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AOP 150: Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty

Short Title: AHR activation to embryolethality

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Status

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Abstract

Interference with endogenous developmental functions of the aryl hydrocarbon receptor (AHR) by sustained exogenous activation causes structural, molecular and functional cardiac abnormalities and altered heart physiology in avian, mammalian and piscine embryos; this cardiotoxicity ultimately leads to severe edema and embryo death in birds and fish and some strains of rat (Carney et al. 2006; Huuskonen et al. 1994; Kopf and Walker 2009). There have been numerous proposed mechanisms of action for this toxicity profile, many of which include the dysregulation of vascular endothelial growth factor (VEGF) as a key event, as it is essential for normal vasculogenesis and therefore cardiogenesis (Ivnitski-Steele and Walker 2005). This AOP describes the indirect suppression of VEGF expression through the sequestration of the aryl hydrocarbon receptor nuclear translocator (ARNT) by AHR. ARNT is common dimerization partner for both AHR and hypoxia inducible factor alpha (HIF-1α), which stimulates angiogenesis through the transcriptional regulation of VEGF (Ivnitski-Steele and Walker 2005); there is considerable cross talk between the two nuclear receptors, leading to the hypothesis that AHR activation leads to sustained AHR/ARNT dimerization and reduced HIF-1α/ARNT dimerization, preventing the adequate transcription of essential angiogenic factors, such as VEGF. The suppression of VEGF thereby reduces cardiomyocyte and endothelial cell proliferation, altering cardiovascular morphology and reducing cardiac output, which ultimately leads to congestive heart failure and death (Lanham et al. 2014).

The biological plausibility of this AOP is strong, and there is significant evidence in the literature to support it; however, there exist some contradictory data regarding the effect of AHR on VEGF, which seem highly dependent on tissue type and life stage. These contradictions and alternate pathways are discussed below. The quantitative

understanding of individual key even relationships (KERs) in this AOP is weak; however, there is a strong correlation between the molecular initiating event (MIE: AHR activation) and adverse outcome (AO: embryolethality), and a quantitative relationship is described for birds.

Background

In 1957, millions of broiler chickens died due to a mysterious chick edema disease characterized by pericardial, subcutaneous and peritoneal edema (SCHMITTLE et al. 1958). This disease was later ascribed to the ingestion of feed contaminated with halogenated aromatic hydrocarbons (HAHs), including 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (Higginbotham et al. 1968; Metcalfe 1972). It has since become evident that TCDD, and other AHR agonists, disrupt the normal development and function of the heart. A general study in the 1980’s found that mothers exposed to herbicides during pregnancy had a 2.8-fold increase in risk of having a baby with congenital cardiovascular malformations (Loffredo et al. 2001). Epidemiological studies have correlated long-term TCDD exposure with ischemic heart disease (Bertazzi et al. 1998; Flesch-Janys et al. 1995); interestingly, and consistent with this AOP, sectioned and stained heart samples from patients with this disease lack epicardial cells (Di et al. 2010). Mammalian studies have confirmed that in utero exposure to TCDD increases susceptibility to cardiovascular dysfunction in adulthood (Aragon et al. 2008; Thackaberry et al. 2005b). The developing heart is highly dependent on oxygen saturation levels; somewhat counterintuitively, a state of hypoxia (relative to adult oxygen tension) drives normal formation and maturation. Deviation from this optimal oxygen level, either above or below normal, hinders myocardial and endothelial development, altering coronary artery connections, ventricle wall thickness and chamber formation (Patterson and Zhang 2010; Wikenheiser et al. 2009). Interestingly, AHR activation (by TCDD), inhibition, and knockdown significantly inhibited the formation of contractile cardiomyocyte nodes during spontaneous differentiation of embryonic stem cells into cardiomyocytes (in vitro) (Wang et al. 2010), indicating that AHR also has an optimal window of expression for normal cardiogenesis. TCDD significantly reduces the degree of myocardial hypoxia that normally occurs during myocyte proliferation and ventricular wall thickening in the developing embryo (Ivnitski-Steele et al. 2004; Lee et al. 2001). This reduction in hypoxia is associated with reduced expression of both HIF-1and the VEGF splice variant, VEGF166 mRNA, which is one of the primary VEGF variants required to mediate coronary vascularization (Ivnitski-Steele et al. 2004). Therefore, it is biologically plausible that sustained AHR activation sequesters ARNT from HIF-1α impairing hypoxia stimulated coronary angiogenesis.

Summary of the AOP

Stressors

Name	Evidence
Dibenzo-p-dioxin	
Polychlorinated biphenyl	
dibenzofuran	

Molecular Initiating Event

Title	Short name
Activation, AHR	Activation, AHR

18: Activation, AHR

Short Name: Activation, AHR

AOPs Including This Key Event

AOP ID and Name	Event Type
21: AhR activation leading to embryo toxicity in fish	MolecularInitiatingEvent
57: AhR activation leading to hepatic steatosis	MolecularInitiatingEvent
131: AhR activation leading to uroporphyria	MolecularInitiatingEvent
150: Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty	MolecularInitiatingEvent

Stressors

Name
Benzidine
Dibenzo-p-dioxin
Polychlorinated biphenyl
Polychlorinated dibenzofurans
Hexachlorobenzene

Evidence for Perturbation of this Molecular Initiating Event by Stressor

The AHR can be activated by several structurally diverse chemicals, but binds preferentially to planar halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons. Dioxin-like compounds (DLCs), which include polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and certain polychlorinated biphenyls (PCBs), are among the most potent AHR ligands[38]. Only a subset of PCDD, PCDF and PCB congeners has been shown to bind to the AHR and cause toxic effects to those elicited by TCDD. Until recently, TCDD was considered to be the most potent DLC in birds[39]; however, recent reports indicate that 2,3,4,7,8-pentachlorodibenzofuran (PeCDF) is more potent than TCDD in some species of birds.[40][13][41][21][42][43] When screened for their ability to induce aryl hydrocarbon hydroxylase (AHH) activity, dioxins with chlorine atoms at a minimum of three out of the four lateral ring positions, and with at least one non-chlorinated ring position are the most active[44]. Of the dioxin-like PCBs, non-ortho congeners are the most toxicologically active, while mono-ortho PCBs are generally less potent[45][9]. Chlorine substitution at ortho positions increases the energetic costs of [45]

assuming the coplanar conformation required for binding to the AHR . Thus, a smaller proportion of mono-ortho PCB molecules are able to bind to the AHR and elicit toxic effects, resulting in reduced potency of these congeners. Other PCB congeners, such as di-ortho substituted PCBs, are very weak AHR agonists and do not likely contribute to dioxin-like effects [9].

The role of the AHR in mediating the toxic effects of planar hydrophobic contaminants has been well studied, however the endogenous role of the AHR is less clear [1]. Some endogenous and natural substances, including prostaglandin PGG2 and the tryptophan derivatives indole-3-carbinol, 6-formylindolo[3,2-b]carbazole (FICZ) and kynurenic acid can bind to and activate the AHR. [6][46][47][48][49] The AHR is thought to have important endogenous roles in reproduction, liver and heart development, cardiovascular function, immune function and cell cycle regulation [50][38][51][52][53][54][46][55][56][57] and activation of the AHR by DLCs may therefore adversely affect these processes.

Biological Organization

Level of Biological Organization
Molecular

The AHR structure has been shown to contribute to differences in species sensitivity to DLCs in several animal models. In 1976, a 10-fold difference was reported between two strains of mice (non-responsive DBA/2 mouse, and responsive C57BL/6 14 mouse) in CYP1A induction, lethality and teratogenicity following TCDD exposure[3]. This difference in dioxin sensitivity was later attributed to a single nucleotide polymorphism at position 375 (the equivalent position of amino acid residue 380 in chicken) in the AHR LBD[30][19][31]. Several other studies reported the importance of this amino acid in birds and mammals[32][30][22][33][34][35][31][36]. It has also been shown that the amino acid at position 319 (equivalent to 324 in chicken) plays an important role in ligand-binding affinity to the AHR and transactivation ability of the AHR, due to its involvement in LBD cavity volume and its steric effect[35]. Mutation at position 319 in the mouse eliminated AHR DNA binding[35].

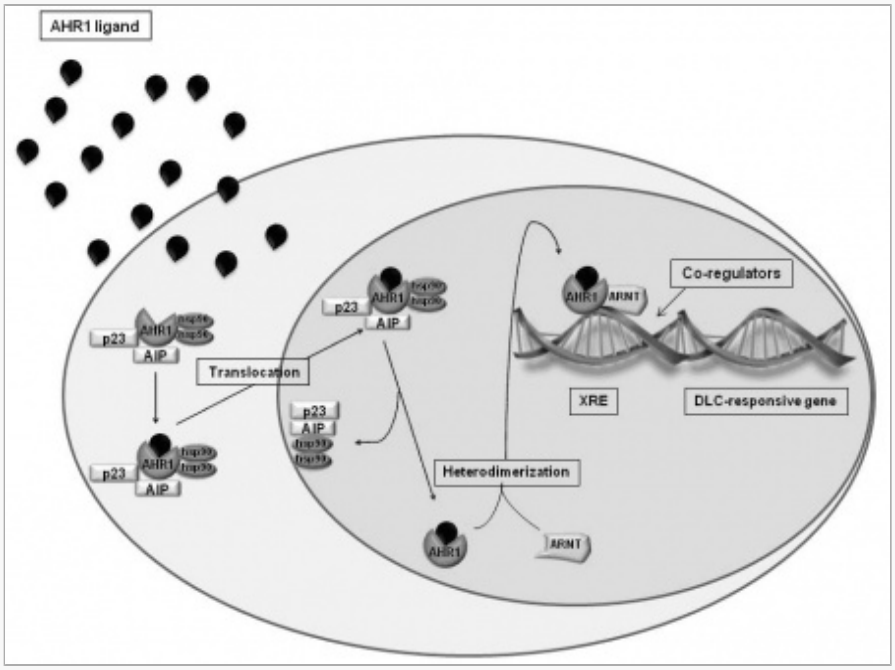
The first study that attempted to elucidate the role of avian AHR1 domains and key amino acids within avian AHR1 in avian differential sensitivity was performed by Karchner ***et al.***[22]. Using chimeric AHR1 constructs combining three AHR1 domains (DBD, LBD and TAD) from the chicken (highly sensitive to DLC toxicity) and common tern (resistant to DLC toxicity), Karchner and colleagues[22], showed that amino acid differences within the LBD were responsible for differences in TCDD sensitivity between the chicken and common tern. More specifically, the amino acid residues found at positions 324 and 380 in the AHR1 LBD were associated with differences in TCDD binding affinity and transactivation between the chicken (Ile324_Ser380) and common tern (Val324_Ala380) receptors[22]. Since the Karchner et al. (2006) study was conducted, the predicted AHR1 LBD amino acid sequences were been obtained for over 85 species of birds and 6 amino acid residues differed among species[14][37] . However, only the amino acids at positions 324 and 380 in the AHR1 LBD were associated with differences in DLC toxicity in ovo and AHR1-mediated gene expression in vitro[14][37][16]. These results indicate that avian species can be divided into one of three AHR1 types based on the amino acids found at positions 324 and 380 of the AHR1 LBD: type 1 (Ile324_Ser380), type 2 (Ile324_Ala380) and type 3 (Val324_Ala380)[14][37][16].

How this Key Event Works

The AHR Receptor

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that belongs to the basic helix-loop-helix Per-ARNT-Sim (bHLH-PAS) superfamily and consists of three domains: the DNA-binding domain (DBD), ligand binding domain (LBD) and transactivation domain (TAD)[1]. Other members of this superfamily include the AHR nuclear translocator (ARNT), which acts as a dimerization partner of the AHR [2][3]; Per, a circadian transcription factor; and Sim, the “single-minded” protein involved in neuronal development [4][5]. This group of proteins shares a highly conserved PAS domain and is involved in the detection of and adaptation to environmental change[4].

The molecular Initiating Event



The molecular mechanism of activation of gene expression by AHR1.

The molecular mechanism for AHR-mediated activation of gene expression is presented in the figure to the right. In its unliganded form, the AHR is part of a cytosolic complex containing heat shock protein 90 (HSP90), the HSP90 co-chaperone p23 and AHR-interacting protein (AIP)[6]. Upon ligand binding, the AHR migrates to the nucleus where it dissociates from the cytosolic complex and forms a heterodimer with ARNT[7]. The AHR-ARNT complex then binds to a xenobiotic response element (XRE) found in the promoter of an AHR-regulated gene and recruits co-regulators such as CREB binding protein/p300, steroid receptor co-activator (SRC) 1, SRC-2, SRC-3 and nuclear receptor interacting protein 1, leading to induction or repression of gene expression[6]. Expression levels of several genes, including phase I (e.g. cytochrome P450 (CYP) 1A, CYP1B, CYP2A) and phase II enzymes (e.g. uridine diphosphate glucuronosyl transferase (UDP-GT), glutathione S-transferases (GSTs)), as well as genes involved in cell proliferation (transforming growth factor-beta, interleukin-1 beta), cell cycle regulation (p27, jun-B) and apoptosis (Bax), are regulated through this mechanism [6][8][7][9].

AHR Isoforms

Two AHR isoforms (AHR1 and AHR2) have been identified in the black-footed albatross (*Phoebastria nigripes*), great cormorant (*Phalacrocorax carbo*) and domestic chicken (*Gallus gallus domesticus*)[10]. AHR1 mRNA levels were similar in the kidney, heart, lung, spleen, brain, gonad and intestine from the great cormorant but were lower in muscle and pancreas. AHR2 expression was mainly observed in the liver, but was also detected in gonad, brain and intestine. AHR1 levels represented a greater proportion (80%) of total AHR levels than AHR2 in the cormorant [10]

liver , and while both AHR isoforms bound to TCDD, AHR2 was less effective at inducing TCDD-dependent transactivation compared to AHR1 in black-footed albatross, great cormorant and domestic chicken[11][10].

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

Transactivation Reporter Gene Assays (recommended approach)

Transient transfection transactivation

Transient transfection transactivation is the most common method for evaluating nuclear receptor activation[12]. Full-length AHR cDNAs are cloned into an expression vector along with a luminescent reporter gene construct (chimeric luciferase, P-lactamase or CAT reporter vectors containing the appropriate response elements for the gene of interest). There are a number of commercially available cell lines that can serve as recipients for these vectors (CV-1, HuH7, FLC-7, LS174T, LS180 MCF-7, HEC1, LLC-PK1, HEK293, HepG2, and Caco-2 cells)[12]. The greatest advantage of using transfected cells, rather than primary cell cultures, is the assurance that the nuclear receptor of interest is responsible for the observed induction. This would not be possible in a primary cell culture due to the co-regulation of different receptors for the same target genes. This model makes it easy to compare the responsiveness of the AHR across multiple species under the same conditions simply by switching out the AHR clone. One disadvantage to the transient transfection assay is the inherent variability associated with transfection efficiency, leading to a movement towards the use of stable cell lines containing the nuclear receptor and reporter gene linked to the appropriate response elements[12].

Luciferase reporter gene (LRG) assay

For demonstrative purposes, a luciferase reporter gene assay used to measure AHR1-mediated transactivation for avian species is described here. A monkey kidney cell line (Cos-7) that has low endogenous AHR1 expression was transfected with the appropriate avian AHR1 clone, cormorant ARNT1, a CYP1A5 firefly luciferase reporter construct and a *Renilla* luciferase vector to control for transfection efficiency. After seeding, the cells were exposed to DLC and luciferase activity was measured using a luminometer. Luminescence, which is proportional to the extent of AHR activation, is expressed as the ratio of firefly luciferase units to *Renilla* luciferase units [13]. This particular assay was modified from its original version to increase throughput efficiency; (a) cells were seeded in 96-well plates rather than Petri dishes or 48- well plates, (b) DLCs were added directly to the wells without changing the cell culture medium, and (c) the same 96-well plates were used to measure luminescence without lysing the cells and transferring to another plate. Similar reporter gene assays have been used to measure AHR1 activation in domestic and wild species of birds, including the chicken, ring-necked pheasant (Phasianus colchicus), Japanese quail (Coturnix japonica), great cormorant, black-footed albatross and peregrine falcon (Falco peregrinus).[14][13][15][11][16][17]

Transactivation in stable cell lines

Stable cell lines have been developed and purified to the extent that each cell contains both the nuclear receptor and appropriate reporter vector, eliminating the variability associated with transfection [12]. A stable human cell line containing a luciferase reporter driven by multiple dioxin response elements has been developed that is useful in identifying AhR agonists and antagonists[18]. An added benefit of this model is the potential to multiplex 3 assays in a single well: receptor activation, cell viability and enzyme activity[12]. Such assays are used extensively in drug

discovery due to their high throughput efficiency, and may serve just as useful for risk assessment purposes.

Ligand-Binding Assays

Ligand binding assays measure the ability of a test compound to compete with a labeled, high-affinity reference ligand for the LBD of a nuclear receptor. It is important to note that ligand binding does not necessitate receptor activation and therefore cannot distinguish between agonists and antagonists; however, binding affinities of AHR ligands are highly correlated with chemical potencies[19] and can explain differences in species sensitivities to DLCs[20][21][22]; they are therefore worth mentioning. Binding affinity and efficacy have been used to develop structure-activity relationships for AHR disruption[20][23] that are potentially useful in risk-assessment. There has been tremendous progress in the development of ligand-binding assays for nuclear receptors that use homogenous assay formats (no wash steps) allowing for the detection of low-affinity ligands, many of which do not require a radiolabel and are amenable to high throughput screening[24][12]. This author however was unable to find specific examples of such assays in the context of AHR binding and therefore some classic radioligand assays are described instead.

Hydroxyapatite (HAP) binding assay

The HAP binding assay makes use of an *in vitro* transcription/translation method to synthesize the AHR protein, which is then incubated with radiolabeled TDCPP and a HAP pellet. The occupied protein adsorbs to the HAP and the radioactivity is measured to determine saturation binding. An additional ligand can also be included in the mixture in order to determine its binding affinity relative to TCDD (competitive binding)[25][22]. This assay is simple, repeatable and reproducible; however, it is insensitive to weak ligand-receptor interactions[22][21][26].

Whole cell filtration binding assay

Dold and Greenlee[27] developed a method to detect specific binding of TCDD to whole mammalian cells in culture and was later modified by Farmahin et al.[21] for avian species. The cultured cells are incubated with radiolabeled TCDD with or without the presence of a competing ligand and filtered. The occupied protein adsorbs onto the filter and the radioactivity is measured to determine saturation binging and/or competitive binding. This assay is able to detect weak ligand-receptor interactions that are below the detection limit of the HAP assay[21].

Protein-DNA Interaction Assays

The active AHR complexed with ARNT can be measured using protein-DNA interaction assays. Two methods are described in detail by Perez-Romero and Imperiale[28]. Chromatin immunoprecipitation measures the interaction of proteins with specific genomic regions *in vivo*. It involves the treatment of cells with formaldehyde to crosslink neighboring protein-protein and protein-DNA molecules. Nuclear fractions are isolated, the genomic DNA is sheared, and nuclear lysates are used in immunoprecipitations with an antibody against the protein of interest. After reversal of the crosslinking, the associated DNA fragments are sequenced. Enrichment of specific DNA sequences represents regions on the genome that the protein of interest is associated with *in vivo*. Electrophoretic mobility shift assay (EMSA) provides a rapid method to study DNA-binding protein interactions in vitro. This relies on the fact that complexes of protein and DNA migrate through a nondenaturing polyacrylamide gel more slowly than free DNA fragments. The protein-DNA complex components are then identified with appropriate antibodies. The EMSA assay was found to be consistent with the LRG assay in chicken hepatoma cells dosed with dioxin-like compounds[29].

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Key Events

Title	Short name
dimerization, AHR/ARNT	dimerization, AHR/ARNT
reduced dimerization, ARNT/HIF1-alpha	reduced dimerization, ARNT/HIF1-alpha
Impairment, Endothelial network	Impairment, Endothelial network
Altered, Cardiovascular development/function	Altered, Cardiovascular development/function
Increase, Pericardial edema	Increase, Pericardial edema
reduced production, VEGF	reduced production, VEGF

944: dimerization, AHR/ARNT

Short Name: dimerization, AHR/ARNT

AOPs Including This Key Event

AOP ID and Name	Event Type
150: Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty	KeyEvent

Biological Organization

Level of Biological Organization

Molecular

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
chicken	Gallus gallus	Strong	NCBI
zebrafish	Danio rerio	Strong	NCBI
mouse	Mus musculus	Strong	NCBI

This mechanism is conserved across species. Mammals possess a single AHR, whereas birds and fish express multiple isoforms, and all three express multiple ARNT isoforms. Not all of the isoforms identified are functionally active. For example, killifish AHR1 and AHR2 are active and display different transcription profiles, whereas zebrafish AHR2 and ARNT2 are active in mediating xenobiotic-mediated toxicity and AHR1 is inactive (Hahn et al. 2006; Prasch et al. 2006).

How this Key Event Works

Upon ligand binding, the aryl hydrocarbon receptor (AHR) migrates to the nucleus where it dissociates from the cytosolic complex and forms a heterodimer with AHR nuclear translocator (ARNT) (Mimura and Fujii-Kuriyama 2003). The AHR-ARNT complex then binds to a xenobiotic response element (XRE) found in the promoter of an AHR-regulated gene and recruits co-regulators such as CREB binding protein/p300, steroid receptor co-activator (SRC) 1, SRC-2, SRC-3 and nuclear receptor interacting protein 1, leading to induction or repression of gene expression (Fujii-Kuriyama and Kawajiri 2010). Expression levels of several genes, including phase I (e.g. cytochrome P450 (CYP) 1A, CYP1B, CYP2A) and phase II enzymes (e.g. uridine diphosphate glucuronosyl transferase (UDP-GT), glutathione S-transferases (GSTs)), as well as genes involved in cell proliferation (transforming growth factor-beta, interleukin-1 beta), cell cycle regulation (p27, jun-B) and apoptosis (Bax), are regulated through this mechanism (Fujii-Kuriyama and Kawajiri 2010; Giesy et al. 2006; Mimura and Fujii-Kuriyama 2003; Safe 1994).

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

The active AHR complexed with ARNT can be measured using protein-DNA interaction assays. Two methods are described in detail by Perez-Romero and Imperiale (Perez-Romero and Imperiale 2007). Chromatin immunoprecipitation measures the interaction of proteins with specific genomic regions in vivo. It involves the treatment of cells with formaldehyde to crosslink neighboring protein-protein and protein-DNA molecules. Nuclear fractions are isolated, the genomic DNA is sheared, and nuclear lysates are used in immunoprecipitations with an antibody against the protein of interest. After reversal of the crosslinking, the associated DNA fragments are

sequenced. Enrichment of specific DNA sequences represents regions on the genome that the protein of interest is associated with in vivo. Electrophoretic mobility shift assay (EMSA) provides a rapid method to study DNA-binding protein interactions in vitro. This relies on the fact that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than free DNA fragments. The protein-DNA complex components are then identified with appropriate antibodies. The EMSA assay was found to be consistent with the luciferase reporter gene assay (in chicken hepatoma cells dosed with dioxin-like compounds (Heid et al. 2001).

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945: reduced dimerization, ARNT/HIF1-alpha

Short Name: reduced dimerization, ARNT/HIF1-alpha

AOPs Including This Key Event

AOP ID and Name	Event Type
150: Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty	KeyEvent

Biological Organization

Level of Biological Organization

Molecular

How this Key Event Works

Sustained dimerization of the aryl hydrocarbon receptor (AHR) and AHR nuclear translocator (ARNT) induced by xenoiotics may sequester ARNT from its other dimerization partners at inappropriate times during embryonic cardiomorphogenesis, disrupting ARNT-dependent cellular functions (Heid et al. 2001; Walker et al. 1997). ARNT serves as a dimerization partner for hypoxia inducible factor 1&alph; (HIF-1α), and this complex is involved in mediating physiological responses to hypoxia. Dimerization between ARNT and HIF-1α forms a transcription factor complex (HIF-1) that binds to hypoxia response enhancer sequences on DNA to activate the expression of genes such as vascular endothelial growth factor (VEGF), which is involved in angiogenesis (Forsythe et al. 1996; Goldberg and Schneider 1994; Jiang et al. 1996; Maxwell et al. 1997; Shweiki et al. 1992).

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

The active HIF1- α complexed with ARNT can be measured using protein-DNA interaction assays. Two methods are described in detail by Perez-Romero and Imperiale (Perez-Romero and Imperiale 2007). Chromatin immunoprecipitation measures the interaction of proteins with specific genomic regions in vivo. It involves the treatment of cells with formaldehyde to crosslink neighboring protein-protein and protein-DNA molecules. Nuclear fractions are isolated, the genomic DNA is sheared, and nuclear lysates are used in immunoprecipitations with an antibody against the protein of interest. After reversal of the crosslinking, the associated DNA fragments are sequenced. Enrichment of specific DNA sequences represents regions on the genome that the protein of interest is associated with in vivo. Electrophoretic mobility shift assay (EMSA) provides a rapid method to study DNA-binding protein interactions in vitro. This relies on the fact that complexes of protein and DNA migrate through a non-denaturing polyacrylamide gel more slowly than free DNA fragments.

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110: Impairment, Endothelial network

Short Name: Impairment, Endothelial network

AOPs Including This Key Event

AOP ID and Name	Event Type
43: Disruption of VEGFR Signaling Leading to Developmental Defects	KeyEvent
150: Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty	KeyEvent

Biological Organization

Level of Biological Organization
Molecular

Blood vessel development utilizes highly conserved molecular pathways that are active across vertebrate species. Anatomically, however, the molecular toolbox for vasculogenesis/angiogenesis has varied themes for arterial, venous, and lymphatic channels, as well as across different organs and species [Tal et al. 2016]. ToxCast high-throughput screening (HTS) data for 25 assays mapping to targets in embryonic vascular disruption signature [Knudsen and Kleinstreuer, 2011] were used to rank-order 1060 chemicals for their potential to disrupt vascular development. The predictivity of this signature is being evaluated in various angiogenesis assays, including tubulogenesis in endothelial cells from zebrafish, chick, mouse and human species [Tal et al. 2016; Vargesson et al. 2003; Knudsen et al. 2016; McCollum et al. 2016; Nguyen et al. 2016]. As an example, a zebrafish embryo vascular model in conjunction with a mouse endothelial cell model identified 28 potential vascular disruptor compounds (pVDCs) from ToxCast. These exposures invoked a plethora of vascular perturbations in the zebrafish embryo, including malformed intersegmental vessels, uncondensed caudal vein plexus, hemorrhages and cardiac edema; 22 pVDCs inhibited endothelial tubulogenesis in an yolk-sac-derived endothelial cell line [McCollum et al. 2016]. The VEGF pathway was implicated across mouse-zebrafish species. Because gene sequence similarity of the ToxCast pVDC signature is comprised of proteins that primarily map to human in vitro and biochemical assays, the U.S. EPA SeqAPASS tool was used to assess the degree of conservation of signature targets between zebrafish and human, as well as other commonly used model organisms in human health and environmental toxicology research [Tal et al. 2016]. This approach revealed that key nodes in the ontogenetic regulation of angiogenesis have evolved across diverse species.

How this Key Event Works

In embryological terms the angiogenic cycle entails a stepwise progression of de novo blood vessel morphogenesis (vasculogenesis), maturation and expansion (angiogenesis), and remodeling [Hanahan, 1997; Chung and Ferrara 2011; Coultas et al. 2005]. These events commence as angioblasts migrate, proliferate, and assemble into a tubular network. With maturation, the endothelial tubules co-opt local stromal cells as pericytes and smooth muscle. Local signals acting on receptor tyrosine kinases (RTKs), G-protein coupled receptors (GPCRs), and glycosyl phosphatidyl-inositol (GPI)-anchored receptors, and later vascular flow-mediated signals. The process of endothelial assembly into a tubular network may be disrupted by environmental agents [Sarkanen et al. 2010; Bondesson et al. 2016; Knudsen et al. 2016; Nguyen et al. 2016; Tal et al. 2016].

How it is Measured or Detected

Endothelial tubule formation (tubulogenesis) can be monitored both qualitatively and quantitatively in vitro using different human cell-based angiogenesis assays that score endothelial cell migration and the degree of tubular network formation, including cell counts, tubule counts, tubule length, tubule area, tubule intensity, and node counts [Muller et al. 2002; Masckauchan et al. 2005; Sarkanen et al. 2010; Knudsen et al. 2016; Nguyen et al. 2016]. Standard practice for reproducible in vitro tubule formation uses endothelial cells co-cultured with stromal cells [Bishop et al. 1999]. Cell types commonly employed are human umbilical endothelial cells (HUVECs) or more recently induced pluripotent stem cells (iPSCs) derived to endothelial cells through various differentiation and purification protocols. The assay is run in agonist or antagonist modes to detect chemical enhancement or suppression of tubulogenesis. Synthetic hydrogels are shown to promote robust in vitro network formation by HUVEC or iPSC-ECs as well as their utilization to detect putative vascular disruptive compounds [Nguyen et al. 2016]. Endothelial networks formed on synthetic hydrogels showed superior sensitivity and reproducibility when compared to endothelial networks formed on Matrigel.

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317: Altered, Cardiovascular development/function

Short Name: Altered, Cardiovascular development/function

AOPs Including This Key Event

AOP ID and Name	Event Type
150: Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty	KeyEvent

Biological Organization

Level of Biological Organization
Organ

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
chicken	Gallus gallus	Strong	NCBI
mouse	Mus musculus	Strong	NCBI
zebrafish	Danio rerio	Strong	NCBI

Birds, fish and mammals are all susceptible to cardiotoxicity following embryonic chemical exposure.

How this Key Event Works

This key event applies to the disruption of cardiogenesis early enough in embryogenesis to result in gross morphological alterations leading to reduced cardiac function.

With respect to dioxin-like compounds that are strong AHR agonists, the malformations that have been observed following embryonic exposure are summarized in table 1.

Table 1: Cardiotoxic effects of strong AHR-agonists

Zebrafish Embryo	Chicken Embryo	Mouse
• Reduced extension of common cardinal vein • Reduced blood flow • Reduced heart rate • Disrupted erythropoiesis • Decreased heart volume • Pericardial edema • Overt heart malformations	• Enlarged left ventricle • Increased heart rate • Increased myosin content • Reduced β-adrenergic responsiveness • Increased ANF mRNA • Arrhythmia • Increased apoptosis • Reduced myocyte proliferation • Pericardial edema • Overt heart malformations	Embryo/Fetus • Reduced heart-to-body weight • Reduced myocyte proliferation • Vascular remodeling 21 Days old • Increased heart-to-body weight • Increased left ventricle weight • Reduced heart rate • Cardiac hypertrophy • Increased ANF mRNA • Increased risk of heart disease

ANF= cardiac atrial natriuretic factor; an indicator of cardiac stress. Source: (Kopf and Walker 2009)

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358: Increase, Pericardial edema

Short Name: Increase, Pericardial edema

AOPs Including This Key Event

AOP ID and Name	Event Type
21: AhR activation leading to embryo toxicity in fish	AdverseOutcome
150: Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty	KeyEvent

Biological Organization

Level of Biological Organization
Organ

Evidence Supporting Applicability of this Event

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
chicken	Gallus gallus	Strong	NCBI
zebrafish	Danio rerio	Strong	NCBI

Birds, fish and mammals are susceptible to pericardial edema.

How this Key Event Works

Severe cardiac dysfunction can result in congestive fetal heart failure (inability of the heart to deliver adequate blood flow to organs) leading to fluid build-up in tissues (in this case, the pericardium) and cavities (edema and effusion, respectively). Fluid buildup exerts a positive pressure on cardiac chambers, which limits the diastolic ventricular filling reserve and diminishes cardiac output (Thakur et al. 2013).

How it is Measured or Detected

In experimental studies, edema is often scored as present or absent rather than being measured quantitatively. The

severity of the edema can be scored based on the area of the pericardial cavity, which can be estimated using CT, ultrasound or MRI equipped with imaging software. This technique has been demonstrated by Prasch et al. (2003) in zebrafish to quantify the pericardial sac area.

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948: reduced production, VEGF

Short Name: reduced production, VEGF

AOPs Including This Key Event

AOP ID and Name	Event Type
150: Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

How this Key Event Works

During vasculogenesis, angioblasts, which express vascular endothelial growth factor (VEGF) receptor 2 (a.k.a. fetal liver kinase; Flk-1), are stimulated to proliferate and differentiate into endothelial cells by VEGF-A. These endothelial cells then assemble into patent capillary tubes via stimulation of VEGF receptor 1 (fms-like tyrosine kinase; Flt-1) by VEGF-A. The endothelial cells then are activated by angiogenic stimuli (such as basic fibroblast growth factor and VEGF-A) to migrate and proliferate, producing new capillary sprouts (Ivnitski-Steele and Walker 2005). Ftl-1 can also act as a decoy receptor to sequester VEGF-A from Flk-1; there is even an alternatively spliced form of this receptor1 (sFlt1) that is secreted (i.e. not membrane bound) and functions primarily as a decoy (Zygmunt et al. 2011).

One potent stimulus of the angiogenic process is tissue hypoxia; insufficient oxygen being delivered to the proliferating tissue leads to the stabilization of the transcription factor hypoxia inducible factor 1α (HIF-1α). Accumulation of HIF-1α leads to the transcriptional induction of angiogenic signaling factors and their receptors,

including VEGF-A and flk-1, and the stimulation of angiogenesis (Ivnitski-Steele and Walker 2005).

How it is Measured or Detected

Methods that have been previously reviewed and approved by a recognized authority should be included in the Overview section above. All other methods, including those well established in the published literature, should be described here. Consider the following criteria when describing each method: 1. Is the assay fit for purpose? 2. Is the assay directly or indirectly (i.e. a surrogate) related to a key event relevant to the final adverse effect in question? 3. Is the assay repeatable? 4. Is the assay reproducible?

VEGF-A protein can be measured by enzyme-linked immunosorbent assay, as described in Ivnitski-Steele et al. (2005).

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Adverse Outcomes

Title	Short name
Increase, Embryolethality	Increase, Embryolethality

947: Increase, Embryolethality

Short Name: Increase, Embryolethality

AOPs Including This Key Event

AOP ID and Name	Event Type
150: Aryl hydrocarbon receptor activation leading to embryolethality via cardiotoxicty	AdverseOutcome

Biological Organization

Level of Biological Organization

Individual

How this Key Event Works

Embryo death at any stage in development prior to birth/hatch is considered embryolethal. In birds and fish it may be identified as failure to hatch or lack of movement within the egg when candled; heartbeat monitors are also available for identifying viable avian eggs. In mammals, stillborn or mummified offspring, or an increased rate of resorptions early in pregnancy are all considered embryolethal.

Scientific evidence supporting the linkages in the AOP

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Activation, AHR	directly leads to	dimerization, AHR/ARNT	Strong	Strong
reduced dimerization, ARNT/HIF1-alpha	directly leads to	reduced production, VEGF	Strong	Moderate
reduced production, VEGF	directly leads to	Impairment, Endothelial network	Strong	Weak
Impairment, Endothelial network	directly leads to	Altered, Cardiovascular development/function	Moderate	Weak
Altered, Cardiovascular development/function	directly leads to	Increase, Pericardial edema	Moderate	Weak
Increase, Pericardial edema	directly leads to	Increase, Embryolethality	Moderate	Weak
Activation, AHR	indirectly leads to	Increase, Embryolethality	Strong	Moderate
dimerization, AHR/ARNT	directly leads to	reduced dimerization, ARNT/HIF1-alpha	Moderate	Weak

Activation, AHR leads to dimerization, AHR/ARNT

How Does This Key Event Relationship Work

Upon ligand binding, the aryl hydrocarbon receptor (AHR) migrates to the nucleus where it dissociates from the cytosolic complex and forms a heterodimer with AHR nuclear translocator (ARNT) (Mimura and Fujii-Kuriyama 2003). The AHR-ARNT complex then binds to a xenobiotic response element (XRE) found in the promoter of an AHR-regulated gene and recruits co-regulators such as CREB binding protein/p300, steroid receptor co-activator (SRC) 1, SRC-2, SRC-3 and nuclear receptor interacting protein 1, leading to induction or repression of gene

expression (Fujii-Kuriyama and Kawajiri 2010). Expression levels of several genes, including phase I (e.g. cytochrome P450 (CYP) 1A, CYP1B, CYP2A) and phase II enzymes (e.g. uridine diphosphate glucuronosyl transferase (UDP-GT), glutathione S-transferases (GSTs)), as well as genes involved in cell proliferation (transforming growth factor-beta, interleukin-1 beta), cell cycle regulation (p27, jun-B) and apoptosis (Bax), are regulated through this mechanism (Fujii-Kuriyama and Kawajiri 2010; Giesy et al. 2006; Mimura and Fujii-Kuriyama 2003; Safe 1994)

Weight of Evidence

Biological Plausibility

The mechanism of AHR-mediated transcriptional regulation is well understood (Fujii-Kuriyama and Kawajiri 2010)

Empirical Support for Linkage

Include consideration of temporal concordance here

ARNT is a necessary dimerization partner for the transcriptional activation of AHR regulated genes (Hoffman et al. 1991; Poland et al. 1976). The AHR/ARNT complex was confirmed following in vitro exposure to halogenated aromatic hydrocarbons using an electrophoretic mobility shift assay; a dose-dependent supershift in DNA-binding was observed using specific antibodies in chicken and human cell lines (Heid et al. 2001)

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reduced dimerization, ARNT/HIF1-alpha leads to reduced production, VEGF

How Does This Key Event Relationship Work

hypoxia inducible factor (HIF-1α) abundance is negatively regulated by a subfamily of dioxygenases referred to as prolyl hydroxylase domain-containing proteins, which use oxygen as a substrate to hydroxylate HIF-1α subunits and hence tag them for rapid degradation. Under conditions of hypoxia, HIF-1α subunits accumulate due to reduced

hydroxylation efficiency and form heterodimers (HIF-1) with AHR nuclear translocator (ARNT) to activate the expression of angiogenic factors including vascular endothelial growth factor (VEGF) (Fong 2009). The HIF-1 complex binds to the VEGF gene promoter, then recruits additional transcriptional factors such as P-CREB and P-STAT3, to the promoter and initiates VEGF transcription (Ahluwalia and Tarnawski 2012). VEGF is the most potent, endothelial specific and fundamental regulator of angiogenesis, and is a key regulator of blood vessel growth (Ahluwalia and Tarnawski 2012). In the absence of HIF-1, VEGF expression and secretion is diminished.

Weight of Evidence

Biological Plausibility

The transcriptional control of VEGF by HIF-1 is well understood (Ahluwalia and Tarnawski 2012; Fong 2009)

Empirical Support for Linkage

Include consideration of temporal concordance here

- In chick embryo development, the oxygen gradient within myocardium induces VEGF mRNA in cardiac myocytes (Cheung 1997).
- ARNT- and HIF-1α- null mice cannot survive gestation due to defects in vasculature development (Iyer et al. 1998; Maltepe et al. 1997)
- Hypoxia increased VEGF expression in AHR+/+ aortic endothelial cells (MAECs) but not in AHR-/- MAECs, suggesting that HIF-1α modulates endothelial VEGF expression in an AHR-dependent manner (Roman et al. 2009)
- HIF-1α protein degradation by 2-methoxyestradio blocked hypoxia induced VEGF expression in AHR+/+ but not AHR-/- MAECs (Roman et al. 2009)
- Exogenous hypoxia significantly increased cardiac VEGF-A mRNA expression and expanded its spatial expression in the myocardium of developing chicks; in contrast, TCDD exposure tended to limit the spatial expression of VEGF-A to ventricular trabeculae (Ivnitski-Steele et al. 2004)
- TCDD reduced myocardial VEGF-A expression in chick embryos and reduced explant VEGF-A secretion (Ivnitski-Steele et al. 2005)

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reduced production, VEGF leads to Impairment, Endothelial network

How Does This Key Event Relationship Work

During vasculogenesis, angioblasts, which express vascular endothelial growth factor (VEGF) receptor 2 (fetal liver kinase; Flk-1), are stimulated to proliferate and differentiate into endothelial cells by VEGF-A. These endothelial cells then assemble into patent capillary tubes via stimulation of VEGF receptor 1 (fms-like tyrosine kinase; Flt-1) by VEGF-A. The endothelial cells then are activated by angiogenic stimuli (such as basic fibroblast growth factor and VEGF-A) to migrate and proliferate, producing new capillary sprouts (Ivnitski-Steele and Walker 2005).

Weight of Evidence

Biological Plausibility

The importance of VEGF for endothelial network formation and integrity is clear (Ivnitski-Steele and Walker 2005); loss of a single VEGF-A allele results in defective vascularization and early embryonic lethality (Carmeliet et al. 1996; Ferrara et al. 1996).

Empirical Support for Linkage

Include consideration of temporal concordance here

- Endothelial tube length (40%±1.7%) and number (36%±3%) significantly reduced in 2,3,7,8-tetrachlorodibenzo-p-dioxin (TDCC) treated chick explants (cell culture derive from treated embryos); exogenous VEGF, or hypoxia increased length and number to control levels (i.e. rescued effect of TCDD). The increase by hypoxia was prevented by VEGF neutralizing antibody (Ivnitski-Steele and Walker 2003)
- Hearts from TCDD treated embryos showed sig. reduction in VEGF mRNA and protein (Ivnitski-Steele and Walker 2003)
- TCDD reduced coronary artery number in chick embryos by 53±8% and reduced tube outgrowth and VEGF-A secretion (43±3%) in vitro (Ivnitski-Steele et al. 2005)
- TCDD reduces human primary umbilical vein endothelial cells (HUVEC) basal proliferation by approx. 50% compared to control and reduces VEGF-A-stimulated proliferation by an additional 30%. In the absence of VEGF-A, HUVECs from control cultures elongate and form linear attachments, while addition of VEGF-A stimulates formation of complex interconnected networks (Ivnitski-Steele and Walker 2005).

Uncertainties or Inconsistencies

Reduced secretion of VEGF is not the sole mechanism responsible for reduced coronary vasculogenesis as TCDD caused a dose-related reduction in tube outgrowth in vitro but all doses reduced VEGF-A secretion equally (Ivnitski-Steele et al. 2005).

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Impairment, Endothelial network leads to Altered, Cardiovascular development/function

How Does This Key Event Relationship Work

The formation of new blood vessels during development occurs via de novo assembly of blood vessels from angioblast precursors (vasculogenesis) and formation of new capillary sprouts from preexisting vessels (angiogenesis) (Ivnitski-Steele and Walker 2005). The epicardium is a single cell layer that spreads over the surface of the heart during embryo development and is the source of angioblasts, which penetrate into the myocardium, providing the endothelial and mural cell progenitor populations that eventually form the entire coronary vasculature (Ivnitski-Steele and Walker 2005; Viragh et al. 1993; Vrancken Peeters et al. 1999). The development of the vasculature into highly branched conduits needs to occur in numerous sites and in precise patterns to supply oxygen and nutrients to the rapidly expanding tissue of the embryo; aberrant regulation and coordination of angiogenic signals during development result in impaired organ development (Chung and Ferrara 2011).

Weight of Evidence

Biological Plausibility

The importance of endothelial cell migration, proliferation and integrity in neovascularization and organogenesis is well documented (Chung and Ferrara 2011; Ivnitski-Steele and Walker 2005).

Empirical Support for Linkage

Include consideration of temporal concordance here

- 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced cardiotoxicity in zebrafish coincides with epicardium formation. Cardiotoxicity begins at 48 hours post fertilization (hpf; start of pre-epicardium formation) and starts to decline at 5 days post fertilization, which is about the time the initial epicardial cell layer is complete. Cardiotoxicity disappears at 2 weeks, after epicardium formation is complete. TCDD prevented the formation of the epicardial cell layer when exposed 4hpf, and blocked epicardial expansion from the ventricle to the atrium following exposure at 96hpf. These effects ultimately result in valve malformation, reduced heart size, impaired development of the bulbus arteriosus, decreased cardiac output, reduced end diastolic volume, decreased peripheral blood flow, edema and death (Plavicki et al. 2013).
- Significant decreases in cardiomyocyte proliferation and thinning of the ventricular wall were observed in

chicken embryos exposed to PCB58 (Carro et al. 2013).
- TCDD inhibition of coronary development is preceded by a decrease in myocyte proliferation and an increase in cardiac apoptosis (Ivnitski et al. 2001).

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Altered, Cardiovascular development/function leads to Increase, Pericardial edema

How Does This Key Event Relationship Work

Changes in heart morphology can result in decreased cardiac output and are associated with myocardial disease, abnormalities in cardiac loading, rhythm disorders, ischemia (restriction in blood supply to tissues, causing a shortage of oxygen and glucose needed for cellular metabolism), and cardiac compression. Severe cardiac dysfunction can result in congestive fetal heart failure (inability of the heart to deliver adequate blood flow to organs) leading to fluid build-up in tissues and cavities (edema and effusion, respectively). Fluid buildup exerts a positive pressure on fetal cardiac chambers, which further limits the diastolic ventricular filling reserve, potentiating the diminished cardiac output and leading to fetal death (Thakur et al. 2013).

Weight of Evidence

Biological Plausibility

Cardiovascular dysfunction accounts for 26% of human fetal hydrops (abnormal amounts of fluid buildup in two or more body areas of a fetus) and is well understood (Thakur et al. 2013). Figure 1 describes the types of general and cardiac causes of hydrops.

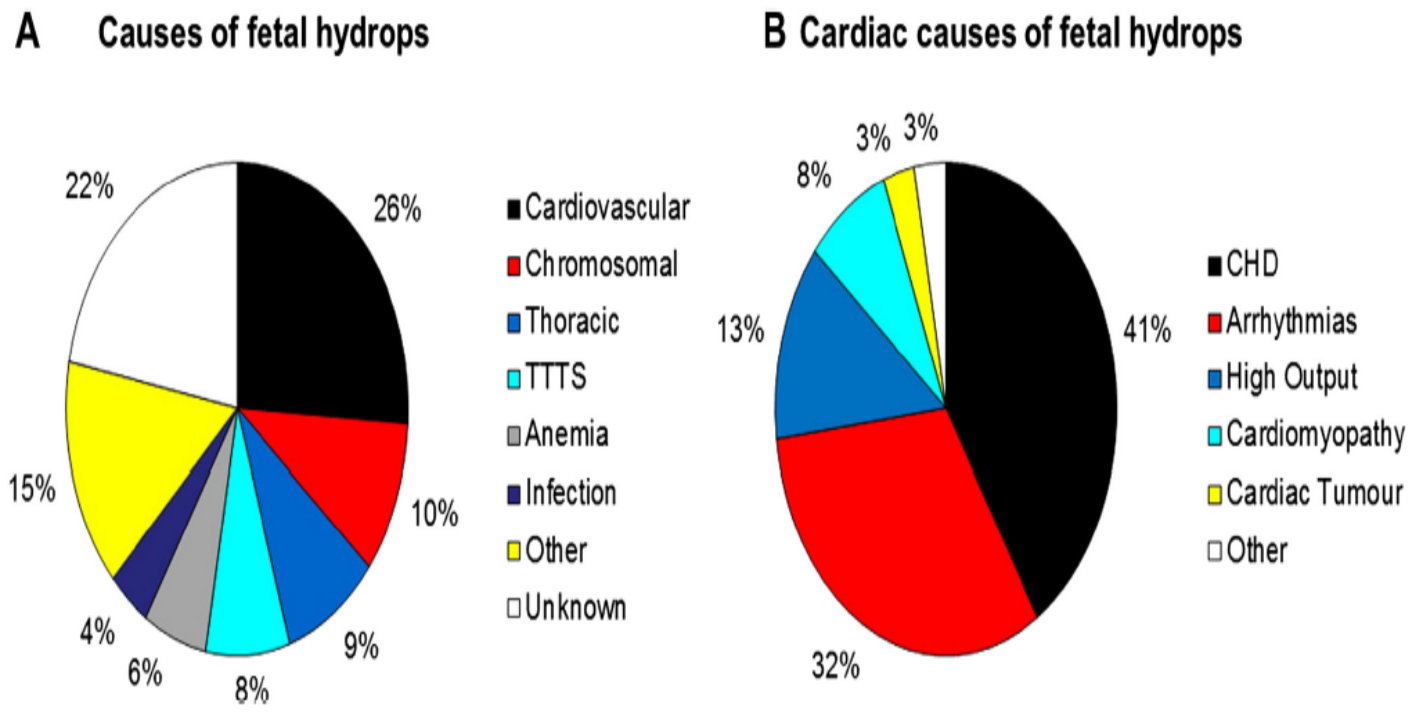

Figure 1. Distribution of the general (**A**) and cardiac (**B**) causes of fetal hydrops. CHD, congenital heart disease; TTTS, twin-twin transfusion syndrome; unknown, causes not established. (Source: Thakur, V. *et al.* (2013). *Can. J Cardiol.* **29**(7), 759-767.)

Empirical Support for Linkage

Include consideration of temporal concordance here

- Edema is a secondary response to changes in cardiac structure. At low doses of dioxin-like compounds, disrupted heart looping (Henshel et al. 1993), congenital heart defects, (Cheung et al. 1981) and impaired contraction of cardiac myocytes (Canga et al. 1993) were observed in chick embryos without the onset of edema. Whereas at higher doses edema and embryo death are increased (Walker et al. 1997).
- Changes in heart morphology consistent with dilated cardiomyopathy (decreased cardiac output and ventricular cavity expansion) were observed in chick embryos exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) followed by progression to congestive heart failure. Dilated ventricles, induction of cardiac atrial natriuretic factor (ANF) mRNA and a decrease in cardiac β-adrenergic responsiveness were observed and proceeded to severe edema (Walker and Catron 2000).
- Pericardial edema is secondary to heart failure in zebrafish as changes in heart morphology and decreases in cardiac output and peripheral blood flow precede heart failure (Antkiewicz et al. 2005; Belair et al. 2001; Henry et al. 1997; Plavicki et al. 2013)
- When mannitol is used as a protective agent against chemical-induced edema in zebrafish, cardiotoxic effects are still observed; therefore, edema is secondary to cardiotoxicity (Antkiewicz et al. 2005; Plavicki et al. 2013)

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Increase, Pericardial edema leads to Increase, Embryolethality

How Does This Key Event Relationship Work

Severe cardiac dysfunction can result in congestive fetal heart failure (inability of the heart to deliver adequate blood flow to organs) leading to fluid build-up in tissues and cavities (edema and effusion, respectively). Fluid buildup exerts a positive pressure on fetal cardiac chambers, which further limits the diastolic ventricular filling reserve, potentiating the diminished cardiac output and leading to fetal death (Thakur et al. 2013).

Weight of Evidence

Biological Plausibility

The connection between edema and diminished cardiac output is well understood in fish, birds and mammals (Antkiewicz et al. 2005; Thakur et al. 2013; Walker 1998; Walker et al. 1997)

Empirical Support for Linkage

Include consideration of temporal concordance here

Edema and hemorrhage are common signs of developmental exposure to cardiotoxic agents in fish, birds and mammals prior to death (Kopf and Walker 2009; Walker and Catron 2000).

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Activation, AHR leads to Increase, Embryolethality

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
chicken	Gallus gallus	Strong	NCBI
Japanese quail	Coturnix japonica	Strong	NCBI
Ring-necked pheasant	Phasianus colchicus	Strong	NCBI
turkey	Meleagris gallopavo	Strong	NCBI
bobwhite quail	Colinus virginianus	Strong	NCBI
American kestrel	Falco sparverius	Strong	NCBI
Double-crested cormorant	Double-crested cormorant	Strong	NCBI
Eastern bluebird	Eastern bluebird	Strong	NCBI

The correlation between AHR-mediated reporter gene activity and embryo death has been demonstrated in avian species as described above.

How Does This Key Event Relationship Work

The aryl hydrocarbon receptor (AHR) structure has been shown to contribute to differences in species sensitivity to dioxin-like compounds (DLCs) in several animal models, with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) being the most potent AHR-agonist. In 1976, a 10-fold difference was reported between two strains of mice (non-responsive DBA/2 mouse, and responsive C57BL/6 14 mouse) in CYP1A induction, lethality and teratogenicity following TCDD exposure (Poland et al. 1976). This difference in dioxin sensitivity was later attributed to a single nucleotide polymorphism at position 375 (the equivalent position of amino acid residue 380 in chicken) in the AHR ligand binding domain (LBD) (Ema et al. 1994; Poland et al. 1994; Poland and Knutson 1982). Several other studies reported the importance of this amino acid in birds and mammals (Backlund and Ingelman-Sundberg 2004; Ema et al. 1994; Karchner et al. 2006; Murray et al. 2005; Pandini et al. 2007; Pandini et al. 2009; Poland et al. 1994;

Ramadoss and Perdew 2004). It has also been shown that the amino acid at position 319 (equivalent to 324 in chicken) plays an important role in ligand-binding affinity to the AHR and transactivation ability of the AHR, due to its involvement in LBD cavity volume and its steric effect (Pandini et al. 2009). Mutation at position 319 in the mouse eliminated AHR DNA binding (Pandini et al. 2009). The first study that attempted to elucidate the role of avian AHR1 domains and key amino acids within avian AHR1 in avian differential sensitivity was performed by (Karchner et al. 2006). Using chimeric AHR1 constructs combining three AHR1 domains (DBD, LBD and TAD) from the chicken (highly sensitive to DLC toxicity) and common tern (resistant to DLC toxicity), Karchner and colleagues (2006), showed that amino acid differences within the LBD were responsible for differences in TCDD sensitivity between the chicken and common tern. More specifically, the amino acid residues found at positions 324 and 380 in the AHR1 LBD were associated with differences in TCDD binding affinity and transactivation between the chicken (Ile324_Ser380) and common tern (Val324_Ala380) receptors. Since the Karchner et al. (2006) study was conducted, the predicted AHR1 LBD amino acid sequences were been obtained for over 85 species of birds and 6 amino acid residues differed among species (Farmahin et al. 2013; Head et al. 2008). However, only the amino acids at positions 324 and 380 in the AHR1 LBD were associated with differences in DLC toxicity in ovo and AHR1-mediated gene expression in vitro (Farmahin et al. 2013; Head et al. 2008; Manning et al. 2012). This would indicate that the AHR1 LBD sequence alone could be used to predict DLC-induced embryolethality in a given bird species. Based on these results, avian species can be divided into one of three AHR1 types based on the amino acids found at positions 324 and 380 of the AHR1 LBD: type 1 (Ile324_Ser380; most sensitive), type 2 (Ile324_Ala380; moderately sensitive) and type 3 (Val324_Ala380; least sensitive) (Farmahin et al. 2013; Head et al. 2008; Manning et al. 2012).

Weight of Evidence

Biological Plausibility

Differences in species sensitivity to DLCs have been associated with differences in the AHR amino acid sequence in mammals, fish and birds; the identity of these amino acids in the AHR LBD affects DLC binding affinity and AHR1-mediated transactivation (Farmahin et al. 2012; Head et al. 2008; Karchner et al. 2006; Mimura and Fujii-Kuriyama 2003; Wirgin et al. 2011). A sampling of bird species and their AHR LBD category is described in table 1. A list of 86 species and their subtype can be found in Farmahin et al. (2013).

Table 1

AHR1 subtypes identified based on predicted amino acid sequences of the avian AHR1 ligand binding domain (LBD). Avian AHR1 LBD sequences from 86 species were obtained from GenBank or were determined from liver or blood samples obtained from the National Wildlife Research Centre, Ottawa, ON, commercial suppliers near Ottawa, ON, or the Tittabawassee River basin, Michigan, USA

AHR 1	LBD amino acid residues						
subtype	256	257	297	324[a]	337	380[a]	Examples[b]
1A	A	A	T	I	V	S	Domestic chicken (*Gallus gallus domesticus*)
1B	A	A	T	I	I	S	European starling (*Sturnus vulgaris*)
1C	A	T	T	I	I	S	Gray catbird (*Dumetella carolinensis*)
2A	A	A	I	I	V	A	Wild turkey (*Meleagris gallopavo*)
2B	A	A	T	I	I	A	Indigo bunting (*Passerina cyanea*)
2C	A	A	V	I	V	A	Rock ptarmigan (*Lagopus muta*)
2D	A	P	T	I	V	A	Spotted sandpiper (*Actitis macularius*)
2E	A	T	T	I	I	A	Tree swallow (*Tachycineta bicolor*)
2F	A	T	T	I	V	A	Black-footed albatross (*Phoebastria nigripes*)
2G	T	A	I	I	V	A	Ring-necked pheasant (*Phasianus colchicus*)
3A	A	A	T	V	V	A	Japanese quail (*Coturnix japonica*)
3B	A	T	T	V	V	A	Herring gull (*Larus argentatus*)
3C	T	T	T	V	V	A	Wood duck (*Aix sponsa*)

[a]Amino acid residues at positions 324 and 380 were used to classify species into three major AHR1 types, which are indicated by white (type 1), light grey (type 2), and dark grey (type 3) shading.

[b]The full list of 86 species is presented in (Farmahin, R. *et al.*(2013). *Toxicol. Sci.* **131**(1), 139-152)

(Source: Manning, G. E. *et al.* (2012). *Toxicol. Appl. Pharmacol.* **263**(3), 390-399)

Empirical Support for Linkage

Include consideration of temporal concordance here

Binding of DLCs to avian AHR1 (Farmahin et al. 2014; Karchner et al. 2006) and AHR1-mediated transactivation measured using luciferase reporter gene (LRG) assays (Farmahin et al. 2012; Farmahin et al. 2013b; Fujisawa et al. 2012; Lee et al. 2009; Manning et al. 2012; Mol et al. 2012) have been demonstrated in domestic and wild species of birds, including the chicken, ring-necked pheasant (Phasianus colchicus), Japanese quail (Coturnix japonica), great cormorant, black-footed albatross and peregrine falcon (Falco peregrinus).

Quantitative Understanding of the Linkage

Is it known how much change in the first event is needed to impact the second? Are there known modulators of the response-response relationships? Are there models or extrapolation approaches that help describe those relationships?

The predictive ability of an LRG assay measuring induction of AHR1-mediated gene expression in cells transfected with different avian AHR1 expression vectors was demonstrated by linear regression analysis comparing log-transformed LD50 values obtained from the literature to log-transformed PC20 values from the LRG assay (Farmahin et al. 2013b; Manning et al. 2012). PC20 values represent the concentration of DLC that elicited 20% of the TCDD maximal response, and were calculated according to the procedure described in OECD guideline 455 (OECD 2009). LD50 values used in regression analyses were obtained from the literature. As shown in the linear regression analysis (Figure 1), logLD50 values were associated with logPC20 and a significant relationship ($R2 = 0.93$, $p < 0.0001$) was observed. Thus, to predict the in ovo LD50 for a given species and DLC, one could use the species' AHR1 LBD sequence to design an AHR1 expression vector, measure the PC20 of the DLC in the LRG assay, and use the regression to obtain an LD50 value.

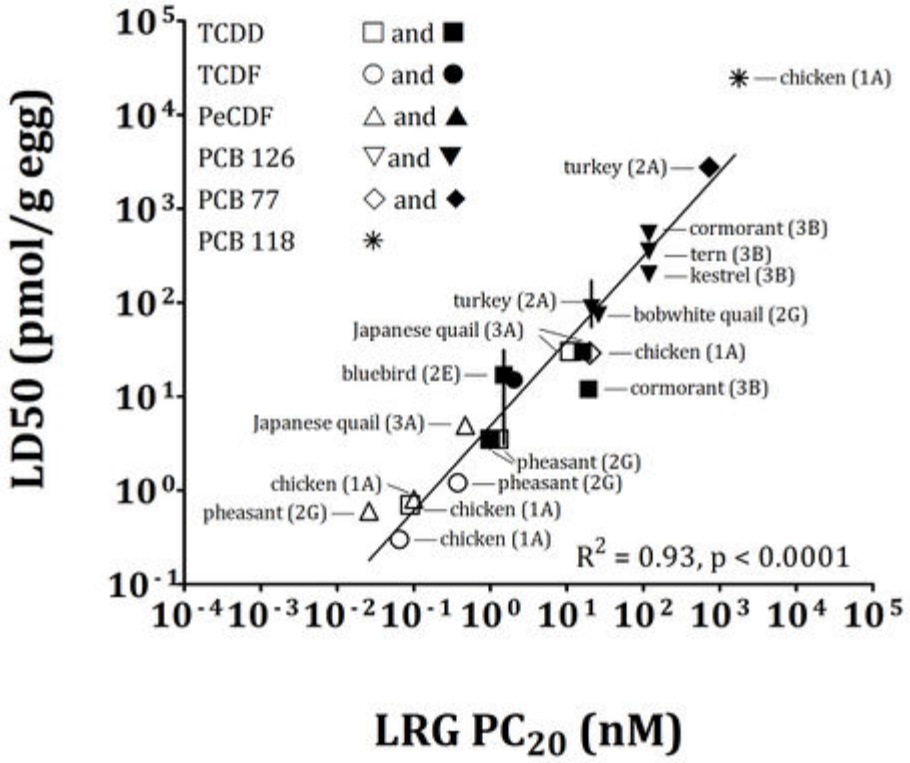

Figure 1. Linear regression analysis comparing LD50 values with PC20 (logLD50 = 0.79logPC20 + 0.51) values derived from luciferase reporter gene (LRG) assay concentration-response curves. Open symbols represent LRG data from wild-type chicken, ring-necked pheasant or Japanese quail AHR1 expression vectors. Closed symbols represent LRG data from mutant AHR1 (Source: Manning, G. E. et al. (2012). *Toxicol. Appl. Pharmacol.* 263(3), 390-399.)

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dimerization, AHR/ARNT leads to reduced dimerization, ARNT/HIF1-alpha

How Does This Key Event Relationship Work

The aryl hydrocarbon receptor nuclear translocator (ARNT) is common dimerization partner for both the aryl hydrocarbon receptor (AHR) and hypoxia inducible factor alpha (HIF-1α). There is considerable cross talk between the two nuclear receptors, leading to the hypothesis that AHR activation leads to sustained AHR/ARNT dimerization and reduced HIF-1α/ARNT dimerization, assuming ARNT is not available in excess (Chan et al. 1999).

Weight of Evidence

Biological Plausibility

The ARNT serves as a dimerization partner for multiple transcription factors including the xenobiotic sensing AHR and HIF1α; therefore, it is plausible that sequestration of ARNT by one receptor would reduce the responsiveness of the other, assuming that ARNT is available in limited quantity. Gel-shift and coimmunoprecipitation experiments have shown that the AHR and HIF1α compete for ARNT in vitro, with approximately equal dimerization efficiencies (Schmidt and Bradfield 1996).

Empirical Support for Linkage

Include consideration of temporal concordance here

- Activation of either AHR (by 2,3,7,8-tetrachlorodibenzo-p-dioxin) or HIF1 (by hypoxia) inhibits the activity of the other, in Hep3B cells (Chan et al. 1999)
- TCDD and hypoxia together reduced the stabilization of HIF1α and HRE-mediated promoter activity when compared to hypoxia alone, in MCF-7 and HepG2 cells (Seifert et al. 2008).
- Hypoxia increased EF5 binding (hypoxic tissue marker) in chicken embryos, whereas it was decreased by TCDD relative to controls (D10 of incubation) (Ivnitski-Steele et al. 2004)
- TCDD reduces the expression of cardiac HIF1α mRNA in chicken embryos (Ivnitski-Steele et al. 2004)
- ARNT overexpression rescued human HepG2 and HaCaT cells from inhibitory effect of hypoxia on XRE-luciferase reporter activity. This indicates that the mechanism of interference between the AHR and HIF1α pathways at least partially dependent on ARNT availability (Vorrink et al. 2014)
- Ischemia-induced upregulation of the expression of HIF1α and ARNT and DNA binding activity of the HIF1α-ARNT complex were enhanced in AHR-null mice (Ichihara et al. 2007).

Uncertainties or Inconsistencies

Although crosstalk between AHR and HIF1α clearly exists, the nature of the relationship is still not clearly defined. It has been suggested that HIF1α and AHR do not competitively regulate each other for hetero-dimerization with ARNT, as ARNT is constitutively and abundantly expressed in cells and does not deplete due to hypoxia or AHR activation (Chan et al. 1999; Pollenz et al. 1999). Nie et al. (2001) hypothesized that the degree of interaction among ARNT-dependent pathways depends on the abundance of ARNT in the cells. They observed crosstalk in Hepa 1 cells but not H4IIE cells, and attributed this to the ratio of AhR to ARNT of 0.3 (i.e. excess ARNT), compared to a ratio of 10 in Hepa 1 cells (Holmes and Pollenz, 1997)

Some studies have shown that the effect of hypoxia on AHR mediated pathways is stronger than effects of a AHR-mediated xenobiotic response on the HIF1α pathway (Gassmann et al. 1997; Gradin et al. 1996; Nie et al. 2001; Prasch et al. 2004); this has been attributed to the stronger binding affinity of HIF1α to ARNT relative to AHR (Gradin et al. 1996).

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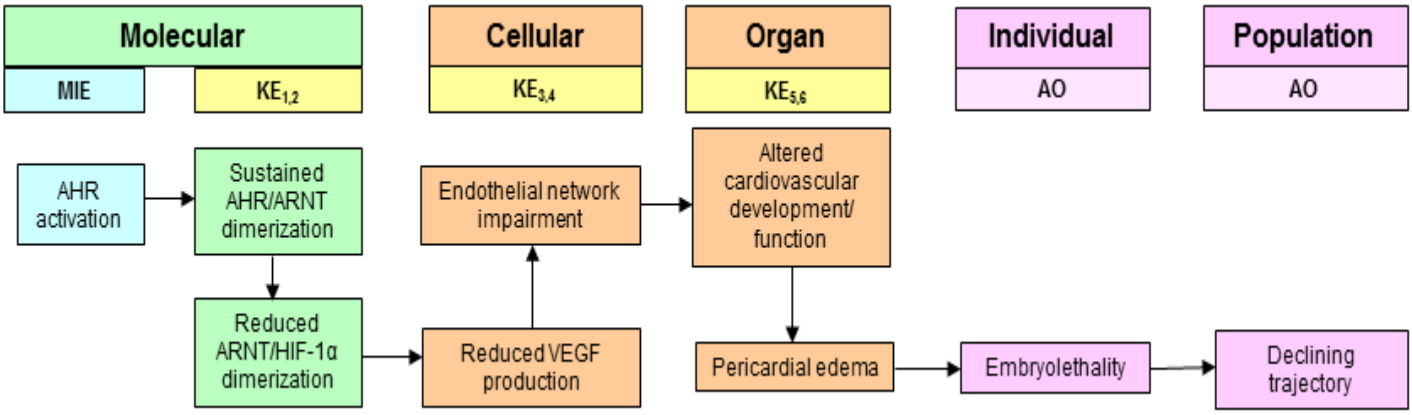
Overall Assessment of the AOP



Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Embryo	Strong

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
chicken	Gallus gallus	Strong	NCBI
zebrafish	Danio rerio	Strong	NCBI
mouse	Mus musculus	Strong	NCBI
Rattus norvegicus	Rattus norvegicus		NCBI

Sex Applicability

Sex	Evidence
Male	Strong
Female	Strong

Life Stage Applicability, Taxonomic Applicability, Sex Applicability

Elaborate on the domains of applicability listed in the summary section above. Specifically, provide the literature supporting, or excluding, certain domains.

Life Stage Applicability: Exposure must occur early in embryo development in utero (mammals) or in ovo (birds and fish). Mammalian studies often dose between gestational days 14.5 and 17.5 as it represents a developmental window of cardiomyocyte proliferation (Kopf and Walker 2009). Cardiotoxicity has been observed in birds dosed on day zero or day 5 of incubation (Ivnitski-Steele et al. 2005; Walker et al. 1997). Zebrafish seem to have a particular sensitive window of cardio-development between 48 hours-post-fertilization (hpf) and 5 days pf, and become

resistant to AHR-mediated cardiotoxicity if exposed after epicardium formation is complete (2 weeks pf) (Plavicki et al. 2013).

Taxonomic Applicability: Early embryonic exposure to AHR-agonists in mice causes cardiotoxicity that persists into adulthood, increasing susceptibility to heart disease (Thackaberry et al. 2005b) and can increased resorptions and late stage fetal death with edema in certain strains of rat (Huuskonen et al. 1994). The resulting cardiac malformations and edema in birds and fish are fatal (Kopf and Walker 2009).

Sex applicability: Embryonic dysfunction is equally robust in males and females, but adult abnormalities of mice exposed in utero are more prevalent in females (Carreira et al. 2015)

Essentiality of the Key Events

Molecular Initiating Event Summary, Key Event Summary

Provide an overall assessment of the essentiality for the key events in the AOP. Support calls for individual key events can be included in the molecular initiating event, key event, and adverse outcome tables above.

Molecular initiating event: AHR activation (Essentiality = Strong)

- Zebrafish AHR2 morphants (transient knock-out of function) are protected against reduced blood flow, pericardial edema, erythrocyte maturation, and common cardinal vein migration (Bello et al. 2004; Carney et al. 2004; Prasch et al. 2003; Teraoka et al. 2003)
- AHR2-/- zebrafish mutants were protected against TCDD toxicity, including pericardial edema and epicardium development (Goodale et al. 2012; Plavicki et al. 2013)
- AHR activation specifically within cardiomyocytes accounts for heart failure (cardiac malformations, loss of circulation, pericardial edema) induced by TCDD as well as non-cardiac toxicity (swim bladder inflation and craniofacial defects) in zebrafish (Lanham et al. 2014).
- AHR-null mice have impaired angiogenesis in vivo: endothelial cells failed to branch and form tube-like structures (Roman et al. 2009).
- Ischemia-induced angiogenesis was markedly enhanced in AHR-null mice compared with that in wild-type animals (Ichihara et al. 2007)

Key Event 1: AHR/ARNT dimerization (Essentiality = Strong)

- ARNT1 is essential for normal vascular and hematopoietic development (Abbott and Buckalew 2000; Kozak et al. 1997; Maltepe et al. 1997)
- zfarnt2-/- mutation is larval lethal, and the mutants have enlarged heart ventricles and an increased incidence of cardiac arrhythmia (Hill et al. 2009)
- ARNT1 morpholono knock-down protected against pericardial edema and reduced blood flow in zebrafish (Prasch et al. 2006)
- ARNT overexpression rescued cells from the inhibitory effect of hypoxia on AHR-mediated luciferase reporter activity; therefore, the mechanism of interference of the signaling cross-talk between AHR and hypoxia pathways is at least partially dependent on ARNT availability (Vorrink et al. 2014).

Key Event 2: Reduced HIF1α/ARNT dimerization (Essentiality = Moderate)

- Both ARNT–/– and HIF1α–/– mice display embryonic lethality with blocks in developmental angiogenesis and cardiovascular malformations (Iyer et al. 1998; Kozak et al. 1997; Maltepe et al. 1997; Ryan et al. 1998) demonstrating that signaling through the HIF-1 pathway is required for normal development of the cardiovascular system.
- The myocardium exhibits a reduced oxygen status during the later stages of coronary vascular development in chick and mouse embryos (Ivnitski-Steele et al. 2004; Lee et al. 2001)
- Rearing fish embryos in a hypoxic environment can modify cardiac activity, organ perfusion, and blood vessel

formation (Pelster 2002)
- TCDD toxicity in fish resembles defects in hypoxia sensing (Prasch et al. 2004)
- Deviation in oxygen levels, below or above normal, during early chick embryogenesis results in abnormal coronary vasculature (Wikenheiser et al. 2009)
- Hypoxia stimulates vasculogenesis and regulates VEGF transcription in vivo and in vitro (Goldberg and Schneider 1994; Levy et al. 1995; Liu et al. 1995) (Goldberg 1994; LEVY 1995A; Liu 1995)
- Hypoxia stimulus can rescue TCDD inhibition of coronary vascular development in chick embryos (Ivnitski-Steele and Walker 2003)

Key Event 3: Reduced VEGF production (Essentiality = Moderate)

- Loss of a single VEGF-A allele in mice results in defective vascularization and early embryonic lethality (Carmeliet et al. 1996; Ferrara et al. 1996).
- Mice lacking VEGF isoforms 164 and 188 exhibit impaired myocardial angiogenesis and reduced contractility leading to ischemic cardiomyopathy (Carmeliet et al. 1999)
- During vasculogenesis, angioblasts are stimulated to proliferate and differentiate into endothelial cells by VEGF-A (Ivnitski-Steele and Walker 2005)
- Migration and assembly of epicardial angioblasts into coronary vessels is regulated by VEGF (Folkman 1992)
- Cardiomyocyte-specific knockout of VEGF in mice results in phenotype similar to TCDD toxicity (thinner ventricular walls, ventricle cavity dilation, and contractile dysfunction) (Giordano et al. 2001; Ivnitski-Steele and Walker 2003)
- Exogenous VEGF rescues the inhibitory effect of TCDD on vasculogenesis (Ivnitski-Steele and Walker 2003)

Key Event 4: Endothelial network impairment (Essentiality = Moderate)

- The epicardium is the source of angioblasts, which penetrate into the myocardium, providing the endothelial and mural cell progenitor populations that eventually form the entire coronary vasculature (Viragh et al. 1993; Vrancken Peeters et al. 1999)
    - TCDD prevents the formation and migration of the epicardial cell layer in zebrafish (Plavicki et al. 2013)
- TCDD exposed chick, zebrafish and mouse embryos have reduced number of cardiac myocytes, which is due to decreased cardiomyocyte proliferation in the chick and mouse (Antkiewicz et al. 2005; Ivnitski et al. 2001; Thackaberry et al. 2005b)
    - Note that myocardial migration is dependent on epithelial integrity (Trinh and Stainier 2004)
- Endothelial tube length (40%±1.7%) and number (36%±3%) were significantly reduced in TDCC treated chick explants (cell culture derive from treated embryos) (Ivnitski-Steele and Walker 2003)
- TCDD reduced coronary artery number in chick embryos (by 53±8%) and reduced tube outgrowth and endothelial cell responsiveness to angiogenic stimuli in chick explants (Ivnitski-Steele et al. 2005)
- TCDD reduces human primary umbilical vein endothelial cells basal proliferation by 50% (Ivnitski-Steele and Walker 2005)
- The phenotype observed in chick embryos following TCDD exposure on day zero of incubation resembles that observed in vertebrate models in which the epicardium fails to form (Ivnitski-Steele and Walker 2005)
- Epicardial cells are missing from the surface of human hearts with ischemic cardiomyopathy (Di et al. 2010)

Key Event 5: Altered cardiovascular development/ function (Essentiality = Strong)

- The most common cause of infant death due to birth defects is congenital cardiovascular malformation (Kopf and Walker 2009)
- The most common heart abnormality observed in nestlings exposed to dioxin-like compounds is thinning of the ventricular wall (Carro et al. 2013); thinning was attributed to reduced cardiomyocyte proliferation in TCDD exposed chick and mouse embryos (Ivnitski et al. 2001; Thackaberry et al. 2005a)
- TCDD reduces blood flow and circulatory function in essentially all fish species studied, including medaka,

lake trout, rainbow trout, brook trout, and zebrafish (Ivnitski-Steele and Walker 2005).

Cardiotoxic effects of strong AHR-agonists

Zebrafish Embryo	Chicken Embryo	Mouse
• Reduced extension of common cardinal vein • Reduced blood flow • Reduced heart rate • Disrupted erythropoiesis • Decreased heart volume • Pericardial edema • Overt heart malformations	• Enlarged left ventricle • Increased heart rate • Increased myosin content • Reduced β-adrenergic responsiveness • Increased ANF mRNA • Arrhythmia • Increased apoptosis • Reduced myocyte proliferation • Pericardial edema • Overt heart malformations	Embryo/Fetus • Reduced heart-to-body weight • Reduced myocyte proliferation • Vascular remodeling 21 Days old • Increased heart-to-body weight • Increased left ventricle weight • Reduced heart rate • Cardiac hypertrophy • Increased ANF mRNA • Increased risk of heart disease

ANF= cardiac atrial natriuretic factor; an indicator of cardiac stress. Source: (Kopf and Walker 2009)

Key Event 6: Increased pericardial edema (Essentiality = Strong)

- Edema is a hallmark sign of developmental toxicity in fish, chick, and mammalian species exposed to strong AHR agonists early in embryogenesis (Carney et al. 2006)
    - Note that it presents as pericardial and yolk sac edema in fish, pericardial, peritoneal and subcutaneous edema on chicks, and peritoneal and subcutaneous edema in mice.
- Edema and hemorrhage are common developmental effects among species exposed to AHR-agonists prior to death; however, edema is a secondary effect rather than a primary target, as cardiotoxicity is observed prior to, or even without, the onset of edema (Walker et al. 1997; Walker and Catron 2000).
- Edema is not observed at sub-lethal doses of TCDD (Walker et al. 1997)

Weight of Evidence Summary

Summary Table

Provide an overall summary of the weight of evidence based on the evaluations of the individual linkages from the Key Event Relationship pages.

Support for Biological Plausibility of KERs	Defining Question	High (Strong)	Moderate	Low (Weak)
	Is there a mechanistic relationship between	Extensive understanding of the KER based on previous	KER is plausible based on analogy to accepted biological relationships, but scientific	Empirical support for association between KEs, but the structural or functional relationship

	KEup and KEdown consistent with established biological knowledge?	documentation and broad acceptance.	understanding is incomplete.	between them is not understood.
MIE => KE1:	Strong	The mechanism of AHR-mediated transcriptional regulation is well understood (Fujii-Kuriyama and Kawajiri 2010). ARNT is a necessary dimerization partner for the transcriptional activation of AHR regulated genes (Hoffman et al. 1991; Poland et al. 1976).		
KE1 => KE2:	Moderate	ARNT is common dimerization partner for both AHR and HIF-1α. Gel-shift and coimmunoprecipitation experiments have shown that the AHR and HIF1α compete for ARNT in vitro, with approximately equal dimerization efficiencies (Schmidt and Bradfield 1996). A number of studies have shown a reduced response to hypoxia following AHR activation (Chan et al. 1999, Seifert et al. 2008, Ivnitski-Steele et al. 2004), however this effect is highly tissue specific; in cells where ARNT is abundant, it does not deplete due to hypoxia or AHR activation (Chan et al. 1999; Pollenz et al. 1999)		
KE2 => KE3:	Strong	The transcriptional control of VEGF by HIF-1 is well understood; The HIF-1 complex binds to the VEGF gene promoter, recruiting additional transcriptional factors and initiating VEGF transcription (Ahluwalia and Tarnawski 2012; Fong 2009)		
KE3 => KE4:	Strong	The importance of VEGF for endothelial network formation and integrity is clear (Ivnitski-Steele and Walker 2005); loss of a single VEGF-A allele results in defective vascularization and early embryonic lethality (Carmeliet et al. 1996; Ferrara et al. 1996).		
KE4 => KE5:	Moderate	The importance of endothelial cell migration, proliferation and integrity in neovascularization and organogenesis is well documented. Development of vasculature into highly branched conduits needs to occur in numerous sites and in precise patterns to supply oxygen and nutrients to the rapidly expanding tissue of the embryo; aberrant regulation and coordination of angiogenic signals during development result in impaired organ development (Chung and Ferrara 2011; Ivnitski-Steele and Walker 2005). The extent to which the observed cardiovascular abnormalities are caused by deregulation of the underlying endothelial network remains unclear.		
KE5 => KE6:	Moderate	Severe cardiac dysfunction can result in congestive fetal heart failure leading to fluid build-up in tissues and cavities (edema and effusion, respectively)(Thakur et al. 2013). Pericardial edema is secondary to heart failure in zebrafish and chicken embryos as changes in heart morphology and decreases in cardiac output precede congestive heart failure (Henshel et al. 1993; Cheung et al. 1981; Canga et al. 1993; Walker et al. 1997; Antkiewicz et al. 2005; Belair et al. 2001; Henry et al. 1997; Plavicki et al. 2013). Furthermore, when mannitol is used as a protective agent against chemical-induced edema in zebrafish, cardiotoxic effects are still		

		observed (Antkiewicz et al. 2005; Plavicki et al. 2013).
KE6 => AO:	Moderate	The connection between edema and diminished cardiac output is well understood in fish, birds and mammals (Antkiewicz et al. 2005; Thakur et al. 2013; Walker 1998; Walker et al. 1997). Fluid buildup exerts a positive pressure on fetal cardiac chambers, further limiting the diastolic ventricular filling reserve, potentiating the diminished cardiac output and leading to fetal death (Thakur et al. 2013). Although pericardial edema consistently precedes embryo death in many fish species (Kopf and Walker 2009; Ivnitski-Steele and Walker 2005), the causal linkage is less clear in avian species. A number of studies report heart malformations leading to embryo death without the observation of pericardial edema (Cheung et al. 1981, Walker et al. 1997, Carro et al. 2013, Wikenheiser et al. 2012); in fact, subcutaneous edema is more often reported in chicken ebryos exposed to AHR agonists (Cheung et al. 1981, Brunstrom 1988, Brunstrom and Anderson 1988, Walker and Catron 2000).
MIE => AO:	Strong	Differences in species sensitivity to dioxin-like compounds have been associated with differences in the AHR amino acid sequence in mammals, fish and birds; the identity of these amino acids in the AHR ligand binding domain affects DLC binding affinity, AHR transactivation and therefore toxicity (Farmahin et al. 2012; Head et al. 2008; Karchner et al. 2006; Mimura and Fujii-Kuriyama 2003; Wirgin et al. 2011). The predictive ability of an LRG assay measuring induction of AHR1-mediated gene expression was demonstrated by linear regression analysis comparing log-transformed LD50 values obtained from the literature to log-transformed PC20 values from the LRG assay (Farmahin et al. 2013; Manning et al. 2012)

Quantitative Consideration

Summary Table

Provide an overall discussion of the quantitative information available for this AOP. Support calls for the individual relationships can be included in the Key Event Relationship table above.

The quantitative understanding of individual KERs in this AOP is weak; however, there is a strong correlation between the molecular initiating event (MIE: AHR activation) and adverse outcome (AO: embryolethality) in birds. This relationship is described in detail in the KER: AHR activation leads to embryolethality, found in the KER summary table. In brief, the AHR1 ligand binding domain (LBD) sequence alone could be used to predict DLC-induced embryolethality in a given bird species. The identity amino acids at two key positions within the LBD dictate the binding affinity of xenobiotics and therefore the strength of induction. AHR-mediated reporter gene induction can be measured using a luciferase reporter gene assay, the strength of which is correlated to the embryo-lethal dose of AHR aginists as shown below.

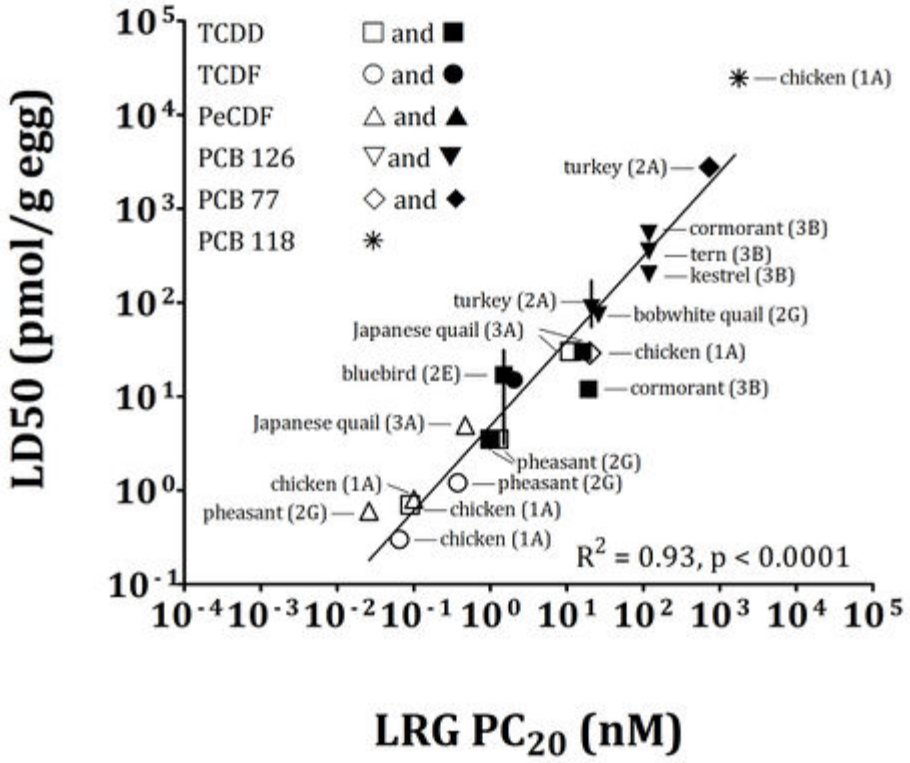


Figure 1. Linear regression analysis comparing LD50 values with PC20 (logLD50 = 0.79logPC20 + 0.51) values derived from luciferase reporter gene (LRG) assay concentration-response curves. Open symbols represent LRG data from wild-type chicken, ring-necked pheasant or Japanese quail AHR1 expression vectors. Closed symbols represent LRG data from mutant AHR1 (Source: Manning, G. E. et al. (2012). *Toxicol. Appl. Pharmacol.* 263(3), 390-399.)

Uncertainties and Inconsistencies

Although crosstalk between AHR and HIF1α clearly exists, the nature of the relationship is still not clearly defined. It has been suggested that HIF1α and AHR do not competitively regulate each other for hetero-dimerization with ARNT, as ARNT is constitutively and abundantly expressed in cells and does not deplete due to hypoxia or AHR activation (Chan et al. 1999; Pollenz et al. 1999). In indirect support of this, a mutant zebrafish model (caAHR-dbd) expressing an AHR that has lost DNA binding ability, but retains other functional aspects (such as dimerization and translocation) showed no signs of cardiotoxicity; this is in contrast to its counterpart (caAHR) in which sever cardiotoxicity was observed, having the same constitutive AHR expression level (Lanham et al. 2014). These results suggest that direct downstream transcription of AHR-regulated genes, not ARNT sequestration, is essential for cardiotoxicity. However, there is also considerable evidence demonstrating the inhibition of either AHR or HIF1α by activation of the other pathway. For example, TCDD inhibited the CoCl2 induction of a hypoxia response element (HRE) driven promoter and CoCl2 inhibited the TCDD induction of a dioxin response element (DRE) driven promoter, in Hep3B cells (Chan et al. 1999). TCDD also reduced HIF1α nuclear-localized staining in most areas of the heart in chick embryos (Wikenheiser et al. 2012), reduced the stabilization of HIF1α and HRE-mediated promoter activity in Hepa 1 cells and reduced hypoxia-mediated reporter gene activity in B-1 cells (Nie et al. 2001), whereas hypoxia inhibited AHR-mediated CYP1A1 induction in B-1 and Hepa 1 cells, but not H4IIE-luc (Nie et al. 2001). Some studies have shown that the effect of hypoxia on AHR mediated pathways is stronger than the reverse (Gassmann et al. 1997; Gradin et al. 1996; Nie et al. 2001; Prasch et al. 2004), which has been attributed to the stronger binding affinity of HIF1α to ARNT relative to AHR (Gradin et al. 1996). Contrary to this pattern, the combined exposure of juvenile orange spotted grouper to benzo[a]pyrine (BaP; an AHR agonist) and hypoxia, enhanced hypoxia-induced gene expression but did not alter BaP-induced gene expression (Yu et al. 2008). All in

all, it appears the effect of cross-talk between AHR and HIF1α is highly dependent on tissue type and life stage, leading to seemingly contradictory results and making it difficult to elucidate a mechanism of action with high confidence.

There is significant evidence suggesting that sustained AHR activation during embryo development results in reduced cardiac VEGF expression (See KER pages for details); however, the opposite relationship has also been observed. In human microvascular endothelial cells, hexachlorobenzene (weak AHR agonist) exposure enhanced VEGF protein expression and secretion. TCDD induced VEGF-A transcription and production in retinal tissue of adult mice and in human retinal pigment epithelial cells (Takeuchi et al. 2009) and induced VEGF secretion from human bronchial epithelial cells (adult) (Tsai et al. 2015). It has been reported that the AHR/ARNT heterodimer binds to estrogen response elements, with mediation of the estrogen receptor (ER), and activates transcription of VEGF-A (Ohtake et al. 2003). The potential involvement of AHR in opposing regulatory cascades (directly inducing VEGF through ER and indirectly suppressing it by ARNT sequestration) helps explain the conflicting results found in the literature. Further complicating the picture is the potential for HIF-1-independent regulation of VEGF, as illustrated in an ARNT-deficient mutant cell line (Hepa1 C4) in which VEGF expression was only partially abrogated (Gassmann et al. 1997).

Alternate Pathways

Altered metabolism of the membrane lipid arachidonic acid (AA) by CYP1A enzymes is another potential mechanism of embryotoxicity. Induction of CYP1A is associated with increased production of AA epoxides that can lead to cytotoxicity and increased susceptibility to injury from oxidative stress due to increased production of oxygen radicals (Toraason et al. 1995). It has been suggested that cyclooxygenase 2 (COX-2) is essential in this toxic response as TCDD-induced morphological defects and edema in the heart were accompanied by COX-2 induction and were prevented with COX-2 inhibitors in fish (Dong et al. 2010; Teraoka et al. 2008). In chick embryos, TCDD-induced mortality, left ventricle enlargement and cardiac stress were prevented by selective COX-2 inhibition (Fujisawa et al. 2014). A non-genomic pathway (ie. ARNT-independent) was suggested as a mechanism for the AHR-mediated induction of COX-2 in which ligand-binding causes a rapid increase in intracellular Ca2+ concentration, activating cytosolic phospholipase A2, inducing COX-2 expression and resulting in an inflammatory response (Matsumura 2009). Interestingly, VEGF-A mRNA was up-regulated 2.7-fold by TCDD and was unaffected by COX-2 inhibition (Fujisawa et al. 2014) Studies investigating the role of CYP1A induction in mediating vascular toxicity have been contradictory, with some studies demonstrating that CYP1A mediates vascular toxicity (Cantrell et al. 1996; Dong et al. 2002; Teraoka et al. 2003), others demonstrating that it does not have an effect (Carney et al. 2004; Hornung et al. 1999), and some showing it to play a protective role (Billiard et al. 2006; Brown et al. 2015). Overall, cardiotoxicity is unlikely a downstream effect of CYP1A induction, but its generation of ROS and therefore oxidative stress likely contributes to the toxicity. Finally, since AHR has a role in heart development that is independent of exogenous ligand-mediated activation, it has been suggested that exogenous AHR ligands sequester it away from its endogenous function (Carreira et al. 2015). Cardiotoxicity may be mediated by Homeobox protein NKX2-5, an essential cardiogenesis transcription factor, as its expression was decreased in AHR-null mice.

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