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Dioxins: diagnostic and prognostic challenges arising from complex mechanisms

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Abstract

Dioxins are ubiquitous environmental challenges to humans, with a pervasiveness that arises from two hundred years of rapid industrialization and mechanization of Western societies and which is now extending into the developing world. Despite their penetrance of the human biota, these compounds are poorly understood in terms of their true physiological potential for harm, and the mechanisms by which they impact cellular and organ level function are only recently becoming clear. Emerging awareness that chronic exposures to toxins may have generational and subtle effects on the outcomes of diseases such as cancer and diabetes, which are already multifactorial and highly complex, creates the context for the current review paper. Here, we summarize dioxin exposure paradigms and the resulting physiological effects that have been documented in animals and humans. Novel insights into potential endogenous end exogenous ligands, as well as the mechanisms by which these ligands impact acute and chronic cellular processes, are discussed. We develop the idea that the diagnosis of dioxin exposure, the subtleties of the cellular effects of the compounds and prognosis of the long term effects of exposure are problems requiring that researchers leverage the power of genomics and epigenetics. However, the continuation of longitudinal epidemiological studies and development of a firmer basis from which to extrapolate animal studies will be critical in ensuring optimal insight from these resource-intensive techniques.

Keywords

Dioxin; TCDD; toxicology; aryl hydrocarbon receptor; Yushchenko; genomics

Dioxins: Structures and Toxicity

Dioxins and dioxin-like compounds comprise a group of polyhalogenated aromatic hydrocarbons which are major environmental contaminants and pollutants. This structurally diverse group contains polychlorinated dibenzodioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and some polychlorinated biphenyls (PCBs) (Sorg, *et al.*, 2009; Van Leeuwen, *et al.*, 2000). Dioxins and dioxin-like compounds are grouped together due to the

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similarity of their physical and chemical properties and their ability to elicit comparable toxicological responses (Schecter, *et al.*, 2006).

While all dioxins consist of two benzene rings connected by two oxygen atoms, they can contain between four to eight chlorines, generating considerable diversity (Figure 1). For example, 75 congeners of PCDD are known, of which 7 have demonstrable cellular toxicity. For both PCDD and PCDF series, toxicity appears limited to compounds with a minimum of four chlorine substitutions at the 2,3,7, and 8 positions (Linden, *et al.*, 2010; Schecter, *et al.*, 2006). While the structure-function relationship of the extensive dioxin chemical families tends to be defined using cellular or *in vivo* toxicity as the metric, this does not exclude the idea that other dioxins may have subtle cellular effects.

A normalized schedule of dioxin toxicity has been defined. Several criteria are required for a compound to be classified into this schedule; (a) a structural relationship to the PCDD and PCDF families; (b) binding to the AHR; (c) eliciting of AHR-mediated biochemical and toxic responses, and (d) environmental persistence and accumulation in the food chain. Based on these characteristics, each qualifying compound is assigned a Toxic Equivalency Factor (TEF). Because the toxicological processes of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are well-studied and considered the most toxic member of the group, it has been assigned a TEF value of 1. All dioxins and dioxin-like compounds are compared to TCDD accordingly, with TEF values ranging from .00001 to 1. These values have been adopted by the Environmental Protection Agency (EPA) and are used routinely to assess risk and exposure levels. In order to gauge toxic dioxin levels in a specimen, the TEF of each dioxin present is multiplied by its respective mass concentration and all values are then added together to create the toxicity equivalence (TEQ). In 1998, in accordance with this system, the EPA determined background levels of dioxins in the U.S. to be 10–30 pg I-TEQ/g lipid in humans. The numbers vary geographically, but the bulk of the population in areas which are even somewhat industrialized will have some level of dioxin exposure. Residents living in less industrialized areas such as Cambodia, Russia and Siberia, and northern Vietnam have detectable background levels of PCDD/PCDF (Schecter, *et al.*, 2006).

Human Exposure to Dioxins

Environmental exposures

The EPA cites five main sources of dioxin contamination into the environment: combustion; metal smelting, processing, and refining; chemical manufacturing; natural sources and processes; and reservoirs such as soil or sediment. Thus dioxin exposure is positively correlated with, but not exclusive to, residence in an industrialized area. Dioxins are released into the air as a product of various industrial processes and accumulate on the surrounding plants and enter the soil and water supply. Subsequently, people are exposed to dioxins through the consumption of commercially-made animal products. Foods with a high fat content, primarily meat and dairy, are particularly important courses of dioxin introduction, and in humans as in other animals, fatty tissues are primary residues of the compounds as they bioaccumulate (WHO, 1998)(Figure 2).

Studies of the U.S. population examined various exposure routes to dioxins, finding that the dose estimates from food sources were significantly higher than other routes, including inhalation and soil and water sources. Food sources accounted for 93% of dioxin levels, which supports the WHO's assertion that 90% of human exposure to dioxins is through their diet (Lorber, *et al.*, 2009). A reassessment conducted by the EPA from 2004–2006 demonstrated that for dioxin-like compounds (17 PCDD/PCDF and 3 PCB), the average background body burden in the United States was 21.7pg TEQ/g lipid weight, while the average daily intake of these compounds was 40.6pg TEQ/day (Lorber, *et al.*, 2009). Taken

together, these data support the assertion that dioxin exposure is indeed constant and ubiquitous in the U.S., spurring significant efforts to understand the public health implications of this environmental challenge.

Poisoning

While chronic, low level, exposure to dioxins is the most usual modality in humans, there are a few documented incidents where individuals or small groups have been exposed to marked levels of dioxins over an acute time period. These poisonings may be informative because they represent the best indications we have of the ultimate outcome of the more common long term cumulative low-dose exposures, although this comparison is imperfect. No acutely lethal dose in humans has ever been established but there are reported instances of acute high level exposures, reviewed below.

Victor Yushchenko was allegedly poisoned with TCDD during his presidential campaign in the Ukraine (September, 2004). Initial symptoms were diagnosed as pancreatitis within 24h, but within 3 weeks his face began to show signs of disfigurement, bloating, and severe pock marking - symptoms commonly associated with chloracne. Three months later, serum samples were analyzed for dioxins and dioxin-like compounds. Only the concentration of TCDD was significantly higher in the samples than in levels observed in the general population, so the analysis focused solely on the presence of this compound (Sorg, *et al.*, 2009). The amount of TCDD present in Yushchenko's blood serum was 108,000 pg/g lipid weight, which is greater than 50,000 times normal levels generally observed in the population (Schecter, *et al.*, 2006; Sorg, *et al.*, 2009). Perhaps because of the high-profile nature of the case, this patient's symptoms have received considerable attention. Currently, this massive dioxin dose appears to have manifested primarily as chloracne, but information on other health effects is not available.

In October 1997, five workers at a textile plant in Vienna, Austria were allegedly poisoned with TCDD (White, *et al.*, 2009). This incident resulted in the highest TCDD level ever reported in humans; 144000 pg/g blood lipid in one female. TCDD levels in the other victims were 26000 pg/g, 856 pg/g, 149 pg/g and 93pg/g blood lipid. Only the two individuals with the highest levels displayed measurable effects of dioxin exposure; the others were asymptomatic (Geusau, *et al.*, 2001). Initially, the primary patient suffered from nausea and vomiting, anemia and amenorrhea. Again, chloracne was the most severe symptom exhibited by the primary victim and manifested in the second victim (Geusau, *et al.*, 2001). Both individuals offered an opportunity for long-term follow up of the consequences of massive dioxin exposure. However, it should be noted that both patients were treated with olestra in an attempt to control the chloracne (in one patient this completely resolved within 2 years) and their TCDD levels have decreased over time. By October 2000, the primary patient had a lowered TCDD concentration of 30,300 pg/g blood lipid (Geusau, *et al.*, 2001).

Both of the above examples focus on a minute population sample. In contrast, a 1976 explosion at a chemical plant near Seveso, Italy released the TCDD intermediate 2,4,5-trichlorophenol into the atmosphere. Seveso and other similar populations, such as U.S. servicemen exposed to herbicides contaminated with dioxin during deforestation operations of the Vietnam War (Operation Ranch Hand), has been studied longitudinally (Cerlesi, *et al.*, 1989; Cypel, *et al.*, 2010; Mocarelli, *et al.*, 1986; Pesatori, *et al.*, 2003). It has proven difficult to establish clear epidemiology of disease based on these populations, and there is little evidence for short term increases in disease/mortality. However, as these longitudinal studies approach several decades, new data about disease linkages, especially to cancers, continue to emerge.

Thousands of exposed individuals in Seveso were studied based on proximity to the plant. Regions surrounding the facility were divided into Zones A (closest to the facility, median immediate TCDD measurement 447 pg/g serum), B, and R (farthest from the facility, median intermediate TCDD measurement 48 pg/g serum), based predominantly on soil dioxin levels and vegetation and animal death in the area (Needham, *et al.*, 1997). A reference zone, Zone non-ABR, was also established.

Twenty-five years following the Seveso incident, a mortality study was conducted on individuals affected by the explosion (n=278,108) and compared to a reference population (Consonni, *et al.*, 2008). The twenty-five year period since exposure was divided into several time intervals, for which a mortality analysis was conducted; 0–4yr, 5–9yr, 10–14yr, 15–19yr, and ≥20yr. The data indicated that there was no increase in all-cause and all-cancer mortality over this extended time period. However, studies conducted at the fifteen-year and twenty-year benchmarks did suggest higher propensities towards particular conditions. At 15 years there was a rise in incidence of non-Hodgkin's lymphoma among Seveso residents, and women residing in Zone A showed significant increase in mortality due to digestive system cancers. In Zone B, women had increased mortality due to stomach cancer and multiple myeloma and men had increased mortality due to rectal cancer and leukemia. Both men and women showed increased mortality from Hodgkin's disease. Despite the prevalence of some of these conditions, no overall increase in all-cancer mortality was found at this particular time (Bertazzi, *et al.*, 1997). At 20 years, there was a non-significant increase in various types of digestive cancers in Zone A. Significant increases in mortality were at this time point derived from two fatal cases of non-Hodgkin's lymphoma and a number of deaths related to COPD. Despite the increases in mortality due to these specified causes, there was no overall increase in all-cause or all-cancer mortality within this population (Consonni, *et al.*, 2008).

A third major population studied longitudinally after intense dioxin exposure consists of Vietnam War veterans who used Agent Orange during combat. Agent Orange is a 1:1 mixture of the herbicides 2,4,5-T and 2.4-D which is highly contaminated with TCDD, thus the effects of dioxins are not being studied in isolation in this population. The effects of the herbicides have often been minimized in discussions of pathology because of their rapid breakdown, however, our growing understanding of epigenetic effects may suggest that this assumption needs to be revisited. In a 2010 review, all-cause mortality and cause-specific mortality were non-significantly higher in the Army Chemical Corps Veterans stationed in Vietnam compared to veterans who did not serve in Southeast Asia. However, there was a significant increase in mortality for the Vietnam cohort from respiratory cancer and non-malignant respiratory disease, including COPD (Cypel, *et al.*, 2010). Additionally, an increase in risk for diabetes, heart disease, hypertension, and chronic respiratory diseases were found in those who sprayed Agent Orange. Although not statistically significant, there was increased mortality due to all types of cancer combined and circulatory, respiratory, and digestive disease when compared to the group of individuals who did not deploy to Vietnam (Cypel, *et al.*, 2010).

Taken together, these studies indicate that all-cause mortality in humans is not strikingly affected by significant dioxin exposures. This is a deceptive oversimplification, since when the symptomatology of exposure is examined, there are clearly a spectrum of health effects that affect quality of life and long term healthcare needs of exposed populations. The classification of TCDD as a group 1 carcinogen has been controversial, but as data continue to emerge from longitudinal studies, novel linkages are emerging. Across the dioxin literature, human exposure to dioxins and dioxin-like compounds has been associated with the following: hepatocellular damage, thymic involution, immune suppression, chloracne, epithelial hyperplasia, teratogenesis, gastrointestinal disruption, increased diabetes risk,

respiratory effects including reduced forced expiratory volume, reproductive hormone disruption, thyroxine and thyroid stimulating hormone suppression, poly-neuropathies and motor skill disruption, tumor promotion and induction of numerous detoxification enzymes including cytochrome P4501A1 (CYP1A1) (Long, *et al.*, 2003; Ovando, *et al.*, 2010; Stevens, *et al.*, 2009). With this spectrum of effects in mind, the obvious experimental step is to examine severity, dose relationship and mechanism of action pertaining to dioxins in model and sentinel organisms. Figure 3 summarizes the outcomes of dioxin-associated pathologies in humans and animals.

Animal exposure to dioxins

LD_{50} have been established in a variety of sentinel and model animal systems. Obviously, there is a more extensive literature that causally links experimental dioxin exposure to symptoms and pathology in animals than in humans. The most common toxicological manifestation observed in animals is the development of wasting syndrome, a condition presenting severe weight loss and retarded metabolism (Schecter, *et al.*, 2006). The duration of wasting syndrome before death is between 2 and 4 weeks in rodents and 6–8 weeks in non-human primates (Van Den Berg, *et al.*, 2000). There are numerous animal studies that support a direct link between oncogenesis and dioxins, but the provision of mechanistic explanations for this link are limited. There is an extensive list of other severe manifestations of dioxin poisoning in various species. Briefly, hyperplasia of the liver and stomach, altered cardiac function, enhanced metabolism, decreased blood insulin and glucose levels, and loss of sebaceous glands have been reported in multiple species (Van Den Berg, *et al.*, 2000). Of particular note are studies showing that constitutive activation of the AHR, modeling chronic ligand availability that would be seem in multi-year exposures, cause hepatocellular carcinomas and stomach tumors (Andersson, *et al.*, 2002; Moennikes, *et al.*, 2004). Extrapolation of these studies to humans would need to address a large number of questions, ranging from dose equivalencies, delivery modality, AHR receptor conservation and differential affinity for ligands, species-specific absorption, distribution, metabolism and extraction (ADME), matching of the specific congeners of dioxin that are administered and secondary metabolism comparisons across systems. The importance of these animal studies as predictive of effects in humans may be somewhat limited, but their importance is uncovering the cellular and molecular mechanisms of dioxin effects cannot be underestimated.

Cellular targets of dioxin

While much progress has been made in defining the cellular targets of TCDD, these data do not yet extend to a clear picture of how toxicity is caused. Since AHR is the major cellular target of TCDD (Beischlag, *et al.*, 2008; Poland, *et al.*, 1982), it is assumed that this receptor mediates most or all of the toxicity associated with TCDD. While this argument does not provide for possible non-AHR effects of secondary metabolites, AHR is unarguably an excellent starting point in the search for mechanism.

AHR itself is a somewhat enigmatic protein. It is ancient, with homologs in invertebrates and insects, although interestingly these do not bind dioxin or other known ligands of the mammalian AHR. The AHR complex (AHRC) consists of a dimer of AHR and AHR nuclear translocator protein (ARNT). These two proteins of each species in human and mouse are approximately 20% identical in amino acid sequence (Hankinson, 1995). Both proteins contain basic helix-loop-helix (bHLH) motifs near their amino termini. These motifs are also found in other transcription factors and function in protein dimerization and DNA binding. An important characteristic of the AHR is that it contains a segment of homology that is also found in regulatory proteins of *Drosophila melanogaster*, PER and

SIM, called the PAS domain (Hankinson, 1995; Swanson, *et al.*, 1993). The PAS domain contains two copies of approximately 50-amino acid degenerate direct repeats, referred to as PAS A and PAS B repeats (Hankinson, 1995). It is poorly conserved and, given its size and diversity in sequence, it can mediate a number of diverse biochemical functions (Pocar, *et al.*, 2005). Unlike other bHLH proteins, AHR activity is dependent upon binding of a ligand (Hankinson, 1995). Upon ligand binding, the cytosolic AHR is translocated to the nucleus, where it dimerizes with the ARNT and interacts with the xenobiotic-responsive element (XRE) in gene promoter regions. The AHR is therefore the only known ligand-activated transcription factor member of the basic helix-loop-helix Per/ARNT/Sim family (Dinatale, *et al.*, 2010; Hankinson, 1995).

If we reason that within the biology of AHR lie the molecular explanations for the dioxin-associated toxicity, then two issues become paramount. First, we need to understand the endogenous activators of AHR, since one path to toxicity may be that dioxin binding to AHR competitively disrupts the physiological signaling of these upstream regulators. Alternatively, the high affinity and slow off rate for TCDD binding to AHR suggest that, in fact, persistence of signaling maybe a contributing factor to pathology (Geyer, *et al.*, 2002; Rifkind, 2006). Second, we need to understand the downstream targets of AHR, since it is likely that inappropriate activation or suppression of AHR targets by following dioxin ligation of the receptor will drive the pathological effects of the ligand.

Endogenous and novel exogenous ligands for AHR

Though the AHR is known as the dioxin receptor (Beischlag, *et al.*, 2008), it is also important to consider that non-toxic dioxins and other exo- or endogenous AHR ligands may have important health effects. Several potential endogenous ligands have been suggested. These include indigoids (Pocar, *et al.*, 2005), indirubin, bilirubin (Sinal, *et al.*, 1997), tryptophan metabolites and indole derivatives which are structurally similar to xenobiotic ligands and able to activate AHR signaling (Dinatale, *et al.*, 2010; Stevens, *et al.*, 2009). Other endogenous ligands such as 7-ketocholesterol have also been suggested (Savouret, *et al.*, 2001), and while low affinity, these ligands may nevertheless be highly significant endogenously. As tryptophan is a precursor in the synthesis of certain AHR ligands, DiNatale et al. examined products generated by the indoleamine-2,3-dioxygenase (IDO) pathway. These tryptophan derivatives, such as kynurenine and kynurenic acid, have regulated synthesis and demonstrate physiological activity, which corresponds with AHR-dependent signaling (Dinatale, *et al.*, 2010; Mezrich, *et al.*, 2010). Intriguingly, there are links between the IDO pathway and T cell proliferation and tolerance (Vogel, *et al.*, 2008), suggesting a potential mechanism for the immunological affects of dioxins that merits further study.

AHR ligand structures are very diverse, including plant flavonoids, indoles, tetrapyroles, arachidonic acid, and toxic or non-toxic PCDDs, PCDFs and PCBs (Long, *et al.*, 2003). There may be numerous, as yet undescribed, AHR ligands. New studies from one of our groups (H. Frokiaer and KMU) are exploring natural products derived from the marine bacterium *Pseudoalteromonas luteoviolacea* as potential AHR ligands. These include violacein and indolmycin (Mansson, *et al.*, 2010). Our data suggest that while violacein is somewhat cytotoxic, indolmycin has some features that are consistent with AHR ligand activity (*Cyp1A1* induction and the causation of autocrine AHR downregulation). Tryptophan is believed to be a precursor for the synthesis of these two compounds and violacein is characterized as a bisindole, generated by the fusion of two L-tryptophan molecules (Ryan, *et al.*, 2008; Ryan, *et al.*, 2009).

Without a full understanding of the activation process for AHR under physiological conditions, it will continue to be a challenge to extrapolate to potential mechanisms of action

for dioxins. Moreover, the mechanisms of toxicity and the search for an endogenous ligand and physiological context of AHR may be more linked than previously thought. One of the effects of chronic exposure to dioxins appear to be chronic AHR down-regulation. This has been demonstrated in laboratory animals (Mimura, *et al.*, 1999; Pollenz, 2002) and in patients from the Seveso incident (Landi, *et al.*, 2005). Here, after a transient increase, transcript and protein levels for AHR have declined steadily and correlate with the decline in blood TCDD concentrations. A key question here is of course whether these levels are declining below pre-exposure levels. However, both in the transient upregulation and chronic downregulation, it seems clear that altered levels of AHR will affect the endogenous signaling pathways in which this transcription factor participates. Thus, within both perturbation of endogenous signaling, and induction of toxin-specific novel pathways, we might find pathology-causing effects of dioxin exposure.

To elucidate the physiological role and developmental importance of AHR, several laboratories have used *Ahr* null mice and found marked phenotypes. These included inability to upregulate the metabolic enzymes of detoxification, resistance to most aspects of dioxin toxicity, smaller livers, abnormal vasculature in the liver, kidney and eye (Fernandez-Salguero, *et al.*, 1995; Lahvis, *et al.*, 2000; Schmidt, *et al.*, 1996; Stevens, *et al.*, 2009). The AHR is also involved in reproduction (Pocar, *et al.*, 2005) and emerging data suggest that AHR is important in inflammation (Casado, *et al.*, 2010). Recently, it has been shown that AHR signaling may play a role in differentiation of regulatory T-cells (Tregs) or TH17 cells, depending on the ligand to AHR (Quintana, *et al.*, 2008). Tregs have been shown to suppress effector cell proliferation and cytokine secretion, in addition to reduce autoimmune and allergic disease, limit the immune response in infectious disease, and inhibit anti-tumour immune responses (Mottet, *et al.*, 2007). These data create the intriguing possibility that dioxin and non-toxic AHR ligands may actually cause beneficial immune modulations. Recent data from our laboratory suggest that in addition to T and B cells, mast cells are affected by exposure to AHR ligands. These pro-inflammatory immunocytes mediate tissue-based inflammatory responses. We assessed known AHR ligands (kynurenine and resveratrol) (Beedanagari, *et al.*, 2010; Mezrich, *et al.*, 2010) as well as IDO-products and putative marine bacterial AHR ligands (violacein, indolmycin) for their effects on multiple parameters of mast cell activation. The effects of these ligands are complex, but variously they altered outcomes of antigen-mediated and basal mast cell activity, including degranulation, IL-6 production and calcium signaling (KMU and HT, manuscript in review). The immunoactivity of AHR ligands creates a clear need for animal studies, and meta-analysis of existing epidemiological data sets, to establish whether there are links between acute or chronic dioxin exposure and immunological outcomes such as susceptibility to infection, autoimmune disease, allergy and cancer. Given the roles of AHR in diverse immunological responses, the possibility exists that exogenous ligands (i.e. resveratrol and even dioxins) could at some level promote beneficial immune responses.

Physiology of AHR targets

The broad tissue distribution of AHR (Hankinson, 1995) suggests that this receptor has utility in multiple cell types and that its role is not only limited to that of the toxicological importance. Besides dimerization with ARNT, the AHR may also bind to other transcription factors to regulate gene expression (Vogel, *et al.*, 2009). This can be seen with the estrogen receptor (Swedenborg, *et al.*, 2008), where most AHR-ER crosstalk leads to anti-estrogenic effects (Ohtake, *et al.*, 2003). Some AHR ligands may also exhibit weak estrogenic activity (Ohtake, *et al.*, 2003; Shanle, *et al.*, 2010). Through physical interaction between the AHR and the estrogen receptor □(ER □), the AHR affects estrogen signalling and the activated AHR can thereby inhibit many ER-dependent responses (Hankinson, 1995; Macpherson, *et al.*, 2010). This can lead to altered levels of thyroxin (Vanbirgelen, *et al.*, 1995) and other

growth factors. Though AHR and ARNT do not constitute the primary response, this inhibition of estrogen actions is dependent on these transcription factors (Hankinson, 1995).

Significant efforts have been made to define the gene targets that are activated by dioxin binding to the AHR. The GEO database contains over 1,000 studies on transcriptional responses to dioxin exposure in experimental systems. As the endogenous ligand is unknown, the usual experimental procedure is to use the xenobiotic challenge of a dioxin or dioxin-like compound. These compounds initiate an adaptive response in the cells, which primarily leads to the upregulation of a group of genes that are involved in the hepatic enzymatic detoxification and metabolism of xenobiotics. These genes include Cyp1A1, Cyp1A2 and Cyp1B1 (phase 1 and phase 2 cytochrome P450 drug metabolizing enzymes, GST (glutathione-S-transferase), UDPGT (uridine diphosphate glucuronosyltransferase) and ALDH (aldehyde dehydrogenase) (Nebert, *et al.*, 1993; Ovando, *et al.*, 2010; Sutter, *et al.*, 1992; Telakowski-Hopkins, *et al.*, 1988; White, *et al.*, 2009). These are known as the 'AHR gene battery', and recent work describes that these are targets of AHR-dependent activation of Nrf2 (Yeager, *et al.*, 2009).

Outside purely detoxification pathways, further microarray studies have identified other AHR gene targets such as Cadps, Exoc3, Serpina7, Slc13a3 and Slc29a1 (trafficking and transport), Ceacam10 and Enpp2 (cell adhesion), Ptprd, Ptprn and Trib3 (cell signaling) and Enpp3 and Srd5a1 (development and differentiation) (Ovando, *et al.*, 2010). These genes are classified as part of the 'toxic response', reflecting the activation of pathways beyond purely cellular adaptation to the presence of a xenobiotic by upregulation of metabolic enzymes. As discussed below, these data are still an over-simplification, since microarray data posted in the GEO database indicates that at least hundreds of genes are AHR responsive in multiple cellular contexts. It is perhaps worth noting that approximately 10% of the GEO studies on dioxin were performed in human cells or tissues, but there are obviously no data from human exposures, accidental or otherwise. However, EWAS and GWAS studies from which associations between exposures to compounds such as TCDD and PCBs and human disease states may yet emerge (Patel, *et al.* 2010).

Dioxin toxicity may also be mediated through a 'non-genomic' pathway. This pathway is initiated by ligand activated AHR, but lacks the requirement for nuclear translocation or for ARNT (White, *et al.*, 2009). In the non-genomic pathway, it was found that TCDD exposure could increase intracellular Ca^{2+} elevations through an undefined mechanism. It seems premature to define this pathway as truly 'non-genomic', since calcium signaling regulates hundreds of transcriptional targets through calcium-dependent transcriptions factors such as NFAT and SRF (Mellstrom, *et al.*, 2008; Savignac, *et al.*, 2007). The downstream targets of calcium that are dioxin-responsive need to be defined. However, at least in cells such a pancreatic cells and myocytes, dioxin does induce extranuclear, calcium-dependent, functional processes such as insulin secretion (Kim, *et al.*, 2009), and myocyte contraction (Vasquez, *et al.*, 2003; Xie, *et al.*, 2006), respectively. Interestingly, our recent data suggest that while AHR ligand effects on acute (sec-min) intracellular free calcium responses in mast cells are minimal, chronic exposure to AHR ligands does alter the intensity of subsequent calcium responses to ionophores or ligation of the high affinity receptor for IgE, Fc RI (HT and KMU, unpublished data). In the pro-inflammatory mast cell, these alterations in responsiveness to antigenic stimuli would be predicted to have a direct effect on the intensity and duration of subsequent inflammatory responses, for example in Type IV hypersensitivity or anaphylaxis. Moreover, these data suggest that AHR ligand exposure is modifying the expression of genes that are involved in calcium signaling. The candidate targets for this effect are obviously numerous and include genes for calcium channels, transporters or their regulatory proteins.

There are also suggestions from the protein biochemistry of AHR that it may interface with cytosolic signaling networks. For example, AHR binds Cyclin D1, providing an obviously attractive set of hypotheses as to the potential for AHR ligands to dysregulate proliferation and differentiation. AHR also complexes with the ubiquitin ligase NEDD1, and may have its own intrinsic activity as an ubiquitin ligase (Ohtake, *et al.*, 2007; Ohtake, *et al.*, 2009), providing the potential for dioxin to interfere with proper protein degradation and ubiquitin-based signaling. Moreover, *in vivo* experiments showed that in rat hepatic membrane, one of the early stage actions of TCDD is to activate a protein kinase; *Src kinase.* Mice deficient in *Src* kinase were less susceptible to the classical wasting syndrome after TCDD exposure than their wild-type counterparts (Matsumura 2008). These data establish a rare insight into a possibly direct mechanism of dioxin toxicity.

Perspectives

Taken together, the cellular effects of dioxin must be the sum of the following: (a) non-genomic signaling via calcium and targets such as *Src*, through pathways that terminate in cellular effects outside transcription; (b) genomic signaling through calcium-dependent genes and genes targeted by signaling pathways in which AHR protein binding partners play a role, and (c) genomic signaling through genes that are targets of either AHR/ARNT or AHR partnered with other transcription factors. Clearly, our level of understanding of each of these pathways is incomplete at best. However, if we are to accurately diagnose and prognose dioxin effects, we must fully assemble the dioxin signaling network. Study of the endogenous ligands will continue to inform predictive efforts as to the effects of toxic ligands. Moreover, the degree to which the toxic effects are mediated through sequestration and perturbation of endogenous pathways, especially through chronic alterations in AHR levels, has yet to be understood. The induction of qualitatively or quantitatively new cellular events by dioxins may be occurring in parallel with a loss-of-function in endogenous pathways that are involved in immune surveillance and beneficial inflammations, with marked consequences for pathology.

Finding a robust and workable transcriptional or epigenetic fingerprint of dioxin exposure in humans is likely to be a critical step in both the diagnostic or prognostic process over the next decade. While current technologies put both of these in reach in terms of assay systems, the remaining challenges are to identify a valid comparison of datasets to compare human samples. For diagnostic purposes such a fingerprint would need to be extrapolated from animal studies, and be robustly related to dose and time of exposure. The latter is especially challenging, because years and decades of exposure may be the most relevant scenario for the bulk of human exposure cases. Moreover, in order to move beyond purely diagnostics work into prognosis, it is necessary to establish causal relationships between biomarkers of exposure and pathophysiological outcomes.

The decline in dioxin emissions in Western countries in recent years may weaken arguments for continuing research resource allocation, especially in fiscally challenging times. However, the population has already accumulated generations with significant lifetime exposure, and in developing economies exposure trend is increasing, not decreasing. Moreover, it seems likely that work on the enigmatic problems of the endogenous ligands and biological roles for AHR, will yield novel insights into the function of major physiological systems.

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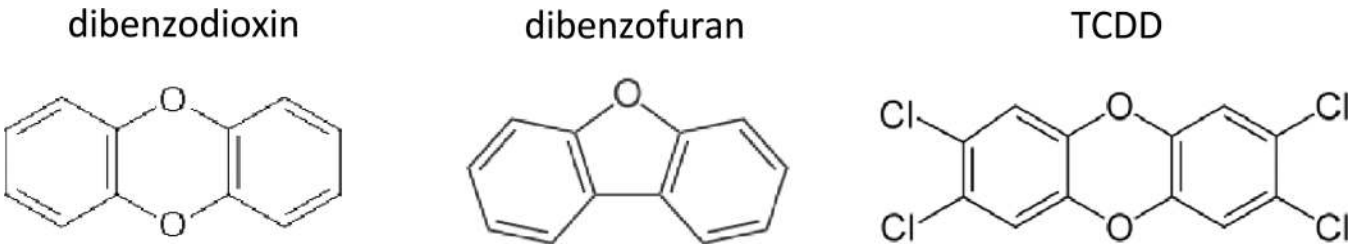

Figure 1.
Examples of different dioxins. Dibenzodioxin, dibenzofuran and 2,3,7,8-tetrachlorobenzo-p-dioxin (TCDD).

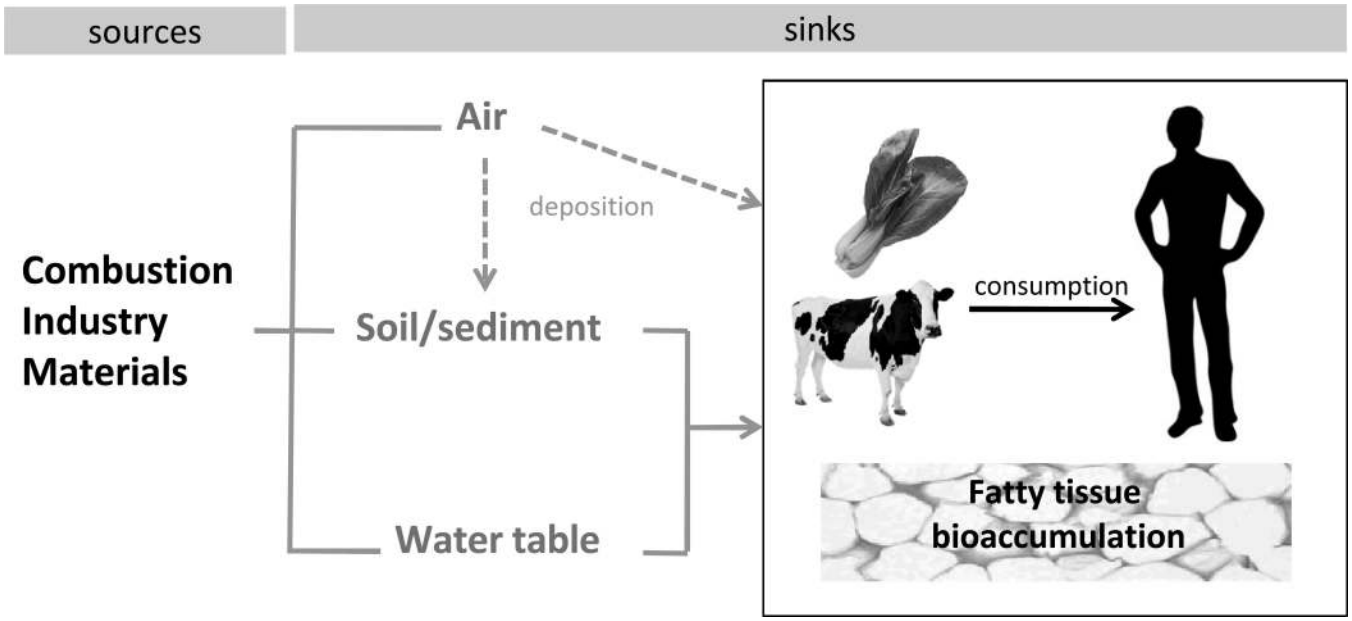

Figure 2.
Dioxin contamination into the environment may arise from sources including combustion, metal smelting and processing from refining industry materials. Dioxins are thereby released into to the air or soil as a product of these industrial processes and accumulate in the surrounding plants and enter the soil and water supply. Subsequently, people are exposed to dioxins through the consumption of commercially-made animal products. Especially foods with a high fat content are important courses of dioxin introduction, and in humans as in other animals, fatty tissues are primary residues of the compounds as they bioaccumulate.

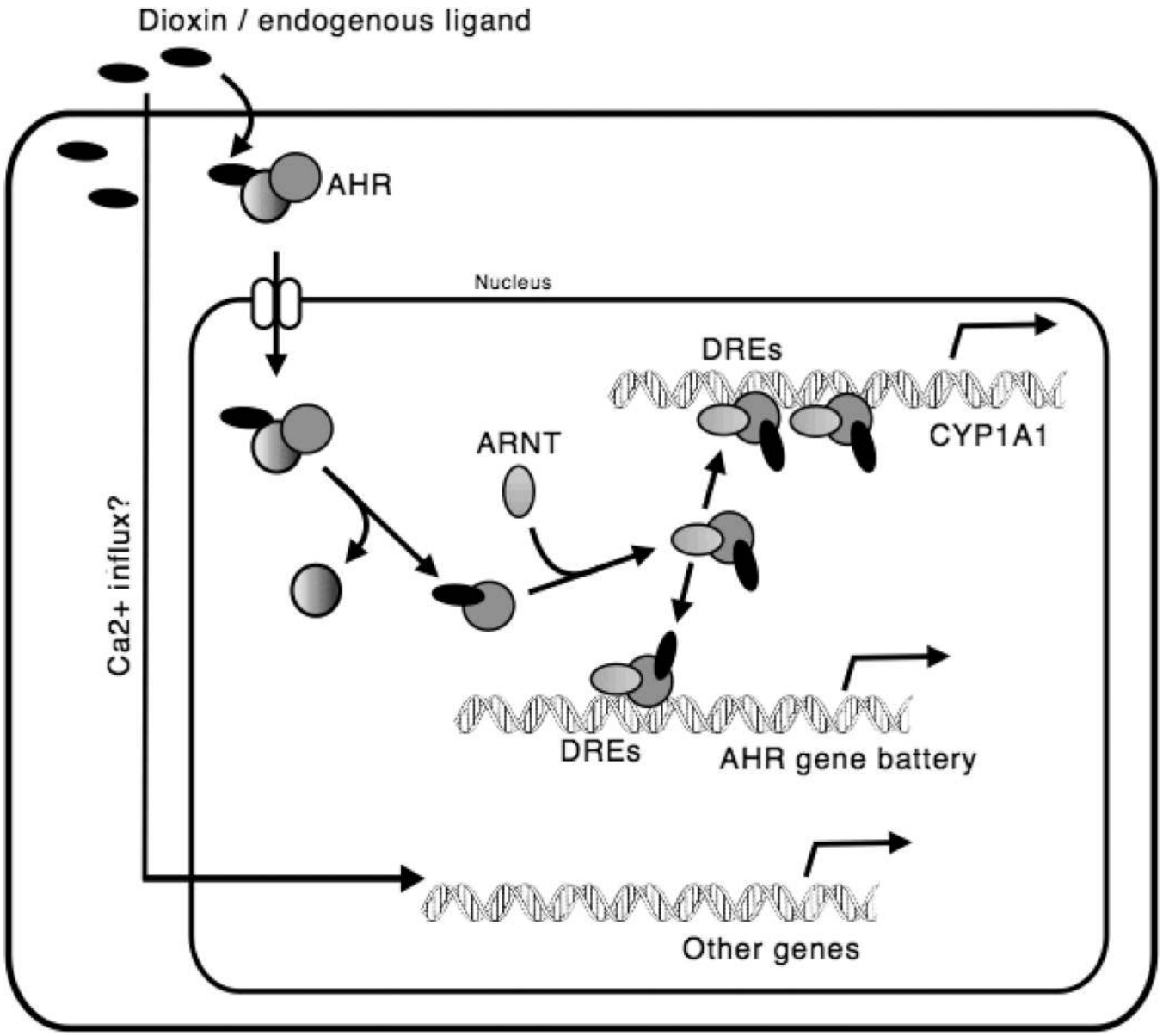

Figure 3.
Physiological effects from TCDD exposures in humans and animals.

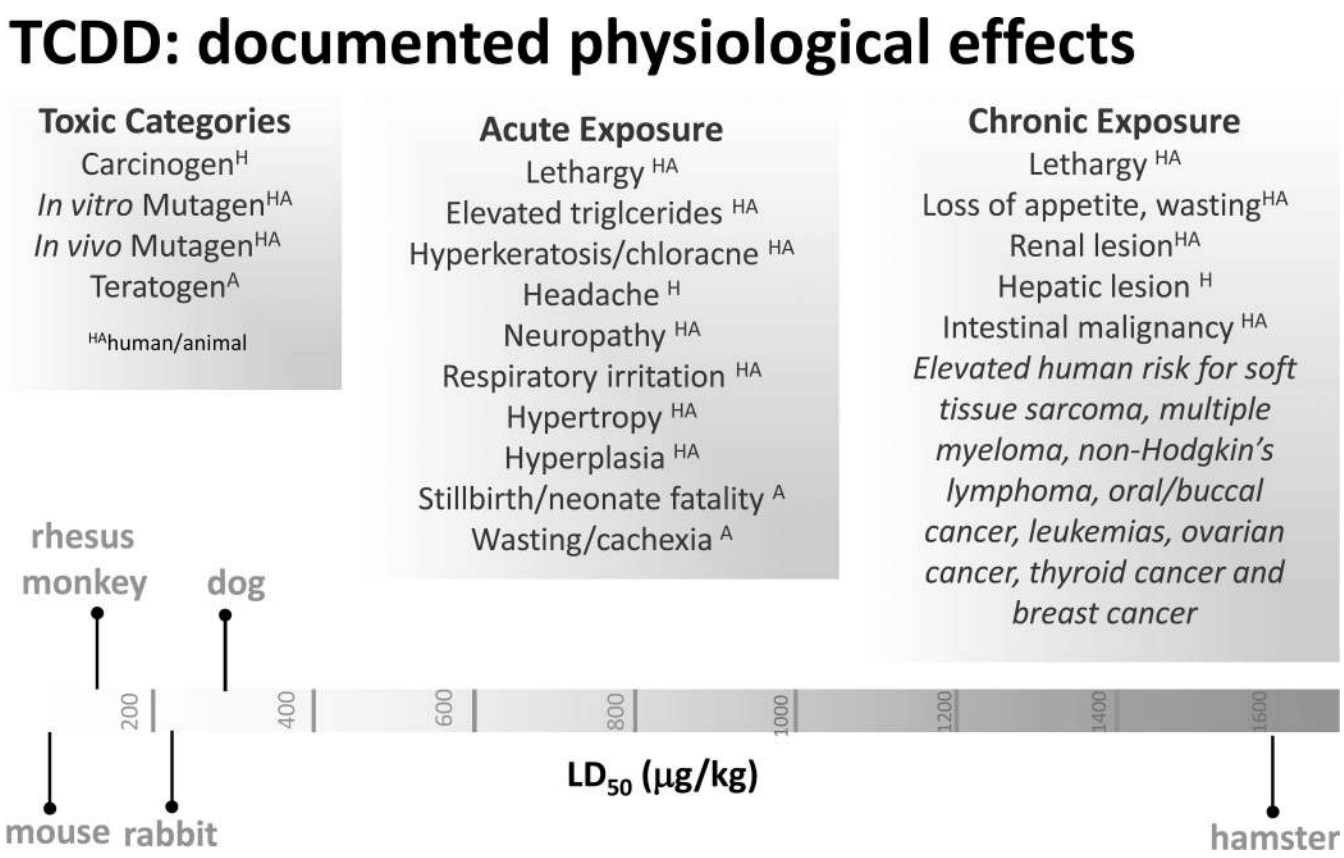

Figure 4.

The AHR signaling pathway. Dioxins or endogenous ligands bind to the AHR in the cytosol, where it is translocated to the nucleus and dimerizes with the ARNT and directs transcription from dioxin response elements (DREs). Alternatively, dioxin signaling can be mediated through a 'non-genomic' pathway. This pathway is initiated by ligand activated AHR, but lacks the requirement for nuclear translocation or ARNT. In this pathway, TCDD exposure can increase intracellular Ca^{2+} elevations through an undefined mechanism.