic fatty acid biosynthesis and lipid metabolism [28]. These results are consistent with accumulating evidence that AhR activation regulates peripheral circadian clocks, resulting in dysregulation of glucose and lipid metabolism [38,39].

B) Indirect Modulation of Signaling Pathways

A more global examination of TCDD-elicited responses has begun to provide insight into the complex interactions between signaling pathways beyond the initial activation of the AhR. For example, TCDD-elicited disruption of iron homeostasis increases production of several signaling molecules including ROS, heme, bilirubin, and CO. TCDD also directly and indirectly affects other signaling pathways through the modulation of growth factors and their receptors, the metabolism of fatty acids, amino acids, and bile acids, and the activation of secondary messenger cascades. Direct effects include the induction of *Tnfa*, *Fgf21*, and *Tgf β* . Indirect effects include altering the composition and continuous remodeling of the extracellular matrix, which not only serves as a structural scaffold but also as a growth factor reservoir [40,41]. Induction of *Cyp1a1*, *1a2*, and *1b1* in the presence of hepatic lipid accumulation facilitates the production of eicosanoids that modulate inflammation, angiogenesis, proliferation, and invasion, while altered lipid metabolism may affect ligand

production for PPAR and LXR [42]. Likewise, TCDD-elicited alterations in the bacterial composition of the gut microbiome affects nutrient absorption and bile acid metabolism, leading to qualitative and quantitative changes in FXR ligand availability and the biosynthesis of hepatotoxic bile acid species [43,44]. In addition, tryptophan-2,3-dioxygenase (TDO)-mediated catabolism of tryptophan yields kynurenine, an endogenous ligand of human AhR that has been associated with immune response suppression and tumor-cell survival [45].

C) Compensatory Metabolic Reprogramming

Not all AhR-mediated responses are associated with an adverse effect. Treatment with TCDD also elicits antioxidant responses including the induction of the AhR-Nrf2 gene battery [46]. More recently, it has been shown that TCDD promotes metabolic reprogramming whereby central carbon metabolism, amino acid metabolism, and the pentose phosphate pathway are redirected to support ROS defenses [47]. An important driving force in this metabolic reprogramming is the dose-dependent induction of pyruvate kinase isoform M2 (*Pkm2*), which lies at the intersection of cell survival and cell proliferation. PKM2 exhibits lower catalytic activity compared to the M1 isoform (PKM1), resulting in decreased glycolytic flux. As a result, accumulating upstream glycolytic intermediates are redirected toward the pentose phosphate pathway and serine/folate biosynthesis, two important NADPH producing pathways required to support glutathione (GSH) biosynthesis and recycling. To compensate for the reduced glycolytic flux, TCDD-treated hepatocytes increase glutaminolysis, thereby ensuring sufficient levels of the intermediates required to maintain the TCA cycle and ATP production. TCDD-elicited induction of glutamine and cysteine transporters, together with increased glycine biosynthesis, also ensures that NADPH and required amino acid precursors are available for GSH biosynthesis. This metabolic reprogramming resembles the Warburg effect typically observed during carcinogenesis and may represent a novel advantageous defense mechanism to increase antioxidant capacity in normal differentiated hepatocytes in response to toxic insults [47].

Concluding Remarks

It is clear that the toxicity of TCDD and related compounds is dependent on AhR activation, which correlates with binding affinity and ligand stability. Beyond the AhR, TCDD and related compounds induce a complex concoction of signaling molecules which can modulate gene expression, protein levels, and enzyme activities, thereby affecting metabolic pathway flux and substrate/product levels. However, specific needs of exposed cells or tissues can often be satisfied through metabolic reprogramming to provide required intermediates. Resolving the significance of any one perturbation is confounded by cross-talk between several adapting signaling pathways that elicit unpredictable outcomes in response to the integration of multiple signal inputs. These adaptive strategies are reminiscent of metabolic changes observed in cancer cells used to ensure survival, support proliferation, and evade host defenses [48]. It is this ability of different signaling pathways to cross-talk, together with the intra- and inter-cellular tug-of-war between adverse and defensive responses, that may be the basis for the spectrum of species-, sex-, age-, tissue-, and cell-specific effects

elicited by TCDD and related compounds. Metabolic pathways and metabolite status may differ between species and sub-populations due to differences in genetic background, sex, age, overall health, nutritional status, and environment. As a result, the cell or tissue may already be primed for toxicity, requiring one additional hit or prolonged exposure to exhaust defensive responses and cause an adverse outcome. For instance, steatosis due to excessive alcohol consumption or a high fat diet may prime the liver for TCDD-induced lipid peroxidation and tissue damage. Alternatively, compromised antioxidant responses or NADPH deficiency may impair the ability of a cell or tissue to mount an effective defense. Given the number of potential metabolic interactions, it is feasible that the status of one or more pathways could attenuate or exacerbate toxicity in a specific cell, tissue, sub-population, or species. Consequently, the toxicity of TCDD and related compounds is not the result of a single mechanism of action, but due to the overall burden of multiple compromised pathways, which overwhelm the capacity to mount sufficient counter responses (Figure 2). It is likely that this tug-of-war between adverse effects and defensive responses is not unique to TCDD and related compounds. Furthermore, cell survival may not always be in the best interest of the organism, as proliferation of surviving mutated cells contributes to tumorigenic potential. As seen with the renaissance of cancer metabolism, the resurgence of biochemical toxicology within the context of ‘omics’ technologies will significantly advance our understanding of both toxicity and cell survival.

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HIGHLIGHTS

Despite AhR conservation, TCDD-elicited gene expression networks are species-specific

AhR-mediated hepatotoxicity involves reprogramming of several metabolic pathways

Dysregulation of iron/heme homeostasis and signaling cascades promote hepatotoxicity

Pkm2-driven metabolic reprogramming supports antioxidant defense responses

The value of applying ‘omics’ technologies to biochemical toxicology is highlighted

Figure 1.

Despite the conservation of the aryl hydrocarbon receptor and its signaling pathway, TCDD-elicited gene expression patterns are largely species-specific with minimal overlap between responsive orthologs (i.e., AhR gene battery). Species-specific target genes and the resulting toxic responses likely account for the reported differences in sensitivity and adverse effects between species.

Figure 2.

Aryl hydrocarbon receptor-mediated hepatotoxicity is a cumulative response to the overall burden of multiple disrupted pathways rather than a single adverse effect. Metabolic reprogramming redirects the pentose phosphate pathway and serine biosynthesis toward NADPH production in support of antioxidant defense, creating a tug-of-war between toxic responses and defensive mechanisms. Red represents factors or adverse effects which promote toxicity, while green represents defensive mechanisms.