AOP ID and Title:

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AOP 131: AhR activation leading to uroporphyria

Short Title: AHR activation-uroporphyria

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Abstract

Hepatic uroporphyria is a disorder where the disturbance of heme biosynthesis results in accumulation and excretion of uroporphyrin, heptacarboxylic acid and hexacarboxylic acid: collectively referred to as highly carboxylated porphyrins (HCPs)[1][2][3]. The disorder can be genetically acquired, due to a dysfunction in any of the 7 enzymes involved in the heme biosynthesis pathway [4], or may be chemically induced, which involves the inhibition of uroporphyrinogen decarboxylase (UROD). This adverse outcome pathway (AOP) describes the linkages leading to chemically induced porphyria through the activation of the aryl hydrocarbon receptor (AHR), a transcription factor that plays important endogenous roles in reproduction, liver and heart development, cardiovascular function, immune function and cell cycle regulation [5][6][7][8][9][10][11][12][13][14]. This AOP was developed in accordance with OECD guidelines and demonstrates a high degree of confidence as a qualitative AOP. The quantitative understanding of this AOP however is not yet complete, preventing the accurate prediction of uroporphyria from lower level key events.

Summary of the AOP

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 assuming the coplanar conformation required for binding to the AHR [45]. 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].

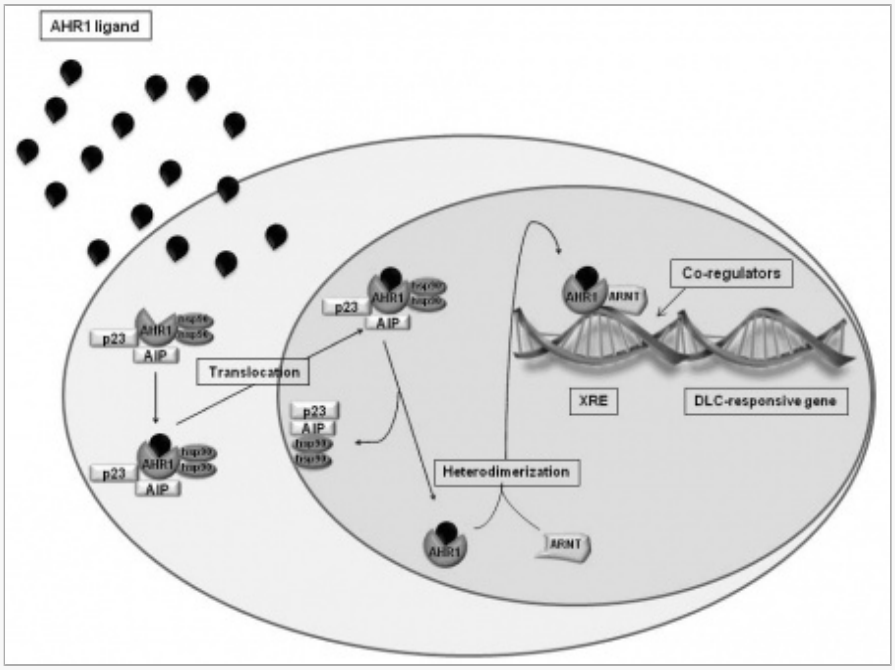
The first study that attempted to elucidate the role of avian AHR1 domains and key amino acids within avian AHR1 in avian differential sensitivity was performed by Karchner *et al.*[22]. Using chimeric AHR1 constructs combining three AHR1 domains (DBD, LBD and TAD) from the chicken (highly sensitive 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 liver[10], 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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1.0 1.1

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Key Events

Title	Short name
Oxidation, Uroporphyrinogen	Oxidation, Uroporphyrinogen
Inhibition, UROD	Inhibition, UROD
Accumulation, Highly carboxylated porphyrins	Accumulation, Highly carboxylated porphyrins
Induction, CYP1A2/CYP1A5	Induction, CYP1A2/CYP1A5

844: Oxidation, Uroporphyrinogen

Short Name: Oxidation, Uroporphyrinogen

AOPs Including This Key Event

AOP ID and Name	Event Type
131: AhR activation leading to uroporphyria	KeyEvent

Biological Organization

Level of Biological Organization
Cellular

How this Key Event Works

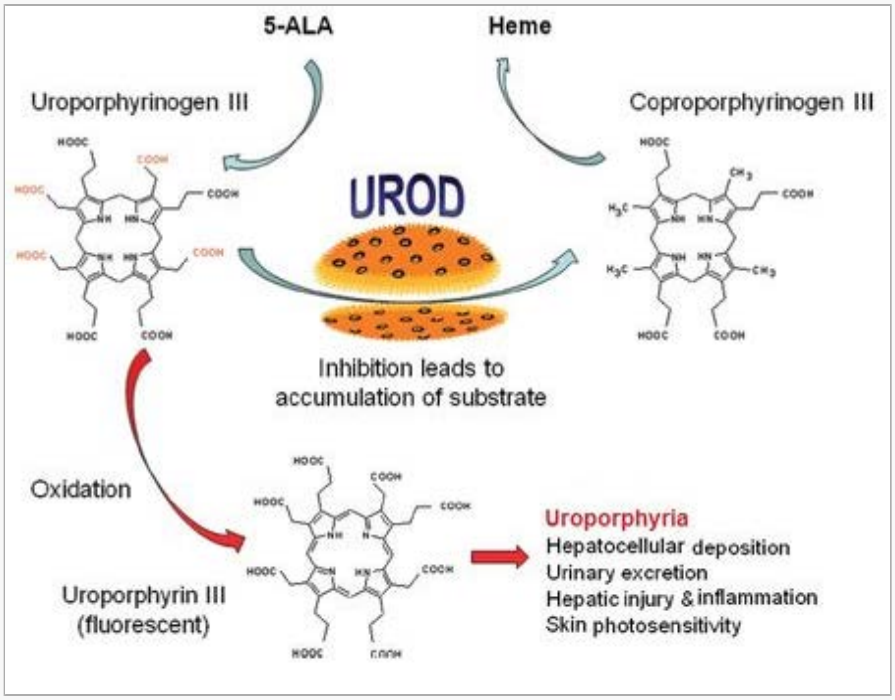

Figure 1:Oxidation of the heme precursor uroporphyrinogen III to uroporphyrin III leading to symptoms of the disorder porphyria. UROD: uroporphyrinogen decarboxylase. (Modified from Smith and Elder (2010) *Chem. Res. Toxicol.* **23** (4), 712-723.

Uroporphyrinogen III is the first cyclic metabolic intermediate in the biosynthesis of heme. Under normal conditions, it is converted into coproporphyrinogen III by the enzyme uroporphyrinogen decarboxylase (UROD), and subsequently processed to heme following three further steps[1]. In the event that UROD activity is reduced (due to genetic disorders or chemical inhibition) uroporphyrinogen III, and other porphyrinogen substrates of UROD, are oxidized to highly stable porphyrins, which accumulation and lead to a heme disorder known as porphyria (Figure 1)[2].

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?

Porphyrins fluoresce red when exposed to UV light; therefore, uroporphyrinogen oxidation (UROX) can be directly measured as uroporororphyrin fluorescence in a spectrophotofluorimeter. UROX has been measured spectrofluorimetrically in avian[3] mammalian[4] and aquatic[5] species.

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845: Inhibition, UROD

Short Name: Inhibition, UROD

AOPs Including This Key Event

AOP ID and Name	Event Type
131: AhR activation leading to uroporphyria	KeyEvent

Biological Organization

Level of Biological Organization
Molecular

How this Key Event Works

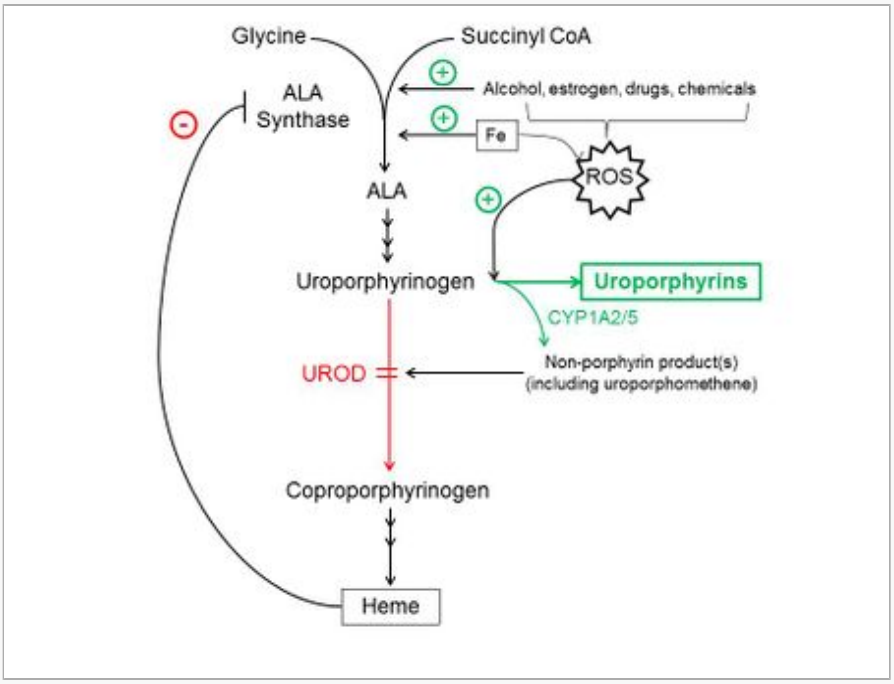

Figure 1: Disruption of the normal heme biosynthesis pathway by uroporphyrinogen decarboxylase (UROD) inhibition. Formation of the inhibitor (suggested as being uroporphomethene) is thought to require the action of the phase I metabolizing enzyme, CYP1A2. Synergistic induction of ALA synthase 1 and increases in oxidative stress (reactive oxygen species (ROS)), caused by alcohol, estrogens and xenobiotics, potentiate the accumulation of porphyrins and therefore the porphyric phenotype. (Modified from Caballes (2012) *Liver Int.* **32** (6), 880-893.)

Uroporphyrinogen decarboxylase (UROD) is the fifth enzyme in the heme biosynthesis pathway and catalyzes the step-wise conversion of uroporphyrinogen to coproporphyrinogen. Each of the four acetic acid substituents is decarboxylated in sequence with the consequent formation of hepta-, hexa-, and pentacarboxylic porphyrinogens as intermediates[1]. Impairment of this enzyme, either due to heterozygous mutations in the UROD gene or chemical inhibition of the UROD protein, leads to accumulation of uroporphyrins (and other highly carboxylated porphyrins) and ultimately a disorder known as uroporphyria[2]. Sufficient overproduction of porphyrins to cause symptoms does not usually occur until hepatic UROD activity is reduced by at least 70%[3].

Several lines of evidence support the concept that a UROD inhibitor is formed within the liver by the oxidation of uroporphyrinogen. Phillips et al.[4] identified this inhibitor as being uroporphomethene using a murine model for porphyria; however, their interpretation of the mass spectroscopy results has been criticized as inaccurate[5], leaving the exact characterization of the UROD inhibitor unresolved. None the less, it has been demonstrated that inhibitor formation is increased by (a) the induction/activation of the phase I metabolizing enzyme cytochrome P4501A2 (orthologous to avian CYP1A5), (b) iron loading, (c) alcohol excess, and (d) estrogen therapy (Figure 1)[6]. A negative-feedback loop exists in which the end-product (heme) represses the enzyme ALA synthase 1 and prevents excess formation of heme. When UROD activity is low, the regulatory heme pool is potentially depleted, causing a repression of the negative feedback loop, thereby increasing levels of precursors and furthering the accumulation of porphyrins.

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?

Due to the high instability of porphyrinogens, they must be synthesized as an integral part of the enzyme assay for use as a substrate. Uroporphyrinogen can either be generated by enzymatic synthesis or chemical reduction[7]. The former makes use of bacterial porphobilinogen deaminase to prepare the porphyrinogen substrate and the latter often utilizes sodium amalgam or sodium borohydride under an inert gas. Chemical reduction however often involves large quantities of mercury or extremely alkaline conditions and requires significant purification before the enzyme assay can be performed. Bergonia and colleagues[8] suggest palladium on carbon (Pd/C) to be the most efficient and environmentally friendly chemical preparation of porphyrinogens as Pd/C is more stable than sodium amalgam and can easily be removed by filtration, eliminating the need for laborious purification.

Once uroporphyrinogen is synthesized it is co-incubated with UROD under standardized conditions. The reaction is then stopped, reaction products and un-metabolized substrate are esterified, and the porphyrin esters are separated and quantified using high performance liquid chromatography[7]. This enzyme assay classically utilizes milliliter quantities but has been modified to a microassay, minimizing cost and enhancing sensitivity[9].

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846: Accumulation, Highly carboxylated porphyrins

Short Name: Accumulation, Highly carboxylated porphyrins

AOPs Including This Key Event

AOP ID and Name	Event Type
131: AhR activation leading to uroporphyria	KeyEvent

Biological Organization

Level of Biological Organization
Organ

How this Key Event Works

Under normal conditions, the heme biosynthesis pathway is tightly regulated and porphyrins (other than protoporphyrin) are only present in trace amounts[1]. However, when the regulatory process is disturbed, a variety of porphyrin precursors of heme accumulate in various organs including the liver and urinary and fecal excretion is elevated[2]). The pattern of porphyrin accumulation in chicken and rodents is similar following exposure to a variety

of chemicals, and can be used to identify which enzyme in the heme pathway is predominately affected. In the case of uroporphyrinogen decarboxylase inhibition, uroporphyrin, hepta-, and hexacarboxylic acid porphyrin (highly carboxylated porphyrins) are elevated[1].

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 hepatic and urinary/fecal porphyrin patters can be determined using a high-performance liquid chromatograph equipped with a fluorescence detector. Kennedy *et al.*[3] describe the method for tissue extraction and porphyrin quantification in detail, which is rapid and highly sensitive.

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850: Induction, CYP1A2/CYP1A5

Short Name: Induction, CYP1A2/CYP1A5

AOPs Including This Key Event

AOP ID and Name	Event Type
131: AhR activation leading to uroporphyria	KeyEvent

Biological Organization

Level of Biological Organization
Molecular

How this Key Event Works

The aryl hydrocarbon receptor (AHR) is involved in the regulation of several genes, including phase I (e.g. CYP1A, CYP1B, CYP2A) and phase II enzymes (e.g. UDP-GT, GSTs)[1][2][3]. Upon ligand binding, the AHR migrates to the nucleus where it dissociates from the cytosolic complex and forms a heterodimer with ARNT[3]. 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[1].

The Cyp1A2/Cyp1A5 gene encodes a member of the cytochrome P450 superfamily of enzymes. The cytochrome P450 proteins are monooxygenases which catalyze many reactions involved in drug metabolism and synthesis of cholesterol, steroids and other lipids. The protein encoded by this gene localizes to the endoplasmic reticulum and its expression is induced by some polycyclic aromatic hydrocarbons (PAHs), some of which are found in cigarette smoke. The enzyme's endogenous substrate is unknown; however, it is able to metabolize some PAHs to carcinogenic intermediates. Other xenobiotic substrates for this enzyme include caffeine, aflatoxin B1, and acetaminophen. [4]

Of all AHR-regulated genes, the CYP1A subfamily of enzymes is the most studied and is often used as a biomarker of Dioxin-like compound (DLC) exposure and toxicity[5][6][7][8]. CYP1A5 is the avian isoform and is orthologous to the mammalian CYP1A2[9]. CYP1A5 is expressed in avian heart, liver and kidney tissues[10][11], and DLC-mediated induction of CYP1A5 mRNA has been measured in avian hepatocyte and cardiomyocyte cultures[12][13][10][14]. Positive correlations between hepatic CYP1A expression and DLC concentrations have also been reported in wild bird species, including bald eagles (*Haliaeetus leucocephalus*)[15], great blue herons (*Ardea herodias*)[16], double-crested cormorants (*Phalacrocorax auritus*)[17], black-crowned night herons (*Nycticorax nycticorax*)[18] and ospreys (*Pandion haliaetus*)[19]. Mouse CYP1A2 is only constitutively expressed in the liver, but is inducible in the liver, lung, and duodenum[20].

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?

Enzyme activity

There are a number of substrates that are preferentially metabolized by Cyp1A2 and CYP1A5 allowing for CYP1A activity to be measured as a function metabolite formation. Methoxyresorufin O-demethylation (MROD) is a classic marker of Cyp1A2/5 activity[21] and is often used due to the ease of fluorometric techniques; however, Burke *et al.*[21] suggest that a ratio of MROD to ethoxyresorufin O-demethylation (EROD) is a better measure of CYP1A2 activity due to the contribution of CYP1A1 to MROD. CYP1A2/5 activity can also be measured as the metabolic rate of arachidonic acid[11], oroporphyrinogen[22], acetanilide 4-hydroxylase and caffeine[23]. Caffeine metabolism has been used in clinical studies as a biomarker for CYP1A2 activity in humans[24].

Quantitative polymerase chain reaction (QPCR)

Levels of CYP1A2/5 messenger RNA can be measured using QPCR. This technique monitors the amplification of a targeted gene during PCR as accumulative fluorescence [25]. For example, Head and Kennedy[26] developed a multiplex QPCR assay utilizing dual-labeled fluorescent probes to measure CYP1A4 and CYP1A5 mRNA levels simultaneously from samples already analyzed for EROD activity. QPCR has high throughput capability and a low detection limit relative to other methods.

Luciferase reporter gene (LRG) assay

An LRG assay can be used to measure AHR1-mediated transactivation of a target gene. This assay is particularly useful as it can measures CYP1A4/5 induction exclusively caused by activation of the AHR, through which many DLCs exert their toxic effects. This assay is easily modified to measure AHR1-mediated transactivation in various species, simply by transfecting the desired AHR cDNA clone and reporter gene construct (containing the appropriate reporter gene) into the chosen cell line. This has been demonstrated to be an efficient high throughput method in various avian and mammalian studies.[27][28][29]

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Adverse Outcomes

Title	Short name

N/A, Uroporphyria	N/A, Uroporphyria

369: N/A, Uroporphyria

Short Name: N/A, Uroporphyria

AOPs Including This Key Event

AOP ID and Name	Event Type
131: AhR activation leading to uroporphyria	AdverseOutcome

Biological Organization

Level of Biological Organization
Individual

Chemical-induced uroporphyria has only been detected in birds[7][1][8] and mammals[5] , including an accidental outbreak in humans due to hexachlorobenzen-contaminated grain in the 1950s[9]. Fish are less susceptible to chemical-induced uroporphyria, but elevated levels of HCP have been documented in highly contaminated environments[10].

Differences in avian species sensitivity to chemical-induced porphyrin accumulation have been observed. Common tern embryo hepatocyte cultures did not accumulate porphyrins in response to dioxin-like compound (DLC) exposure and were 50 to over 1600 times less sensitive than chicken embryo hepatocyte cultures to DLC-mediated CYP1A induction[11]. Porphyrin accumulation in ring-necked pheasant embryo hepatocyte cultures following PCDD, PCDF and PCB exposure was also significantly less than that observed in chicken embryo hepatocytes[12]. These results are consistent with the predicted species sensitivity based on AHR1 sequence, as ring-necked pheasants are type 2 (Ile324_Ala380; moderately sensitive), and common terns are type 3 (Val324_Ala380; least sensitive) species[13][14][15].

How this Key Event Works

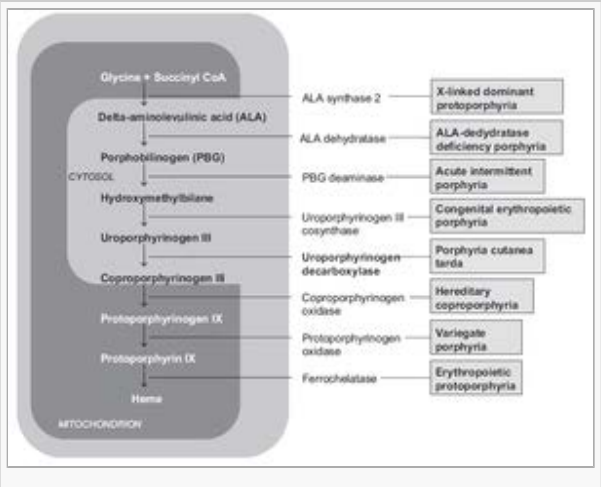

Figure 1: The heme biosynthetic pathway. Deficiency in a particular gene along the pathway results in the indicated form of porphyria: 8 separate disorders that are characterized by hepatic accumulation and increased excretion of porphyrins. Source: Frank, J., and Poblete-Gutierrez, P. (2010) Porphyria cutanea tarda--when skin meets liver. *Best. Pract. Res. Clin Gastroenterol.* **24** (5), 735-745.

Porphyria is a disorder in which the disturbance of heme biosynthesis results in accumulation and excretion of porphyrins[1]. A variety of porphyrias exist depending on which enzyme in the pathway is deficient (Figure 1). In the case of chemically induced porphyria, uroporphyrinogen decarboxylase (UROD), which converts uroporphyrinogen to coproporphyrinogen, is inhibited. The phase I metabolizing enzyme CYP1A2/CYP1A5 is believed to catalyzes the oxidation of uroporphyrinogen to uroporphyrin, leading to uroporphyrin accumulation and liver damage[2][3]. In humans, this disorder is known as porphyria cutanea tarda (PCT) and may be caused by chemical exposure or a hereditary deficiency in UROD[4]. The accumulation of porphyrins in the liver causes cirrhosis, mild fatty infiltration, patchy focal necrosis, and inflammation of portal tracts. When the activity of UROD is reduced to less than 30% of normal, the disorder manifests as an overt skin disease; the accumulation of porphyrins in the skin causes photosensitization that is characterized by fragile skin, superficial erosions, sub-epidermal bullae, hypertrichosis, patchy pigmentation and scarring[5].

How it is Measured or Detected

Porphyria is easily confirmed through a urinary or fecal analysis to measure the levels and pattern of excreted porphyrins. Samples are quantified using a high-performance liquid chromatograph equipped with a fluorescence detector[6]. Frank and Poblete-Gutiérrez[4] illustrate how the types of porphyria can be differentiated by the relative abundance of different porphyrins (Figure 2). Uroporphyria is the animal model equivalent to human porphyria cutanea tard [5]

Type of Porphyria	Enzyme Defect	Enzyme Assay Available	Biochemical Findings – Increased Levels Seen in Affected Patients			
			Urine	Feces	Erythrocytes	Plasma
Aminolevulinic acid dehydratase deficiency porphyria	Aminolevulinic acid dehydratase	Yes	Aminolevulinic acid, Coproporphyrin	NA	Zinc protoporphyrin	NA
Acute intermittent porphyria	Porphobilinogen deaminase	Yes	Porphobilinogen, Aminolevulinic acid, Uroporphyrin, Coproporphyrin*	Uroporphyrin*	NA	Uroporphyrin*
Hereditary coproporphyria	Coproporphyrinogen oxidase	No	Coproporphyrin, Porphobilinogen, Aminolevulinic acid	Coproporphyrin III Coproporphyrin III/ Coproporphyrin I ratio	NA	Coproporphyrin*
Variegate porphyria	Protoporphyrinogen oxidase	No	Coproporphyrin, Porphobilinogen, Aminolevulinic acid	Protoporphyrin > Coproporphyrin III Coproporphyrin III/ Coproporphyrin I ratio	NA	Coproporphyrin, Protoporphyrin*
X-linked dominant protoporphyria	Aminolevulinic acid synthase 2	No	NA	Protoporphyrin*	Zinc protoporphyrin, Free protoporphyrin	Protoporphyrin
Congenital erythropoietic porphyria	Uroporphyrinogen III synthase	Yes	Uroporphyrin, Coproporphyrin	Coproporphyrin I	Uroporphyrin, Coproporphyrin, Zinc protoporphyrin	Uroporphyrin, Coproporphyrin
Porphyria cutanea tarda	Uroporphyrinogen decarboxylase	Yes	Uroporphyrin, Heptacarboxyl-porphyrin	Isocoproporphyrin, Heptacarboxyl-porphyrin III	NA	Uroporphyrin, Heptacarboxyl-porphyrin
Hepatoerythropoietic porphyria	Uroporphyrinogen decarboxylase	Yes	Uroporphyrin, Heptacarboxyl-porphyrin	Isocoproporphyrin, Heptacarboxyl-porphyrin III, Coproporphyrin I,	Zinc protoporphyrin	Uroporphyrin, Heptacarboxyl-porphyrin
Erythropoietic protoporphyria	Ferrochelatase	No	NA	Protoporphyrin	Free protoporphyrin	Protoporphyrin

Informative Biochemical Findings in Porphyria

*sometimes

(Source: http://www.mayomedicallaboratories.com/articles/communique/2015/03-porphyria-testing/; Accessed December 9, 2015.)

Figure 2: Biochemical characteristics of the porphyrias in urine, stool, and blood (plasma and erythrocytes). Source: http://www.mayomedicallaboratories.com/articles/communique/2015/03-porphyria-testing/; Accessed December 9, 2015

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Scientific evidence supporting the linkages in the AOP

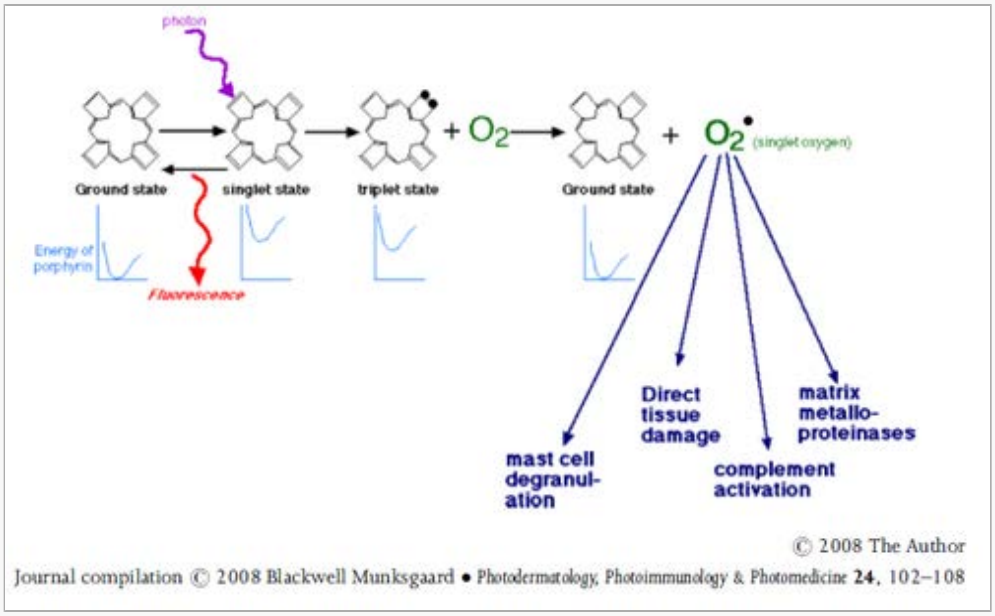
Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Accumulation, Highly carboxylated porphyrins	directly leads to	N/A, Uroporphyria	Strong	Strong
Induction, CYP1A2/CYP1A5	directly leads to	Oxidation, Uroporphyrinogen	Moderate	Weak
Activation, AHR	directly leads to	Induction, CYP1A2/CYP1A5	Strong	Strong
Oxidation, Uroporphyrinogen	directly leads to	Inhibition, UROD	Moderate	Weak
Inhibition, UROD	directly leads to	Accumulation, Highly carboxylated porphyrins	Moderate	Moderate

Accumulation, Highly carboxylated porphyrins leads to N/A, Uroporphyria

This relationship exists in birds[3] and mammals, including humans[4].

How Does This Key Event Relationship Work

Accumulation of porphyrins causes both physical and chemical damage to tissues, resulting in what is generally termed porphyria. The ability of porphyrins to absorb light of 400–410 nm (the Soret band) is the key factor in producing the photocutaneous lesions observed on sun exposed areas in affected individuals. The porphyrins absorb this light and enter a high energy state, which is then transferred to molecular oxygen resulting in reactive oxygen species (ROS). These ROS cause phototoxic damage and further catalyze the oxidation of porphyrinogens to porphyrins. Some porphyrins, mainly uroporphyrin and heptacarboxyl porphyrin, form needle-shaped crystals resulting in hydrophilic cytoplasmic inclusions[1]. Porphyrins demonstrate a range of water solubilities, and therefore show unique tissue and cellular distributions, resulting in different patterns of phototoxic damage histologically and cytologically[2].



Violet light excites the delocalized electrons in porphyrins. If the energy is not given out as red fluorescent light, it is passed onto oxygen to form tissue damaging free radicals. (Source: Sarkany, R. P. (2008).Photodermatol. Photoimmunol. Photomed. 24(2), 102-108.)

Weight of Evidence

Biological Plausibility

The mechanism by which porphyrins cause tissue damage is well understood[1][2]

Empirical Support for Linkage

Include consideration of temporal concordance here

Uroporphyria is defined as the accumulation and excretion of uroporphyrin, heptacarboxylic acid and hexacarboxylic acid: collectively referred to as highly carboxylated porphyrins (HCPs)[3]. It is the animal model equivalent to the human disorder, porphyria cutanea tarda[4].

Uncertainties or Inconsistencies

No current inconsistencies to report.

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?

According to the U.S. National Library of Medicine[5], red blood cell porphyrin levels in healthy individuals should lie within the following ranges:

- Total porphyrin levels: 16 to 60 mcg/dL
- Coproporphyrin level: < 2 mcg/dL
- Protoporphyrin level: 16 to 60 mcg/dL
- Uroporphyrin level: < 2 mcg/dL

The European Porphyria Network details the minimum laboratory requirements necessary for diagnosing each type of porphyria [6].

Excessive accumulation of porphyrins can lead to the neuropsychiatric symptoms of hereditary hepatic porphyrias; Andrade *et al*.[7] demonstrate that a linear combination of urinary prophyrin levels in rats exposed to heavy metals can predict the magnitude of the resulting neurotoxicity.

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Induction, CYP1A2/CYP1A5 leads to Oxidation, Uroporphyrinogen

CYP1A2 catalyzes UROX in mice, rats and humans[1][2][11], as does CYP1A5 in chickens[3], but may not be essential for UROX in humans[11].

How Does This Key Event Relationship Work

The oxidation of uroporphorynogen to its corresponding porphyrin (UROX) is preferentially catalyzed by the phase one metabolizing enzyme, CYP1A2, in mammals[1][2] and CYP1A5 in birds[3]. Uroporphyrinogen, an intermediate in heme biosynthesis, is normally converted to coproporphyrinogen by uroporphyrinogen decarboxylase (UROD)[4]; induction of CYP1A2 expression translates to increased protein levels and increased oxidation of uroporphyrinogen, preventing its conversion to coproporphyrinogen and leading to uroporphyrin accumulation.

Weight of Evidence

Biological Plausibility

Uroporphyrinogen has clearly been identified as a substrate of CYP1A2/5, which results in its oxidation to uroporphyrin[1][2][3].

Empirical Support for Linkage

Include consideration of temporal concordance here

UROX activity is increased by inducers of the CYP1A subfamily[4][1] and inhibited by substrates of CYP1A2[2], indicating that uroporphorynogen binds to the active site of CYP1A2. Furthermore, mice with a higher endogenous level of CYP1A2 are more susceptible to porphyrin accumulation[5] and CYP1A2 knock-out prevents chemical-induced uroporphyria all-together[6][7][8]; therefore, CYP1A2 is essential for UROX. A mild porphyric response was observed in the presence of iron loading and 5-aminolevulinic acid (ALA; a heme precursor) in AHR-/- mice, indicating that CYP1A2 induction is not absolutely necessary, but that constitutive CYP1A2 levels are sufficient for UROX under certain conditions[9].

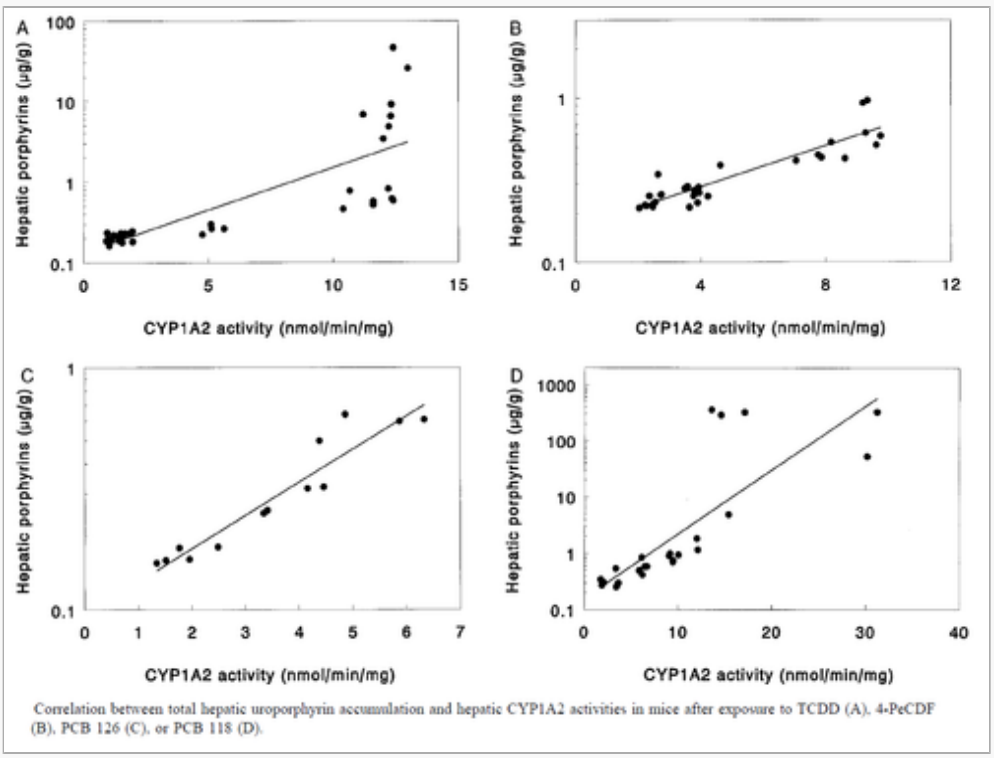
Uncertainties or Inconsistencies

Although CYP1A2 plays an essential role in UROX in animal models of porphyria, it seems to be less significant in human development of porphyria cutanea tarda. UROX activity in human liver microsomes was not correlated with CYP1A2 content. Experiments with different expression systems confirmed that human CYP1A2 catalyzes UROX, but with a lower specific activity than that of the mouse orthologue[10].

It is also worth noting that there exists a secondary, CYP1A2-independent pathway to UROX that is hypothesized to depend solely on iron. Phillips et al.[11] were able to generate uroporphyria in a Cyp1A2-/- mouse model that is genetically predisposed (Hfe-/-, Urod-/+) to develop porphyria in the absence of external stimuli; CYP1A2 knockout alone prevented porphyrin accumulation, but with the addition of iron and ALA to the triple knockout, modest porphyria was observed. Therefore, under extreme porphyric conditions, UROX can occure in the absence of the CYP1A2 enzyme.

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?



Correlation between total hepatic uroporphyrin accumulation and hepatic CYP1A2 activities in mice after exposure to TCDD (A), 4-PeCDF (B), PCB 126 (C), or PCB 118 (D). (Source: van Birgelen *et al.* (1996). Toxicol. Appl.

Pharmacol. **138** (1), 98-109.)

UROX is positively correlated with CYP1A2/5 activity[12] but this relationship has not been quantitatively describes. It has been noted however, that a CYP1A2 induction of just 2-fold dramatically induces porphyrin accumulation in iron-loaded mice[5].

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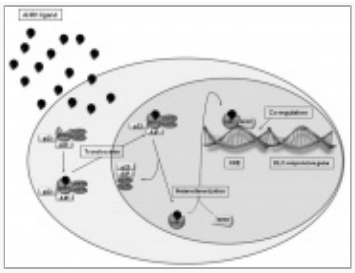
Activation, AHR leads to Induction, CYP1A2/CYP1A5

How Does This Key Event Relationship Work



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)[1]. Upon ligand binding, the AHR migrates to the nucleus where it dissociates from the cytosolic complex and forms a heterodimer with ARNT[2]. 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 of gene expression[1].

Weight of Evidence

Biological Plausibility

There is a strong mechanistic understanding of AHR-mediated induction of CYP1A genes[1].

Empirical Support for Linkage

Include consideration of temporal concordance here

It is well established that the extent of CYP1A induction is directly proportional to the strength of ligand binding to the AHR[3][4][5]. Two sites within the ligand binding domain (LBD)of the AHR have been identified (positions 375 and 319 in mammals; equivalent to positions 380 and 324 in birds) as being responsible for the range of binding affinities of dioxin-like compound (DLCs) and their corresponding efficacy (transactivation potential).[4][3][6][7][8][9][10] A similar investigation in sturgeon (fish) revealed that the residue at position 388 of the LBD of AHR2 was responsible for differences in sensitivity between White Sturgeon and Lake Sturgeon, both of which are endangered species[11]. Furthermore, Hestermann et al.[12] described that compounds with a high intrinsic efficacy demonstrate a 1:1 relationship between AHR binding affinities and CYP1A protein induction.

Uncertainties or Inconsistencies

There are no knowledge gaps or inconsistencies/conflicting lines of evidence for this KER.

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 relationship between chemical structure and response potencies of AHR ligands has been well studied. With additional knowledge of intrinsic efficacy, it is possible to build a computational model of receptor action to predict transactivation potential, as demonstrated by Hestermann et al.[12]. This model was able to explain the less-than additive effect of some DLC mixtures on AHR activation. Quantitative structure-activity relationships (QSARs) have also been developed[13][14].

As mentioned above, the identity of two amino acids within the LBD of the AHR can also be used to predict transactivation sensitivity. This quality has been studied extensively in birds, resulting in the categorization of bird

species into 3 groups: type 1, high sensitivity (e.g. chicken); type 2, moderate sensitivity (e.g. ring-necked pheasant); and type 3, low sensitivity (e.g. Japanese quail)[5][4][15]. Furthermore, a non-invasive method for RNA extraction using plucked feathers has been determined[16], making it possible to predict the sensitivity of any bird species to AHR agonists, and mediating the selection of priority species for risk assessment purposes.

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Oxidation, Uroporphyrinogen leads to Inhibition, UROD

A hepatically generated UROD inhibitor has been detected in porphyric mice[7] and rats[6], and humans with porphyria cutanea tarda[1]).

How Does This Key Event Relationship Work

One of the oxidation products of uroporphyrinogen is believed to be a competitive inhibitor of uroporphyrinogen decarboxylase (UROD). This inhibitor binds to the active site of UROD preventing the normal synthesis of heme and attenuating the accumulation of hepatic porphyrins[1]. The formation of this inhibitor is increased by iron, a well-known oxidant, by activity of cytochrome P-4501A2, by alcohol excess and by oestrogen therapy[2].

Weight of Evidence

Biological Plausibility

Reduced UROD enzyme activity, not protein levels, is characteristic of uroporphyria in humans and rats[3][4][5], indicating that disrupted decarboxylation is due to an enzyme inhibitor rather that a reduction in protein synthesis. Early reports confirmed the presence of a UROD inhibitor in porphyric animal models that was not present in animals resistant to chemical-porphyria under the same conditions[6][7]. The identity of this UROD inhibitor is not yet agreed upon, but there is a general consensus among the scientific community that it is an oxidation product of uroporphyrinogen or hydroxymethylbilane (the tetrapyrrole precursor of uroporphyrinogen)[2].

Empirical Support for Linkage

Include consideration of temporal concordance here

Phillips *et al*.[1] identified uroporphomethene, a compound in which one bridge carbon in the uroporphyrinogen macrocycle is oxidized, as a potent UROD inhibitor derived from the liver or porphyric mice.

Uncertainties or Inconsistencies

The characterization of the inhibitor isolated by Phillips *et al*.[1] has been criticized by Danton and Lim[8]. Namely, they claim that the high-performance liquid chromatography/electrospray ionization tandem mass spectrometry results were interpreted incorrectly. They analyzed the fragmentation pattern themselves, and concluded that the compound is not a tetrapyrrole or an uroporphyrinogen or uroporphyrin related molecule, but rather a poly(ethylene glycol) structure.

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?

This linkage has not been quantitatively characterized.

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Inhibition, UROD leads to Accumulation, Highly carboxylated porphyrins

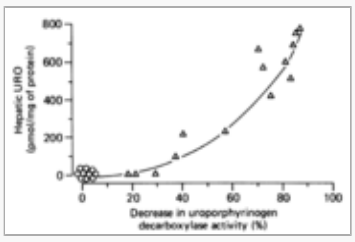
Chemical induces porphyrin accumulation has been demonstrated in, rats, mice and chicken[18][4][2]. Human porphyria cutanea tarda is also characterized biochemically by an increase in porphyrinogen oxidation leading to accumulation of porphyrins[15]. The correlation between reduced UROD activity and HCP accumulation in mammals is well defined[15][16][17] but is less consistent in avian models[14].

How Does This Key Event Relationship Work

Through the normal heme biosynthesis pathway, uroporphyrinogen is converted to coproporphyrinogen by uroporphyrinogen decarboxylase (UROD)[1]. In the event that UROD activity is reduced (due to genetic disorders or chemical inhibition) uroporphyrinogen, and other porphyrinogen substrates of UROD, are preferentially oxidized to highly stable porphyrins by the phase one metabolizing enzyme CYP1A2 (in mammals;CYP1A5 in birds)[2][3][4] . Uroporphyrin and hepta- and hexa-carboxylic acid porphyrins (highly carboxylated porphyrins)[5] accumulate in the liver, kidneys, spleen, skin and blood leading to a heme disorder known as porphyria [6][7].

Weight of Evidence

Biological Plausibility



Hepatic uroporphyrinogen accumulation versus inhibition of uroporphyrinogen decarboxylase activity from individual mice treated with iron and HCB.

Control: ○, Treated: Δ. (Source: Lambrecht, R.W. *et al.* (1988) Biochem. J. **253** (1), 131-138.)

It is well established that porphyrin accumulation, which is a result of uroporphyrin oxidation (UROX), and UROD inhibition go hand in hand[8]. Because CYP1A2/5 binds a broad range of substrates, significant UROX only occurs when there is an excess of uroporphorynogen, which occurs when UROD is inhibited. Each of the four acetic acid substituents of porphyrinogen is decarboxylated in sequence with the consequent formation of hepta-, hexa-, and pentacarboxylic porphyrinogens as intermediates[9]. Oxidation of these intermediates results in their corresponding, highly stable porphyrins.

Empirical Support for Linkage

Include consideration of temporal concordance here

A number of studies have demonstrated that increased UROD inhibition results in higher hepatic porphyrin accumulation[10][11][12].

Uncertainties or Inconsistencies

Uroporphyrin accumulation in avian models is less consistently accompanied by decreased UROD activity, and when it does occur, it is less marked than in mammals[13][14]. Although numerous studies show both a decrease in UROD activity and porphyrin accumulation in avian species, Lambrecht et al.[14] reported the accumulation of porphyrins in chicken embryo hepatocytes and japanese quail liver without a decrease in UROD activity. They also note that the modest reduction in UROD activity (often less than 50%) is not enough to explain the extent of porphyrin accumulation observed and suggests there may be another mechanism at play.

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?

A reduction in UROD activity of at least 70% is required to achieve a makeable increase in hepatic porphyrins, in mammals.[15][16][17]

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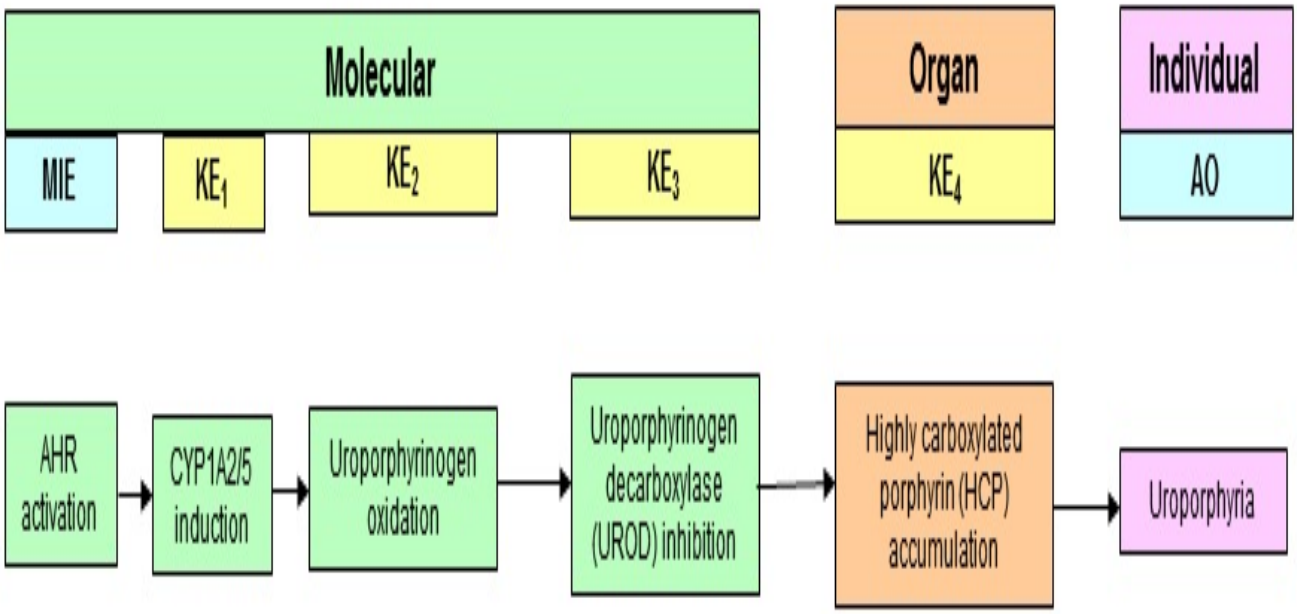
Graphical Representation



Overall Assessment of the AOP

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adult	Strong
Juvenile	Strong

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
mouse	Mus musculus	Strong	NCBI
rat	Rattus norvegicus	Strong	NCBI
human	Homo sapiens	Strong	NCBI

chicken	Gallus gallus	Strong	NCBI
herring gull	Larus argentatus	Strong	NCBI
Japanese quail	Coturnix japonica	Strong	NCBI
Common Starling	Common Starling	Moderate	NCBI

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: Uroporphyria occurs following chemical exposure in juvenile or adult individuals. Fetal exposure to dioxin-like compounds causes developmental abnormalities and embryolethality rather than HCP accumulation[15][16][17][18][19]. Turkish children under the age of two that were exposed to HCB through breastmilk passed away from a condition called "pink sore"[20].

Taxonomic Applicability: Although the AHR is highly conserved in evolution[21], chemical-induced uroporphyria has only been detected in birds[1][2][3] and mammals[22] , including an accidental outbreak in humans due to hexachlorobenzen-contaminated grain in the 1950s[20]. Fish are less susceptible to chemical-induced uroporphyria, but elevated levels of HCP have been documented in highly contaminated environments[23].

Sex Applicability: Although this AOP applies broadly to both males and females, sexual dimorphism for uroporphyria has been observed in rats exposed to hexachlorobenzene (HCB). Hepatic uroporphyrin III was markedly increased in female rats exposed to HCB whereas exposed males showed levels of hepatic porphyrins similar to controls[24].

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.

Every Key event in this AOP is absolutely essential for downstream events to occur. A summary of evidence for essrntiality of each key event is given below.

Molecular Initiating Event: AHR activation (Essentiality=strong)

- Mice with a high-affinity Ahr allele (C57BL/6J) are much more sensitive to uroporphyria than mice with low-affinity Ahr allele (DBA/2)[25][26][27][28][29];
- The Ah locus influences the susceptibility of C57BL/6J mice to HCB-induced porphyria[30];
- Ahr knockout mice (C57BL/6) are resistant to development of porphyria, even in the presence of iron loading[25];
- Primary hepatocytes of avian species indicate that species that are highly sensitive to AHR activation are more sensitive to uroporphyrin accumulation than species with lower sensitivity to AHR activation[31].

Key Event 1: CYP1A2/Cyp1A5 induction (Essentiality=strong)

- CYP1A2 knockout in mice prevents chemical-induced uroporphyria[32][33][34];
- CYP1A2 knockout prevents porphyria in genetically predisposed mice (Hfe-/-, Urod-/+) that normally develop porphyria in absence of external stimuli[35];
- CYP1A2 levels are correlated with the extent of urophorphyrin accumulation in mice[36];
- 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and non-ortho substituted PCBs that are potent inducers of

CYP1A4/5 cause accumulation of only HCPs in chicken embryonic hepatocytes cultures, whereas PCBs that do not induce CYP1A4/5 cause a porphyrin pattern that is not consistent with inhibition of UROD[37];
- Common tern (Sterna hirundo) embryonic hepatocyte cultures, which are ~50 to > 1600 times less sensitive than chicken embryonic hepatocyte cultures to CYP1A5 induction by TCDD and PCBs, do not accumulate HCPs upon chemical exposure[31].

It should be noted that a recent study by Davies et al.[25] found that both C57BL/6J mice (susceptible to chemical-induced porphyria) and DBA/2 mice (resistant to porphyria due to polymorphism in AHR gene) showed increased expression of CYP1A2 when exposed to TCDD, even though the DBA/2 strain did not develop porphyria. Furthermore AHR-/- mice showed a mild uroporphyric response in the presence of iron loading and 5-aminolevulinic acid (a heme precursor). These findings suggest that the induction of CYP1A2 is not crucial for chemical-induced porphyria, but a basal level of expression is absolutely essential.

Key Event 2: Uroporphyrinogen oxidation (UROX) (Essentiality=strong)

- Uroporphyria is characterized biochemically by increased formation of HCPs derived by oxidation of the porphyrinogen substrates of uroporphyrinogen decarboxylase (UROD); secondary to decreased activity of this enzyme in the liver[22];
- Uroporphomethane, derived from oxidizing a single carbon bridge in uroporphyrinogen, has been identified as the UROD inhibitor that leads to chemically- and genetically-induced uroporphyria in mice[38];
- UROX activity is positively correlated with uroporphyrin levels in mice[36].

Key Event 3: Uroporphyrinogen decarboxylase (UROD) inhibition (Essentiality=strong)

- Mutations in the UROD gene that reduce or eliminate UROD activity lead to porphyria in mammals; a decrease in hepatic UROD activity of at least 70% is necessary to observe symptoms from overproduction of porphyrins[22];
- A marked progressive decrease in UROD enzyme activity is a common feature in animal models of chemical-induced porphyria[22][34][39][40][41];
- Liver cytosol UROD activity in female rats exposed to HCB was decreased more than 70% and correlated with elevated hepatic uroporphyrin levels, whereas male rats, which did not develop porphyria, showed UROD activity similar to controls[24];
- UROD activity is inversely proportional to uroporphyrin levels in mice[36];
- In chicken hepatocytes, the strongest inducers of porphyrin accumulation were also the strongest inhibitors of UROD activity[41];
- Reduced UROD enzyme activity, not protein levels, is characteristic of uroporphyria in humans and rats[24][42][43].

Key Event 4: Highly carboxylated porphyrin (HCP) accumulation (Essentiality=strong)

- Under normal heme biosynthesis, porphyrins are only present in trace amounts in the liver; however, in the absence of UROD activity, the oxidation of Uroporphorynogen to uroporphyrins dominates, leading to an accumulation of HCPs;
- Porphyrins are strongly fluorescent compounds resulting in a characteristic red fluorescence of hepatic tissue under UV light that is proportional to the level of porphyrins[44][45]. Increased urinary excretion of porphyrins is also indicative of their accumulation and can lead to dark red/brown urine[22]. HCPs also accumulate in the skin causing solar hypersensitivity and increased skin fragility[46];
- HCP accumulation was observed in avian embryo hepatocyte cultures following exposure potent AHR agonists (dioxin-like compounds)[37][47][48][49] and in the livers of Japanese quails and chickens exposed to PCBs[50][51][52];
- HCP accumulation was evident in mice treated with polyhalogenated aromatic compounds[36] and TCDD-treated rats[25].

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.

Dose concordance

Table 1 demonstrates that upstream KEs (monooxygenase activity/quantity) are significantly affected at lower doses than downstream KEs (porphyrin levels). After a 6 month recovery period, CYP450 and hepatic porphyrin levels were dramatically reduced, however, they did not return to normal. Furthermore, urinary porphyrin excretion remained maximally elevated[53]

Table 1: Porphyria in Female Sprague-Dawley rats chronically exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) over 16 weeks.

Total exposure (µg/kg)	AHH activity	Hepatic CYP450 (nmol/g)	Relative porphyrin level (hepatic)	Incidence of porphyria
0		22		
0.16	3-fold	26	1	0/8
1.6	16-fold[a]	34[a]	5	1/8
16	48-fold[a]	42[a]	1000[a] (urinary uroporphyrin ↑ 120-fold[a])	7/8
16 (after 6 month recovery)	2-fold[a]	20[a] (Control =25)	100[a] (urinary uroporphyrin = 100-fold[a])	

[a]p<0.05 relative to control; AHH= aryl hydrocarbon hydroxylase; general measure of monooxygenase activity. (Goldstein *et al.* 1982)

Temporal Concordance

Table 2 demonstrates that upstream KEs (CYP1A2 expression and UROD inhibition) are significantly affected at earlier time-points than downstream KEs (porphyrin levels). These studies also show that upstream KEs are more sensitive to change than downstream KEs; ddY mice showed a 44% reduction in UROD activity but did not develop uroporphyria[25][54].

Table 2: Characterization of porphyria over time susceptible (C57BL/6 and C57BL/6J) or resistant (ddY and DBA/2) mice

Strain	Time (weeks)	CYP1A2 expression fold change	UROD (nmol/hr/g)	Relative Uroporphyrin level	Reference
C57BL/6	Control		21.4	1	(Seki *et al.* 1987)
	1		7.4	9	
	2		8.5	16	
	3		4.4	2100	
ddY	Control		32.8	1	
	1		30.3	3	
	3		31.8	3	
	6		21.1	14	
	10		18.4	13	
DBA/2	0.5 (3 days)	0		<1	(Davies *et al.* 2008)
	2	0		<1	
	5	0		<1	
C57BL/6J	0.5 (3 days)	0		<1	
	2	56		20[a] (nmol/g liver)	
	5	677		270[a] (nmol/g liver)	

C57BL/6 and ddY mice were exposed to 500 ppm of Kanechlor-500 and DBA/2 and C57BL/6J mice were exposed to 75µ/kg TCDD; [a]Values estimated from figure.

Key Events Relationships

Table 3 shows a sampling of the literature that demonstrates changes in KEs at multiple levels of organization leading to uroporphyria. The use of animal models resistant to porphyria (low AHR affinity or AHR/CYP1A2 knockout) illustrates the essentiality of these KEs in for downstream effects.

Table 3: Sampling of literature supporting essentiality of key events and demonstrating key events relationships

	MROD (pmol/ min per mg	MDOD/ EROD	CYP1A2 (relative protein level)	UROX (pmol/ min per mg	UROD (pmol/ min per mg	Hepatic Porphyrins (nmol/ g)		Liver Toxicity	Ref.
Female C57BL/6J wild-type mice 500 mg Fe/kg + 300 ppm of 3,4,5,3',4',5'-hexachlorobiphenyl in feed for 3 weeks									(Hahn *et al.* 1988)
weeks						Hepatic	Urinary (nmol/ day)		
8							6.3		
9						75.4±57.6	12		
10							30		
11							60		
12							140		
13							145		
14							180		
15			12-fold		30%	1110±393	265		
Female C57BL/6J AHR non-responsive mice									
13							3		
14							6		
15			4-fold		80%	17.6±14.5	6.3		
16							10		
17						48.5±31.6	13		
Male C57BL/6J Wild-type mice pre-treated with Iron									
Control	71±7	1.9+±0.1		8.1±0.9		3-310 (162±111)			(Sinclair *et al.* 1998)
75 mg/kg MC +ALA	240±210	1.3±0.6		15±6		50-260			
100 mg/kg HCBZ			2-3-fold			270-350			(Sinclair *et al.* 2000)
Male C57BL/6J CYP1A2(-/-) mice pre-treated with Iron									
Control	18±2	0.4±0.02		3.4±0		<0.8			(Sinclair *et al.* 1998)
75 mg/kg MC +ALA	300±150	0.2±0.2		2.6±0.7		<0.8			
100 mg/kg HCBZ	ND		NC			<0.8			(Sinclair *et al.* 2000)
C57BL/6J Wild-type mice pre-treated with Iron									
Control	54±6	1±0	ND		100%	0.4±0.1		-	(Smith *et al.* 2001)
75 mg/kg TCDD	800±170	0.50±0.1	↑		10%	1150±690		+++	
C57BL/6J CYP1A2(-/-) mice pre-treated with Iron									
Control	8±3	0.18±0.02	ND		100%	0.6±0.6 0.2		-	
75 mg/kg TCDD	336±30	0.13±0.01	ND		95%	0.3±0.2		+	
Male C57BL/6J mice 75 µ/kg TCDD + 800 mg Fe/kg + 2 mg/mL drinking water ALA									(Davies *et al.* 2008)
AHR(+/+)	860	0.38				400 (964 fold)		+++	
AHR(+/-)	940	0.38				10 (60 fold)		+	
AHR(-/-)	40	0.4				<1 (0 fold)		-	
Male C57BL/6J mice 500 mg Fe/kg + 2 mg/mL drinking water ALA									(Gorman *et al.* 2002)
						4 wks	8 wks		
CYP1A2(-/-)	10±2			3.4±0.4		<1	<1		
CYP1A2(-/+)	48±5			5.5±0.7		<1	19±9		
CYP1A2(+/+)	65±10			9.0±1.0		33±30	194±88		
CYP1A2(+/+) /PCB 126	140±35			19±3.9		205±81	NA		

Liver Toxicity=hepatocellular hypertrophy and degeneration, necrosis, and focal chronic inflammation; Fe= iron; ALA= 5-aminolevulinic acid; ND=Not detected; NC= No change; ↑=Induced relative to control.

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 overall quantitative understanding of this AOP is poor. Quantitative models have been developed that predict the AHR transactivation potential of various compounds [55][56][57], but the extent of AHR activation necessary to produce porphyria is not known. It has been established that a reduction in UROD activity of at least 70% is required to lead to overt uroporphyrin in mammals[58][24][54]. Additionally, numerous in vitro systems have been developed to study porphyrin accumulation and UROD inhibition simultaneously; therefore, this KER provides the most feasible target for a predictive, quantitative model. However, care must be taken when reading across to other species; UROD inhibition is not always observed in avian models of porphyria, and when it is, it is less pronounced[59][60][47].

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