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FORUM

Perspectives on the Potential Involvement of the Ah Receptor-Dioxin Axis in Cardiovascular Disease

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The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that mediates the induction of the CYP1 family of cytochrome P450s and of several phase II detoxification enzymes. Although induction of these genes is the best characterized AHR function, it does not adequately explain the diversity of AHR-mediated effects. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) is the prototypical AHR ligand and dioxin congener and a model for many environmentally relevant organochlorinated compounds. Research over the course of the last 30 years has made it evident that AHR activation in response to TCDD and other xenobiotic agonists directly affects multiple metabolic pathways, leading to the identification of many AHR-directed effects of dioxin involved in regulation of growth factor signaling, cell cycle proliferation, differentiation, arrest, and apoptosis. There is ample evidence that TCDD causes persistent cardiac defects in zebrafish, chickens, mice, and likely humans and is associated with human cardiovascular disease. The question that I address here is whether exposure to TCDD during early development perturbs the concerted differentiation patterns of cardiovascular cell lineages and tissues and leads to cardiac malformations and long-term cardiovascular disease. Research to define the mechanisms responsible for the lifelong cardiovascular malformations resulting from TCDD exposure during embryonic development will be highly significant to the prevention of environmental cardiovascular injury.

Key Words: **Ah receptor; cardiovascular disease; NKX2.5; dioxin; development.**

The aryl hydrocarbon receptor (abbreviated hereafter as AHR, as per the Rules of Genetic Nomenclature of the International Committee on Standardized Genetic Nomenclature for Mice) mediates the teratogenic and carcinogenic effects of environmental planar aromatic hydrocarbons, among which the most potent xenobiotic agent, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), is the causative agent of chloracne, a long-lasting hyperkeratotic skin disease in humans (Zugerman, 1990). The AHR is a ligand-activated transcription factor that belongs to a protein family characterized by the presence of a basic helix-loop-helix/PER-ARNT-SIM (PAS) domain (Burbach *et al.*, 1992; Ema *et al.*, 1992). The unliganded AHR resides in a cytosolic protein complex with the 90-kDa heat shock protein hsp90, a p23 protein, and the immunophilin homolog, XAP2 (Carver *et al.*, 1994; Kazlauskas *et al.*, 1999; Perdew, 1988). Upon ligand-mediated activation, the complex translocates into the nucleus, where the partner proteins dissociate and the AHR dimerizes with the AH receptor nuclear translocator (ARNT), also a member of the PAS protein family (Reyes *et al.*, 1992). The AHR-ARNT heterodimer binds to the AHR, dioxin, or xenobiotic response element (AHRE) core consensus 5′-TNGCGTG-3′ located in the promoter region of AHR target genes (Fujisawa-Sehara *et al.*, 1987) and recruits coactivators and chromatin remodeling proteins that are essential components in the transcriptional induction of AHR target genes (Schnekenburger *et al.*, 2007; Taylor *et al.*, 2009). Well-known AHR target genes encode drug-metabolizing enzymes belonging to the cytochrome P450 CYP1 family and several phase II detoxification enzymes (Nebert *et al.*, 2004). Although induction of these genes is the best characterized AHR function, it does not adequately explain the diversity of AHR-mediated effects. Work from our laboratory and many others has led to the identification of many AHR-directed effects of dioxin exposure involved in regulation of growth factor signaling, cell cycle proliferation, differentiation, arrest, and apoptosis that result from various forms of cross talk between AHR and other regulatory proteins (reviewed in Barouki *et al.*, 2007; Bock and Kohle, 2006; Puga *et al.*, 2009).

THE DUAL ROLES OF THE AHR

Emerging evidence summarized here implicates the role of AHR not only in the response to xenobiotic intoxication and detoxification but also in developmental and cell proliferation processes. Beyond its role as mediator of xenobiotic toxicity,

the normal role of the AHR in a number of biological processes is just beginning to be recognized, associating its target genes in signaling pathways with a fundamental role in cell cycle regulation and development. The AHR is dispensable but has an important function in controlling the balance among processes involved in cell proliferation, death, and differentiation that, if deregulated, contribute to disease initiation, promotion, and progression, ultimately leading to regulatory alterations of gene expression in a multiplicity of biological processes (Gasiewicz *et al*., 2008; Puga *et al*., 2005, 2009).

Mice with a homozygous ablation of the *Ahr* gene are viable and resistant to dioxin toxicity (Fernandez-Salguero *et al*., 1996; Shimizu *et al*., 2000) but suffer numerous age-related pathologies involving multiple organ systems, including reduced liver size, diminished reproductive capabilities, immunosuppression, and epidermal hyperplasia (Fernandez-Salguero *et al*., 1995, 1997). In the absence of a xenobiotic inducer, AHR knockout mice suffer from an impaired cardiovascular phenotype in which fetal vascular structures in the liver and eye fail to undergo apoptosis (Lahvis *et al*., 2000). The inescapable conclusion from these observations is that the AHR has physiological functions in addition to metabolic detoxification of xenobiotics and, albeit indirectly, strongly argue in favor of the existence of AHR endogenous functions that do not require activation by xenobiotic ligands and that play an important role in maintenance of cellular homeostasis (Bock and Kohle, 2006).

The known functions of the AHR can be broadly understood as pertaining to either of these dual roles in development or in adaptive and toxic responses to environmental injury. How these two roles cross talk and interact is the critical question that must be answered to arrive at a full understanding of AHR physiology. The ancestral function of the AHR appears to be the regulation of specific aspects of embryonic development having acquired the ability to bind xenobiotic compounds only during the evolution of vertebrates (Hahn, 2002) because invertebrate AHR homologues do not respond to the exogenous ligands of the vertebrate AHR and their functions are more closely related to embryonic development and differentiation. However, the invertebrate AHR protein also functions as a transcription factor and binds to the same dimerization partner and *cis*-acting AHREs as the vertebrate protein, but it does not respond to any of the environmental agonists recognized by the vertebrate receptor. Instead, it regulates diverse developmental processes that are independent of toxicant or of exogenous ligand exposure. Spineless (ss), the *Drosophila* AHR homolog, is involved in neurite morphogenesis (Kim *et al*., 2006) and development of distal segments of leg and antenna (Emmons *et al*., 1999). AHR-1, the AHR homolog in the nematode *Caenorhabditis elegans*, regulates the cell fate specification of GABAergic motor neurons (Huang *et al*., 2004; Qin and Powell-Coffman, 2004). Interestingly, differentiation of GABAergic neurons in the ventral telencephalon appears to be also a function of the murine AHR (Gohlke *et al*., 2009). Given the role of the mammalian AHR in development and craniofacial, renal, and cardiovascular morphogenesis (Birnbaum *et al*., 1989; Fernandez-Salguero *et al*., 1997; Lahvis *et al*., 2005) alluded to earlier, it is reasonable to raise the key question of whether the AHR ancestral function has been retained during vertebrate evolution.

AHR ACTIVATION BY DIOXIN EXPOSURE TO THE ADULT CAUSES HEART DISEASE, WHEREAS DISRUPTION OF AHR SIGNALING AFFECTS DEVELOPMENT AND CARDIOGENESIS

Long-term epidemiologic studies have established a strong link between occupational exposure to high doses of TCDD and ischemic heart disease (Flesch-Janys *et al*., 1995). A highly significant dose-dependent relationship between death from ischemic heart disease and dioxin exposure (relative risk 2.5, 95% confidence interval 1.3–4.7, for the most and the least exposed groups) was found in the study of a cohort of 1189 male workers in a chemical plant in Hamburg, Germany, who had produced phenoxy herbicides, chlorophenols, and other herbicides known to be contaminated with TCDD and other chlorinated dioxins and furans. Interestingly, the body burden at which these effects were observed in humans ranged from 110 to 4000 ng/kg of blood fat compared with a parallel cohort of nonexposed German workers who had a mean blood fat dioxin level of 3.2 (1.3–6.5) ng/kg (Kogevinas *et al*., 1997) and well below the body burden of TCDD known to induce cancer in rodents (1000–140,000 ng/kg) (DeVito *et al*., 1995), suggesting that heart disease might be a low-dose effect of dioxin exposure. A similar association between dioxin exposure and heart disease was found in exposed individuals from the Seveso accident (Pesatori *et al*., 1998) and in an analysis of the International Agency for Research in Cancer international study, comprising 36 cohorts from 12 countries followed from 1939 to 1992, of noncancer mortality among phenoxyacid herbicide and chlorophenol production workers and sprayers (Vena *et al*., 1998). Other studies have found no significant association between TCDD exposure and any of several cardiovascular outcomes, including myocardial infarction, angina, cardiac arrhythmias, hypertension, and abnormal peripheral arterial flow, even though in a total of 281 workers and 260 unexposed referents, the workers had substantial exposure to TCDD, as demonstrated by a significantly elevated mean serum TCDD concentration of 220 ng/kg of lipid, compared with 7 ng/kg of lipid among the referents. However, a recent analysis of all English language epidemiologic studies and their citations in PubMed regarding dioxin exposure and cardiovascular disease mortality found consistent and significant dose-related increases in ischemic heart disease mortality and more modest associations with all cardiovascular disease mortality and concluded that dioxin exposure in humans is associated with mortality from both ischemic heart

disease and all cardiovascular disease, although more strongly with the former (Humblet *et al.*, 2008).

In experimental animals, the heart is a sensitive target of TCDD toxicity and the AHR is a major contributor to cardiovascular homeostasis in all species that have been studied. In fish embryos, TCDD reduces blood flow and circulatory functions associated with sc hemorrhage and pericardial edema (Ivnitski-Steele and Walker, 2005). In avian embryos, TCDD induces dilated cardiomyopathy, myocardial hypoxia, increased VEGF-A expression, and coronary vascularization (Ivnitski-Steele *et al.*, 2005). The mouse heart is also a TCDD target during fetal development, showing many of the TCDD-induced effects observed in fish and avian embryos, including reduced cardiomyocyte proliferation and altered fetal heart size. In mice, TCDD disrupts neoangiogenesis (Ivnitski-Steele and Walker, 2005). Microarray analysis showed significant AHR-dependent changes in cardiac gene expression, particularly in genes involved in extracellular matrix remodeling, such as matrix metalloproteinases 9 and 13 and endothelin-1 (ET-1), and cardiac hypertrophy, such as atrial natriuretic peptide, β-myosin heavy chain, and osteopontin. It is noteworthy that all the cardiac genes that are induced by TCDD in the fetus, except for ET-1, remain induced in the hearts of adult male offspring. This persistence long after the removal of the inducing agent is consistent with the hypothesis that an epigenetic mechanism is responsible for the change (Aragon *et al.*, 2008; Thackaberry *et al.*, 2005a).

In hyperlipidemic *Apoe*$^{-/-}$ mice, TCDD exposure exacerbated the severity of ischemic heart disease compared with their normal *Apoe*$^{+/+}$ counterparts. TCDD caused changes in levels of vasoactive eicosanoids followed by an initial vasoconstriction and subsequent hypertension. In these mice, TCDD partitioned into low-density lipoprotein particles and accelerated the development of atherosclerotic plaques (Dalton *et al.*, 2001). This work showed for the first time that TCDD exposure in mice causes the two primary risk factors, dyslipidemia and high blood pressure, characteristic of atherosclerosis in humans.

More recent findings from the Walker laboratory have shown that *in utero* exposure to TCDD increases the susceptibility to cardiovascular dysfunction in adult life (Aragon *et al.*, 2008; Thackaberry *et al.*, 2005b). Consistent with the concept that the AHR may be a major player in cardiac function, knockout of the *Ahr* gene in mice also disrupts cardiovascular homeostasis, causing significant cardiac hypertrophy, as determined by heart weight, echocardiography, pressure overload, and elevated levels of ET-1, angiotensin-2, β-myosin heavy chain, and atrial natriuretic factor expression (Lund *et al.*, 2003). AHR ablation also causes increased cardiac fibrosis, as determined by increased osteopontin and collagen I messenger RNA expression. Elevated levels of ET-1 cause the activation of ET(A) receptors and mediate an increase in ROS initiated via increased NAD(P)H oxidase activity (Lund *et al.*, 2005) that is associated with cardiac hypertrophy. These findings establish ET-1 and the ET(A) receptor as primary determinants of hypertension and cardiac pathology in AHR null mice (Lund *et al.*, 2003, 2006).

Paradoxically, it appears that cardiovascular disease is a common end point of AHR activation by dioxin and of AHR loss by gene ablation. Because sustained AHR activation leads to its degradation (Davarinos and Pollenz, 1999; Pollenz, 1996), one intriguing possibility that would resolve this paradox is that cardiovascular disease might be the consequence of depletion of a functional AHR, which would be the common consequence of both gene ablation and protein downregulation.

THE AHR-TCDD AXIS AND CONGENITAL HEART DISEASE IN MICE AND HUMANS

In humans, there is ample evidence that several forms of congenital cardiovascular malformations result from interactions between genetic and environmental factors (Goldberg *et al.*, 1990; Kuehl and Loffredo, 2005, 2006). Two congenital heart defects, valvular stenosis and hypoplastic left heart syndrome, are the most common forms of birth defects in humans, occurring in eight newborns from every 1000 live births and constituting 25–30% of all cases of human cardiovascular malformation (Armstrong and Bischoff, 2004). Overall, these two congenital heart defects are the leading cause of neonatal and infant death and a major cause of adult cardiac insufficiency (Tanner *et al.*, 2005; Tennstedt *et al.*, 1999), thus linking fetal and adult cardiovascular disease. Hypoplastic left heart, more common in males than in females, results from the incomplete development of mitral valve, left ventricle aortic valve, and aorta on the left side of the heart (Armstrong and Bischoff, 2004). The condition develops during embryonic development and causes the heart to fail unless a shunt connecting right and left sides of the heart is established soon after birth. Interestingly, 10% of patients with hypoplastic left heart have other birth defects.

The main known risk factors for these abnormalities in cardiac development are genetic inheritance and maternal exposure to hazardous chemicals (Ferencz, 1990; Ferencz and Boughman, 1993; Ferencz and Neill, 1992; Ferencz *et al.*, 1989), but the precise molecular etiologies remain elusive. The *NKX2.5* gene, which encodes a homeobox transcription factor with a central role in the early events of cardiogenesis, is the main known genetic determinant of hypoplastic left heart syndrome. It is genetically upstream of multiple genes essential for heart development (Jay *et al.*, 2004; Pashmforoush *et al.*, 2004; Tanaka *et al.*, 1999), and mutations in this gene are causally associated with diverse congenital heart malformations that include septal defects, cardiomyopathy, outflow tract defects, hypoplastic left heart, and associated arrhythmias in both humans and mice (Elliott *et al.*, 2003; Jay *et al.*, 2004; McElhinney *et al.*, 2003; Pashmforoush *et al.*, 2004; Schott *et al.*, 1998).

From an environmental perspective, organochlorinated compounds are epidemiologically associated with these same cardiac malformations. Infants born to mothers living near incinerators that emitted complex mixtures of dioxins, furans, particulates, and heavy metals exhibited a higher incidence of lethal congenital heart diseases (Dummer *et al*., 2003). In independent studies, the incidence of hypoplastic left heart syndrome was epidemiologically associated (Odds Ratio = 3.0, $p < 0.005$) with maternal exposure to halogenated hydrocarbons, dioxins, and polychlorinated biphenyls during pregnancy (Kuehl and Loffredo, 2005, 2006).

Despite compelling genetic and epidemiological data, our understanding of the mechanisms by which dioxins exert their cardiotoxic effects in humans is limited, and a direct causal link between congenital heart disease and fetal exposure to dioxins has yet to be established. Acquisition of such data is confounded by the fact that many congenital malformations result in spontaneous abortions or undetected miscarriages that are overlooked from inclusion in epidemiological surveys (Dolk and Vrijheid, 2003; Sheiner *et al*., 1998). We have recently found that treatment of differentiating mouse embryonic stem (ES) cells with TCDD represses *Nkx2*.5 gene expression and other cardiac markers as a consequence of AHR activation (Wang *et al*., 2010). When we followed the expression trajectories of cardiomyocyte markers during ES cell differentiation in the presence of TCDD, we found that TCDD silenced the expression of *Nkx2.5* and of several cardiomyocyte-specific genes, including the genes coding for cardiac troponin-T and α- and β-myosin heavy chains, and inhibited the formation of beating cardiomyocytes, a characteristic phenotype of differentiating mouse ES cells. Based on chromatin immunoprecipitation data, AHR was the key mediator of these TCDD effects (Wang *et al*., 2010).

Human cardiac malformations because of environmental (organochlorinated compounds) or genetic (*NKX2.5* mutations) causes can be experimentally recapitulated by a combination of ES cell work and subsequent follow up experiments *in vivo*. Inhibition of cardiomyocyte differentiation resulting from interactions between AHR, TCDD, and NKX2.5 can be a relevant animal model to study the molecular mechanisms of the human disease. Repression of NKX2.5 by TCDD-mediated AHR activation recapitulates the loss of function in humans resulting from mutations or from dioxin exposure, with the additional advantage that this animal model helps us to identify the regulatory loops controlling the NKX2.5 functions that determine embryonic identity and progression of cardiac tissue differentiation and how these functions are silenced by the activated AHR. Furthermore, dose relationships between TCDD doses *in utero* and cardiac malformations in adulthood will be amenable to study. Although *Nkx2.5* knockout chimeric embryos tolerate the loss of 15–20% of their cardiomyocytes (Tanaka *et al*., 1999), there is a good likelihood that these losses would have a significant effect on predisposing the adult to cardiac disease. In fact, these congenital heart defects in humans are also a major cause of adult cardiac insufficiency (Tanner *et al*., 2005; Tennstedt *et al*., 1999).

It is critical to identify the potential gene-X-environment pathways that organochlorinated compounds follow to act as developmental teratogens in conjunction with the *AHR* gene, causing congenital cardiovascular malformations. Knowledge of these pathways will ultimately provide the molecular targets that will help us understand the basis of AHR-mediated cardiotoxicity. A significant number of genes other than *NKX2.5* play important and selective roles in cardiac development, converging on a finite number of signaling pathways that regulate endothelial, smooth muscle, cardiac, and mesenchymal cell proliferation and differentiation in the developing and postnatal heart. Many of these genes are constituents of signaling pathways, such as VEGF, NFATc1, BMP10, Notch, WNT/β-catenin, TGF-β, and others (Armstrong and Bischoff, 2004; Pashmforoush *et al*., 2004) that cross talk with the AHR signaling pathway (Puga *et al*., 2009). The relative contribution of AHR-dependent effects to these other cardiac development pathways will likely become a further area of relevance and research.

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