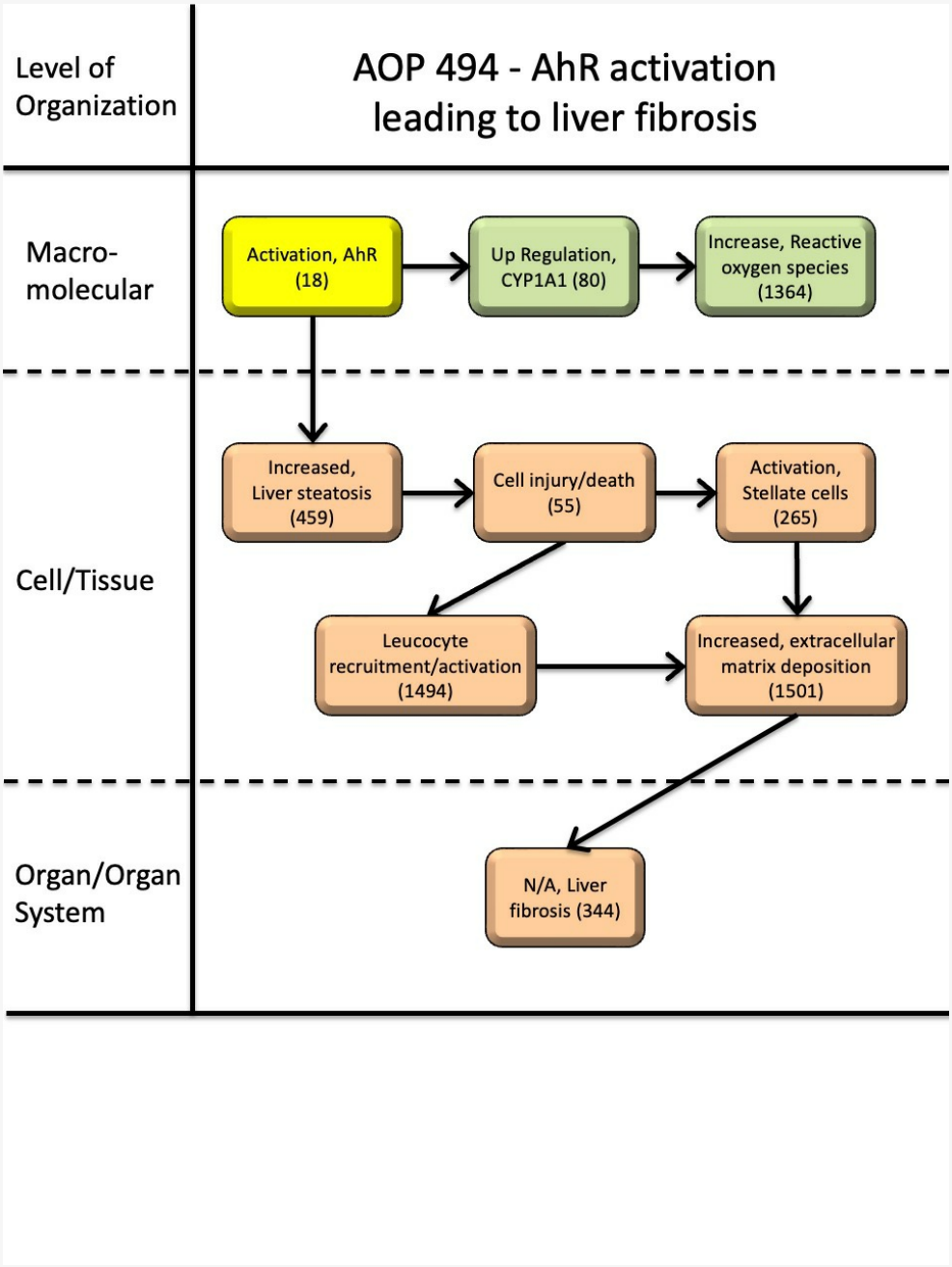
AOP ID and Title:

AOP 494: AhR activation leading to liver fibrosis
Short Title: AhR and chronic liver diseases

Graphical Representation



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Status

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Abstract

Liver fibrosis, characterized by excessive accumulation of extracellular matrix proteins, represents a significant health burden worldwide. The Ah receptor (AhR), a ligand-activated transcription factor primarily known for its involvement in xenobiotic metabolism, has emerged as a key player in various physiological processes, including liver homeostasis and inflammation. Recent studies have implicated the AhR signaling pathway in the development and progression of liver fibrosis. This AOP provides a comprehensive overview of the molecular mechanisms underlying the association between AhR activation and liver fibrogenesis. AhR activation by endogenous ligands, such as tryptophan metabolites and environmental toxins, triggers a cascade of events leading to hepatic stellate cell activation, inflammation, and fibrogenesis. Understanding the intricate interplay between AhR and liver fibrosis offers novel insights into the pathogenesis of chronic liver diseases and highlights AhR as a potential therapeutic target for the management of liver fibrosis.

Background

Understanding the biological link between Ah receptor (AhR) activation and liver fibrosis holds significant relevance due to its implications in the pathogenesis of various liver diseases. Liver fibrosis represents a common pathological process underlying the progression of chronic liver disorders, including hepatitis, alcoholic liver disease, and non-alcoholic fatty liver disease (NAFLD). Notably, NAFLD, characterized by hepatic steatosis, inflammation, and fibrosis, has become a global health concern, closely associated with obesity, metabolic syndrome, and insulin resistance. Given that AhR activation has been implicated in the regulation of lipid metabolism and inflammation, elucidating its role in liver fibrosis provides valuable insights into the molecular mechanisms driving NAFLD progression. Moreover, the interconnected nature of liver diseases underscores the importance of investigating AhR-mediated pathways as potential therapeutic targets for the management of liver fibrosis and its comorbidities, including hepatic steatosis. Therefore, establishing a biological link between AhR activation and liver fibrosis not only enhances our understanding of disease pathogenesis but also offers promising avenues for the development of targeted therapies for liver-related disorders.

Summary of the AOP

Events

Molecular Initiating Events (MIE), Key Events (KE), Adverse Outcomes (AO)

Sequence	Type	Event ID	Title	Short name
	MIE	18	Activation, AhR	Activation, AhR
	KE	80	Up Regulation, CYP1A1	Up Regulation, CYP1A1
	KE	1364	Increase, Reactive oxygen species	Increase, ROS
	KE	55	Increase, Cell injury/death	Cell injury/death
	KE	459	Increased, Liver Steatosis	Increased, Liver Steatosis
	KE	265	Activation, Stellate cells	Activation, Stellate cells
	KE	1494	Leukocyte recruitment/activation	Leukocyte recruitment/activation
	KE	1501	Increased, extracellular matrix deposition	Increased extracellular matrix deposition
	AO	344	N/A, Liver fibrosis	N/A, Liver fibrosis

Key Event Relationships

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Activation, AhR	adjacent	Up Regulation, CYP1A1	High	High
Up Regulation, CYP1A1	adjacent	Increase, Reactive oxygen species	High	High
Activation, AhR	adjacent	Increased, Liver Steatosis	High	Moderate
Increased, Liver Steatosis	adjacent	Increase, Cell injury/death	Moderate	Moderate
Increase, Cell injury/death	adjacent	Activation, Stellate cells	Moderate	Moderate
Increase, Cell injury/death	adjacent	Leukocyte recruitment/activation	Moderate	Moderate
Leukocyte recruitment/activation	adjacent	Increased, extracellular matrix deposition	Moderate	Moderate
Activation, Stellate cells	adjacent	Increased, extracellular matrix deposition	High	High

Upstream Event	Relationship Type	Downstream Event	Evidence	Quantitative Understanding
Increased, extracellular matrix deposition	adjacent	N/A, Liver fibrosis	High	High

Stressors

Name	Evidence
2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)	

Overall Assessment of the AOP

The *biological plausibility* of KERs is defined by the OECD as the « understanding of the fundamental biological processes involved and whether they are consistent with the causal relationship being proposed in the AOP ». The biological plausibility is strong due to the presence of overwhelming evidence present in different studies. A minor setback would be the difficulty to dismiss alternative mechanisms caused by the ligands used for AhR activation.

The *essentiality of KEs* refers to « experimental data for whether or not downstream KEs or the AO are prevented or modified if an upstream event is blocked ». The essentiality of KEs is strong: most works converge to imply the AhR in fibrotic processes. One setback would be that AhR knockout mice also develop a specific liver fibrosis. We propose that exogenous ligands alter the activity of endogenous ligands and therefore contribute just like the knockout to the occurence of liver fibrosis. The AhR activation needs to be considered then as the binding of exogenous ligands (xenobiotics) counteracting on the physiological processes which regulate the physiological functions.

Finally, the *empirical support* of KERs, is often « based on toxicological data derived by one or more reference chemicals where dose–response and temporal concordance for the KE pair can be assessed ». The overall assessment of the empirical support of our KERs is also strong. There is evidence in human cell lines and mice showing a dose–response and temporal concordance for severity of our KEs and the adverse outcomes (for example, a dose-dependant effect of TCDD on the development of liver fibrosis in mice).

Domain of Applicability

Life Stage Applicability

Life Stage	Evidence
Adults	Moderate

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Homo sapiens	Homo sapiens	Moderate	NCBI
Mus musculus	Mus musculus	High	NCBI

Sex Applicability

Sex	Evidence
Mixed	Moderate

References

Appendix 1

List of MIEs in this AOP

Event: 18: Activation, AhR

Short Name: Activation, AhR

Key Event Component

Process	Object	Action
aryl hydrocarbon receptor activity	aryl hydrocarbon receptor	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:21 - Aryl hydrocarbon receptor activation leading to early life stage mortality, via increased COX-2	MolecularInitiatingEvent
Aop:57 - AhR activation leading to hepatic steatosis	MolecularInitiatingEvent
Aop:131 - Aryl hydrocarbon receptor activation leading to uroporphyria	MolecularInitiatingEvent
Aop:150 - Aryl hydrocarbon receptor activation leading to early life stage mortality, via reduced VEGF	MolecularInitiatingEvent
Aop:310 - Embryonic Activation of the AHR leading to Reproductive failure, via epigenetic down-regulation of GnRHR	MolecularInitiatingEvent
Aop:151 - AhR activation leading to preeclampsia	MolecularInitiatingEvent
Aop:414 - Aryl hydrocarbon receptor activation leading to lung fibrosis through TGF-β dependent fibrosis toxicity pathway	MolecularInitiatingEvent
Aop:415 - Aryl hydrocarbon receptor activation leading to lung fibrosis through IL-6 toxicity pathway	MolecularInitiatingEvent
Aop:416 - Aryl hydrocarbon receptor activation leading to lung cancer through IL-6 toxicity pathway	MolecularInitiatingEvent
Aop:417 - Aryl hydrocarbon receptor activation leading to lung cancer through AHR-ARNT toxicity pathway	MolecularInitiatingEvent
Aop:418 - Aryl hydrocarbon receptor activation leading to impaired lung function through AHR-ARNT toxicity pathway	KeyEvent
Aop:419 - Aryl hydrocarbon receptor activation leading to impaired lung function through P53 toxicity pathway	KeyEvent
Aop:420 - Aryl hydrocarbon receptor activation leading to lung cancer through sustained NRF2 toxicity pathway	MolecularInitiatingEvent
Aop:439 - Activation of the AhR leading to metastatic breast cancer	MolecularInitiatingEvent
Aop:455 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced impeded craniofacial development	MolecularInitiatingEvent
Aop:456 - Aryl hydrocarbon receptor activation leading to early life stage mortality via sox9 repression induced cardiovascular toxicity	MolecularInitiatingEvent
Aop:458 - AhR activation in the liver leading to Subsequent Adverse Neurodevelopmental Outcomes in Mammals	MolecularInitiatingEvent
Aop:494 - AhR activation leading to liver fibrosis	MolecularInitiatingEvent
Aop:459 - AhR activation in the thyroid leading to Subsequent Adverse Neurodevelopmental Outcomes in Mammals	MolecularInitiatingEvent

Stressors

Name
Benzidine
Dibenzo-p-dioxin
Polychlorinated biphenyl
Polychlorinated dibenzofurans
Hexachlorobenzene
Polycyclic aromatic hydrocarbons (PAHs)

Biological Context

Level of Biological Organization

Molecular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
zebra danio	Danio rerio	High	NCBI
Gallus gallus	Gallus gallus	High	NCBI
Pagrus major	Pagrus major	High	NCBI
Acipenser transmontanus	Acipenser transmontanus	High	NCBI
Acipenser fulvescens	Acipenser fulvescens	High	NCBI
rainbow trout	Oncorhynchus mykiss	High	NCBI
Salmo salar	Salmo salar	High	NCBI
Xenopus laevis	Xenopus laevis	High	NCBI
Ambystoma mexicanum	Ambystoma mexicanum	High	NCBI
Phasianus colchicus	Phasianus colchicus	High	NCBI
Coturnix japonica	Coturnix japonica	High	NCBI
mouse	Mus musculus	High	NCBI
rat	Rattus norvegicus	High	NCBI
human	Homo sapiens	High	NCBI
Microgadus tomcod	Microgadus tomcod	High	NCBI
Homo sapiens	Homo sapiens		NCBI

Life Stage Applicability

Life Stage	Evidence
Embryo	High
Development	High
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

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].

- Little is known about differences in binding affinity of AhRs and how this relates to sensitivity in non-avian taxa.
- Low binding affinity for DLCs of AhR1s of African clawed frog (*Xenopus laevis*) and axolotl (*Ambystoma mexicanum*) has been suggested as a mechanism for tolerance of these amphibians to DLCs (Lavine et al 2005; Shoots et al

2015).
- Among reptiles, only AhRs of American alligator (*Alligator mississippiensis*) have been investigated and little is known about the sensitivity of American alligator or other reptiles to DLCs (Oka et al 2016).
- Among fishes, great differences in sensitivity to DLCs are known both for AhRs and for embryos among species that have been tested (Doering et al 2013; 2014).
- Differences in binding affinity of the AhR2 have been demonstrated to explain differences in sensitivity to DLCs between sensitive and tolerant populations of Atlantic Tomcod (*Microgadus tomcod*) (Wirgin et al 2011).
    - This was attributed to the rapid evolution of populations in highly contaminated areas of the Hudson River, resulting in a 6-base pair deletion in the AHR sequence (outside the LBD) and reduced ligand binding affinity, due to reduces AHR protein stability.
- Information is not yet available regarding whether differences in binding affinity of AhRs of fishes are predictive of differences in sensitivity of embryos, juveniles, or adults (Doering et al 2013).

The AhR is a very conserved and ancient protein (95) and the AhR is present in human and mice (96–98).The AhR is present in human physiology and pathology. The AhR is highly expressed at several important physiological barriers such as the placenta, lung, gastrointestinal system, and liver in human (Wakx, Marinelli, Watanabe). In these tissues, the AhR is involved in both detoxication processes involving xenobiotic metabolizing enzymes such as cytochromes P450, and in immune functions translating chemical signals into immune defence pathways (Marinelli, Stobbe). Moreover, it has a regulatory role in human dendritic cells and myelination (Kado, Shackleford). The lung constitutes another barrier exposed to components of air pollution such as particles and hydrocarbons (air pollution, cigarette smoke). The AhR detects such hydrocarbons and protects the pulmonary cells from their deleterious effects through metabolization. The regulatory effect on blood cells of the AhR, balancing different related cell types, can be extended to the megakaryocytes and their precursors; indeed, StemRegenin 1 (SR1), an antagonist of the AhR increases the human population of CD34+CD41low cells, a fraction of very efficient precursors of proplatelets (Bock). The occurrence of a nystagmus has been subsequently diagnosed in humans bearing a AhR mutation (Borovok).

In human cancer, the AhR has either a pro or con tumor effect depending on the tissue, the ligand, and the duration of the activation (Zudaire, Chang, Litzenburg, Gramatzki, Lin, Wang). In human breast cancer, the AhR is thoughts to be responsible of its progression (Goode, Kanno, Optiz, Novikov, Hall, Subramaniam, Barhoover). In human mammary benign cells, Brooks et al. noted that a high level of AhR was associated with a modified cell cycle (with a 50% increase in population doubling time in cells expressing the AhR by more than 3-fold) and EMT including increased cell migration. Narasimnhan et al. found that suppression of the AhR pathway had a pro-tumorigenic effect in vitro (EMT, tumor migration) in triple negative breast cancer.

Many endogenous and exogenous ligands are present for the AhR in human (Optiz, Adachi, Schroeder, Rothhammer). Indoles, such as indole-3-carbinol or one of its secondary metabolites, 3-3'- Diindolylmethane, are degradation products found in cruciferous vegetables and characterized as AhR ligands (Ema, Kall, Miller) they are also inducers of the human and rat CYP1A1 (Optiz). FICZ is the most potent AhR ligand known to date: it has a stronger affinity than TCDD for the human AhR (TCDD Kd=0.48 nM/FICZ Kd=0.07 nM) (Coumoul).

Key Event Description

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].

Investigations of invertebrates possessing early homologs of the AhR suggest that the AhR evolutionarily functioned in regulation of the cell cycle, cellular proliferation and differentiation, and cell-to-cell communications (Hahn et al 2002). However, critical functions in angiogenesis, regulation of the immune system, neuronal processes, metabolism, development of the heart and other organ systems, and detoxification have emerged sometime in early vertebrate evolution (Duncan et al., 1998; Emmons et al., 1999; Lahvis and Bradfield, 1998).

The molecular Initiating Event

Figure 1: The molecular mechanism of activation of gene expression by AHR.

The molecular mechanism for AHR-mediated activation of gene expression is presented in Figure 1. 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

- Over time the AhR has undergone gene duplication and diversification in vertebrates, which has resulted in multiple clades of AhR, namely AhR1, AhR2, and AhR3 (Hahn 2002).
- Fishes and birds express AhR1s and AhR2s, while mammals express a single AhR that is homologous to the AhR1 (Hahn 2002; Hahn et al 2006).
- The AhR3 is poorly understood and known only from some cartilaginous fishes (Hahn 2002).
- Little is known about diversity of AhRs in reptiles and amphibians (Hahn et al 2002).
- In some taxa, subsequent genome duplication events have further led to multiple isoforms of AhRs in some species, with up to four isoforms of the AhR (α, β, δ, γ) having been identified in Atlantic salmon (*Salmo salar*) (Hansson et al 2004).
- Although homologs of the AhR have been identified in some invertebrates, compared to vertebrates these AhRs have differences in binding of ligands in the species investigated to date (Hahn 2002; Hahn et al 1994).

Roles of isoforms in birds:

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].

- AhR1 and AhR2 both bind and are activated by TCDD*in vitro* (Yasui et al 2007).
- AhR1 has greater binding affinity and sensitivity to activation by TCDD relative to AhR2 (Yasui et al 2007).
- AhR1 is believed to mediate toxicities of DLCs, while AhR2 has no known role in toxicities (Farmahin et al 2012; Farmahin et al 2013; Manning et al 2012).

Roles of isoforms in fishes:

- AhR1 and AhR2 both bind and are activated by TCDD*in vitro* (Bak et al 2013; Doering et al 2014; 2015; Karchner et al 1999; 2005).
- AhR1 has greater sensitivity to activation by TCDD than AhR2 in red seabream (*Pagrus major*), white sturgeon (*Acipenser transmontanus*), and lake sturgeon (*Acipenser fulvescens*) (Bak et al 2013; Doering et al 2014; 2015)
- AhR2 has greater binding affinity or activation by TCDD than AhR1 in zebrafish (*Danio rerio*) and mummichog (*Fundulus heteroclitus*) (Karchner et al 1999; 2005).
- AhR2 is believed to mediate toxicities in fishes, while AhR1 has no known role in toxicities. Specifically, knockdown of AhR2 protects against toxicities of dioxin-like compounds (DLCs) and polycyclic aromatic hydrocarbons (PAHs) in zebrafish (*Danio rerio*) and mummichog (*Fundulus heteroclitus*), while knockdown of AhR1 offers no protection (Clark et al 2010; Prasch et al 2003; Van Tiem & Di Giulio 2011).

Roles of isoforms in amphibians and reptiles:

- Less is known about AhRs of amphibians or reptiles.
- AhR1 is believed to mediate toxicities in amphibians (Hahn 2002; Lavine et al 2005; Oka et al 2016; Shoots et al 2015). However, all AhRs of amphibians that have been investigated have very low affinity for TCDD (Hahn 2002; Lavine et al 2005; Oka et al 2016; Shoots et al 2015).
- Both AhR1s and AhR2 of American alligator (*Alligator mississippiensis*) are activated by agonists with comparable sensitivities (Oka et al 2016). AhRs of no other reptiles have been investigated.

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 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

The described luciferase reporter gene (LRG) assays have been used to investigate activation of AhRs of:

- Humans (*Homo sapiens*) (Abnet et al 1999)
- Species of birds, namely chicken (*Gallus gallus*), ring-necked pheasant (*Phasianus colchicus*), Japanese quail (*Coturnix japonica*), and common tern (*Sterna hirundo*) (Farmahin et al 2012; Manning et al 2013), Mutant AhR1s with ligand binding domains resembling those of at least 86 avian species have also been investigated (Farmahin et al 2013). AhR2s of birds have only been investigated in black-footed albatross (*Phoebastria nigripes*) and common cormorant (*Phalacrocorax carbo*) (Yasio et al 2007).
- American alligator (*Alligator mississippiensis*) is the only reptile for which AhR activation has been investigated (Oka et al 2016), AhR1A, AhR1B, and AhR2 of American alligator were assayed (Oka et al 2016).
- AhR1 of two amphibians have been investigated, namely African clawed frog (*Xenopus laevis*) and salamander (*Ambystoma mexicanum*) (Lavine et al 2005; Shoots et al 2015; Ohi et al 2003),
- AhR1s and AhR2s of several species of fish have been investigated, namely Atlantic salmon (*Salmo salar*), Atlantic tomcod (*Microgadus tomcod*), white sturgeon (*Acipenser transmontanus*), rainbow trout (*Onchorhynchys mykiss*), red seabream (*Pagrus major*), lake sturgeon (*Acipenser fulvescens*), and zebrafish (*Danio rerio*) (Andreasen et al 2002; Abnet et al 1999; Bak et al 2013; Doering et al 2014; 2015; Evans et al 2005; Hansson & Hahn 2008; Karchner et al 1999; Tanguay et al 1999; Wirgin et al 2011).

For demonstrative purposes, a luciferase reporter gene assay used to measure AHR1-mediated transactivation for avian species is described here. However, comparable assays are utilized for investigating AHR1s and AHR2s of all taxa. 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].

In silico Approaches

In silico homology modeling of the ligand binding domain of the AHR in combination with molecular docking simulations can provide valuable insight into the transactivation-potential of a diverse array of AHR ligands. Such models have been developed for multiple AHR isoforms and ligands (high/low affinity, endogenous and synthetic, agonists and antagonists), and can accurately predict ligand potency based on their structure and physicochemical properties (Bonati et al 2017; Hirano et al 2015; Sovadinova et al 2006).

References

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List of Key Events in the AOP

Event: 80: Up Regulation, CYP1A1

Short Name: Up Regulation, CYP1A1

Key Event Component

Process	Object	Action

Process	Object	Action
gene expression	cytochrome P450 1A1	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:57 - AhR activation leading to hepatic steatosis	KeyEvent
Aop:494 - AhR activation leading to liver fibrosis	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Cell term

Cell term

hepatocyte

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Acipenser transmontanus	Acipenser transmontanus	High	NCBI
Oncorhynchus mykiss	Oncorhynchus mykiss	High	NCBI

Event: 1364: Increase, Reactive oxygen species

Short Name: Increase, ROS

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:325 - Thermal stress leading to population decline (2)	KeyEvent
Aop:396 - Deposition of ionizing energy leads to population decline via impaired meiosis	KeyEvent
Aop:481 - AOPs of amorphous silica nanoparticles: ROS-mediated oxidative stress increased respiratory dysfunction and diseases.	MolecularInitiatingEvent
Aop:494 - AhR activation leading to liver fibrosis	KeyEvent

Biological Context

Level of Biological Organization

Molecular

Event: 55: Increase, Cell injury/death

Short Name: Cell injury/death

Key Event Component

Process	Object	Action

Process	Object	Action
cell death		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:48 - Binding of agonists to ionotropic glutamate receptors in adult brain causes excitotoxicity that mediates neuronal cell death, contributing to learning and memory impairment.	KeyEvent
Aop:13 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development induces impairment of learning and memory abilities	KeyEvent
Aop:38 - Protein Alkylation leading to Liver Fibrosis	KeyEvent
Aop:12 - Chronic binding of antagonist to N-methyl-D-aspartate receptors (NMDARs) during brain development leads to neurodegeneration with impairment in learning and memory in aging	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis	KeyEvent
Aop:17 - Binding of electrophilic chemicals to SH(thiol)-group of proteins and /or to seleno-proteins involved in protection against oxidative stress during brain development leads to impairment of learning and memory	KeyEvent
Aop:278 - IKK complex inhibition leading to liver injury	KeyEvent
Aop:281 - Acetylcholinesterase Inhibition Leading to Neurodegeneration	KeyEvent
Aop:273 - Mitochondrial complex inhibition leading to liver injury	KeyEvent
Aop:377 - Dysregulated prolonged Toll Like Receptor 9 (TLR9) activation leading to Multi Organ Failure involving Acute Respiratory Distress Syndrome (ARDS)	KeyEvent
Aop:265 - Uncoupling of oxidative phosphorylation leading to growth inhibition via increased cytosolic calcium	KeyEvent
Aop:264 - Uncoupling of oxidative phosphorylation leading to growth inhibition via ATP depletion associated cell death	KeyEvent
Aop:266 - Uncoupling of oxidative phosphorylation leading to growth inhibition via decreased Na-K ATPase activity	KeyEvent
Aop:268 - Uncoupling of oxidative phosphorylation leading to growth inhibition via mitochondrial swelling	KeyEvent
Aop:479 - Mitochondrial complexes inhibition leading to left ventricular function decrease via increased myocardial oxidative stress	KeyEvent
Aop:490 - Co-activation of IP3R and RyR leads to socio-economic burden through reduced IQ and non-cholinergic mechanisms	KeyEvent
Aop:494 - AhR activation leading to liver fibrosis	KeyEvent
Aop:530 - Endocytotic lysosomal uptake leads to intestinal barrier disruption	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term

eukaryotic cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI

Term	Scientific Term	Evidence	Links
human and other cells in culture	human and other cells in culture	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Cell death is an universal event occurring in cells of any species (Fink and Cookson,2005).

Key Event Description

Two types of cell death can be distinguished by morphological features, although it is likely that these are two ends of a spectrum with possible intermediate forms. Apoptosis involves shrinkage, nuclear disassembly, and fragmentation of the cell into discrete bodies with intact plasma membranes. These are rapidly phagocytosed by neighbouring cells. An important feature of apoptosis is the requirement for adenosine triphosphate (ATP) to initiate the execution phase. In contrast, necrotic cell death is characterized by cell swelling and lysis. This is usually a consequence of profound loss of mitochondrial function and resultant ATP depletion, leading to loss of ion homeostasis, including volume regulation, and increased intracellular Ca2+. The latter activates a number of nonspecific hydrolases (i.e., proteases, nucleases, and phospholipases) as well as calcium dependent kinases. Activation of calpain I, the Ca2+-dependent cysteine protease cleaves the death-promoting Bcl-2 family members Bid and Bax which translocate to mitochondrial membranes, resulting in release of truncated apoptosis-inducing factor (tAIF), cytochrome c and endonuclease in the case of Bid and cytocrome c in the case of Bax. tAIF translocates to cell nuclei, and together with cyclophilin A and phosphorylated histone H2AX (γH2AX) is responsible for DNA cleavage, a feature of programmed necrosis. Activated calpain I has also been shown to cleave the plasma membrane Na+–Ca2+ exchanger, which leads to build-up of intracellular Ca2+, which is the source of additional increased intracellular Ca2+. Cytochrome c in cellular apoptosis is a component of the apoptosome.

DNA damage activates nuclear poly(ADP-ribose) polymerase-1(PARP-1), a DNA repair enzyme. PARP-1 forms poly(ADP-ribose) polymers, to repair DNA, but when DNA damage is extensive, PAR accumulates, exits cell nuclei and travels to mitochondrial membranes, where it, like calpain I, is involved in AIF release from mitochondria. A fundamental distinction between necrosis and apoptosis is the loss of plasma membrane integrity; this is integral to the former but not the latter. As a consequence, lytic release of cellular constituents promotes a local inflammatory reaction, whereas the rapid removal of apoptotic bodies minimizes such a reaction. The distinction between the two modes of death is easily accomplished in vitro but not in vivo. Thus, although claims that certain drugs induce apoptosis have been made, these are relatively unconvincing. DNA fragmentation can occur in necrosis, leading to positive TUNEL staining (see explanation below). Conversely, when apoptosis is massive, it can exceed the capacity for rapid phagocytosis, resulting in the eventual appearance of secondary necrosis.

Two alternative pathways - either extrinsic (receptor-mediated) or intrinsic (mitochondria-mediated) - lead to apoptotic cell death. The initiation of cell death begins either at the plasma membrane with the binding of TNF or FasL to their cognate receptors or within the cell. The latter is due to the occurrence of intracellular stress in the form of biochemical events such as oxidative stress, redox changes, covalent binding, lipid peroxidation, and consequent functional effects on mitochondria, endoplasmic reticulum, microtubules, cytoskeleton, or DNA. The intrinsic mitochondrial pathway involves the initiator, caspase-9, which, when activated, forms an “apoptosome” in the cytosol, together with cytochrome c, which translocates from mitochondria, Apaf-1 and dATP. The apoptosome activates caspase-3, the central effector caspase, which in turn activates downstream factors that are responsible for the apoptotic death of a cell (Fujikawa, 2015). Intracellular stress either directly affects mitochondria or can lead to effects on other organelles, which then send signals to the mitochondria to recruit participation in the death process (Fujikawa, 2015; Malhi et al., 2010). Constitutively expressed nitric oxide synthase (nNOS) is a Ca2+-dependent cytosolic enzyme that forms nitric oxide (NO) from L-arginine, and NO reacts with the free radical such as superoxide (O2−) to form the very toxic free radical peroxynitrite (ONOO−). Free radicals such as ONOO−, O2 − and hydroxyl radical (OH−) damage cellular membranes and intracellular proteins, enzymes and DNA (Fujikawa, 2015; Malhi et al., 2010; Kaplowitz, 2002; Kroemer et al., 2009).

How it is Measured or Detected

Necrosis:

Lactate dehydrogenase (LDH) is a soluble cytoplasmic enzyme that is present in almost all cells and is released into extracellular space when the plasma membrane is damaged. To detect the leakage of LDH into cell culture medium, a tetrazolium salt is used in this assay. In the first step, LDH produces reduced nicotinamide adenine dinucleotide (NADH) when it catalyzes the oxidation of lactate to pyruvate. In the second step, a tetrazolium salt is converted to a colored

formazan product using newly synthesized NADH in the presence of an electron acceptor. The amount of formazan product can be colorimetrically quantified by standard spectroscopy. Because of the linearity of the assay, it can be used to enumerate the percentage of necrotic cells in a sample (Chan et al., 2013).

The MTT assay is a colorimetric assay for assessing cell viability. NAD(P)H-dependent cellular oxidoreductase enzymes may reflect the number of viable cells present. These enzymes are capable of reducing the tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan, which has a purple color. Other closely related tetrazolium dyes include XTT, MTS and the WSTs. Tetrazolium dye assays can also be used to measure cytotoxicity (loss of viable cells) or cytostatic activity (shift from proliferation to quiescence) of potential medicinal agents and toxic materials. MTT assays are usually done in the dark since the MTT reagent is sensitive to light (Berridgeet al.,2005).

Propidium iodide (PI) is an intercalating agent and a fluorescent molecule used to stain necrotic cells. It is cell membrane impermeant so it stains only those cells where the cell membrane is destroyed. When PI is bound to nucleic acids, the fluorescence excitation maximum is 535 nm and the emission maximum is 617 nm (Moore et al.,1998)

Alamar Blue (resazurin) is a fluorescent dye. The oxidized blue non fluorescent Alamar blue is reduced to a pink fluorescent dye in the medium by cell activity (O'Brien et al., 2000) (12).

Neutral red uptake, which is based on the ability of viable cells to incorporate and bind the supravital dye neutral red in lysosomes (Repetto et al., 2008)(13). Moreover, quantification of ATP, signaling the presence of metabolically active cells, can be performed (CellTiter-Glo; Promega).

ATP assay: Quantification of ATP, signaling the presence of metabolically active cells (CellTiter-Glo; Promega).

Apoptosis:

TUNEL is a common method for detecting DNA fragmentation that results from apoptotic signalling cascades. The assay relies on the presence of nicks in the DNA which can be identified by terminal deoxynucleotidyl transferase or TdT, an enzyme that will catalyze the addition of dUTPs that are secondarily labeled with a marker. It may also label cells that have suffered severe DNA damage.

Caspase activity assays measured by fluorescence. During apoptosis, mainly caspase-3 and -7 cleave PARP to yield an 85 kDa and a 25 kDa fragment. PARP cleavage is considered to be one of the classical characteristics of apoptosis. Antibodies to the 85 kDa fragment of cleaved PARP or to caspase-3 both serve as markers for apoptotic cells that can be monitored using immunofluorescence (Li, Peng et al., 2004).

Hoechst 33342 staining: Hoechst dyes are cell-permeable and bind to DNA in live or fixed cells. Therefore, these stains are often called supravital, which means that cells survive a treatment with these compounds. The stained, condensed or fragmented DNA is a marker of apoptosis (Loo, 2002; Kubbies and Rabinovitch, 1983).

Acridine Orange/Ethidium Bromide staining is used to visualize nuclear changes and apoptotic body formation that are characteristic of apoptosis. Cells are viewed under a fluorescence microscope and counted to quantify apoptosis.

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Event: 459: Increased, Liver Steatosis

Short Name: Increased, Liver Steatosis

Key Event Component

Process	Object	Action
Hepatic steatosis		increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:58 - NR1I3 (CAR) suppression leading to hepatic steatosis	AdverseOutcome
Aop:60 - NR1I2 (Pregnane X Receptor, PXR) activation leading to hepatic steatosis	AdverseOutcome
Aop:61 - NFE2L2/FXR activation leading to hepatic steatosis	AdverseOutcome
Aop:62 - AKT2 activation leading to hepatic steatosis	AdverseOutcome
Aop:36 - Peroxisomal Fatty Acid Beta-Oxidation Inhibition Leading to Steatosis	AdverseOutcome
Aop:213 - Inhibition of fatty acid beta oxidation leading to nonalcoholic steatohepatitis (NASH)	KeyEvent
Aop:285 - Inhibition of N-linked glycosylation leads to liver injury	KeyEvent
Aop:318 - Glucocorticoid Receptor activation leading to hepatic steatosis	AdverseOutcome
Aop:517 - Pregnane X Receptor (PXR) activation leads to liver steatosis	AdverseOutcome
Aop:518 - Liver X Receptor (LXR) activation leads to liver steatosis	AdverseOutcome
Aop:529 - Perfluorooctanesulfonic acid (PFOS) binding to peroxisome proliferator-activated receptors (PPARs) causes dysregulation of lipid metabolism and subsequent liver steatosis	AdverseOutcome
Aop:232 - NFE2/Nrf2 repression to steatosis	AdverseOutcome
Aop:57 - AhR activation leading to hepatic steatosis	AdverseOutcome
Aop:494 - AhR activation leading to liver fibrosis	KeyEvent

Biological Context

Level of Biological Organization
Organ

Organ term

Organ term
liver

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
Vertebrates	Vertebrates	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	High

Sex Applicability

Sex	Evidence
Unspecific	High

Steatosis is the result of perturbations in well-known metabolic pathways that are well-studied and well-known in many taxa.

Life Stage: The life stage applicable to this key event is all life stages with a liver. Older individuals are more likely to manifest this adverse outcome pathway (adults > juveniles) due to accumulation of triglycerides.

Sex: This key event applies to both males and females.

Taxonomic: This key event appears to be present broadly in vertebrates, with most representative studies in mammals (humans, lab mice, lab rats).

Key Event Description

Biological state: liver steatosis is the inappropriate storage of fat in hepatocytes. *Four major pathways for triglyceride accumulation are: 1. Increased fatty acid uptake; 2. Increased De Novo FA and Lipid Synthesis; 3. Decreased FA Oxidation; 4. Decreased Lipid Efflux (Angrish et al. 2016). Chemical stressors can increase gene expression of key genes involving these pathways, leading to increased accumulation of triglycerides (Aguayo-Orozco et al. 2018). In addition, excessive dietary compounds of fatty compounds can also increase likelihood of accumulation of triglycerides (Nguyen et al. 2008).*

Biological compartment: steatosis is generally an organ-level diagnosis; however, the pathology occurs within the hepatocytes.

Role in biology: steatosis is an adverse endpoint.

Description from EU-ToxRisk:

Activation of stellate cells results in collagen accumulation and change in extracellular matrix composition in the liver causing fibrosis. (Landesmann, 2016; Koo et al 2016)

How it is Measured or Detected

Steatosis is measured by lipidomics approaches *(e.g. Yang and Han 2016)*that measure lipid levels, or by histology. *Concentrations of triglycerides, cholesterols, fatty acids, and related compounds are measured biochemically include high throughput enzymatic analyses, analytical ultracentrifuging, gradient gel electrophoresis, Nuclear Magnetic Resonance, and other direct assessment techniques (Schaefer et al. 2016).*

Regulatory Significance of the AO

Steatosis is a regulatory endpoint and has been used as an endpoint in many US EPA assessments, including IRIS assessments.

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NOTE: Italics symbolize edits from John Frisch

Event: 265: Activation, Stellate cells

Short Name: Activation, Stellate cells

Key Event Component

Process	Object	Action
hepatic stellate cell activation	hepatic stellate cell	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:38 - Protein Alkylation leading to Liver Fibrosis	KeyEvent
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis	KeyEvent
Aop:494 - AhR activation leading to liver fibrosis	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Cell term

Cell term
hepatic stellate cell

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
human and other cells in culture	human and other cells in culture	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
mouse	Mus musculus	High	NCBI
pigs	Sus scrofa	High	NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Human: Friedman, 2008

Rat: George et al.,1999

Mouse: Chang et al., 2014

Pig: Costa et al., 2001

Key Event Description

Stellate cell activation means a transdifferentiation from a quiescent vitamin A–storing cell to a proliferative and contractile myofibroblast. Multiple cells and cytokines play a part in the regulation of hepatic stellate cell (HSC) activation that consists of discrete phenotype responses, mainly proliferation, contractility, fibrogenesis, matrix degradation, chemotaxis, and retinoid loss.

HSCs undergo activation through a two-phase process. The first step, the initiation phase, is triggered by injured hepatocytes, reactive oxygen speecies (ROS) and paracrine stimulation from neighbouring cell types (Kupffer cells (KCs), Liver sinusoidal endothelial cells (LSECs), and platelets) and make HSCs sensitized to activation by up-regulating various

receptors. The perpetuation phase refers to the maintenance of HSC activation, which is a dynamic process including the secretion of autocrine and paracrine growth factors (such as TGF-β1), chemokines, and the up-regulation of collagen synthesis (mainly type I collagen). In response to growth factors (including Platelet-derived Growth Factor (PDGF) and Vascular Endothelial Growth Factor (VEGF)) HSCs proliferate. Increased contractility (Endothelin-1 and NO are the key opposing counter-regulators that control HSC contractility, in addition to angiotensinogen II, and others) leads to increased portal resistance. Driven by chemoattractants their accumulation in areas of injury is enhanced. TGF-β1 synthesis promotes activation of neighbouring quiescent hepatic stellate cells, whereas the release of HGF (hepatocyte growth factor) stimulates regeneration of adjacent hepatocytes. The release of chemoattractants (monocyte chemoattractant protein-1(MCP-1) and colony-stimulating factors (CSFs)) amplifies inflammation (Lee and Friedman; 2011; Friedman, 2010; 2008; 2000; Bataller and Brenner, 2005; ↑ Lotersztain et al., 2005; Poli, 2000). Activated HSCs (myofibroblasts) are the primary collagen producing cell, the key cellular mediators of fibrosis and a nexus for converging inflammatory pathways leading to fibrosis. Experimental inhibition of stellate cell activation prevents fibrosis (Li, Jing-Ting et al.,2008; George et al. (1999).

How it is Measured or Detected

Alpha-smooth muscle actin (α-SMA) is a well-known marker of hepatic stellate cells activation. Anti-alpha smooth muscle Actin [1A4] monoclonal antibody reacts with the alpha smooth muscle isoform of actin.

Gene expression profiling confirmed early changes for known genes related to HSC activation such as alpha smooth muscle actin (Acta2), lysyl oxidase (Lox) and collagen, type I, alpha 1 (Col1a1). Insulin-like growth factor binding protein 3 (Igfbp3) was identified as a gene strongly affected and as marker for culture-activated HSCs and plays a role in HSC migration (Morini et al., 2005; Mannaerts et al., 2013).

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Event: 1494: Leukocyte recruitment/activation

Short Name: Leukocyte recruitment/activation

Key Event Component

Process	Object	Action
cell activation involved in immune response	leukocyte	increased

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis	KeyEvent

AOP ID and Name	Event Type
Aop:293 - Increased DNA damage leading to increased risk of breast cancer	KeyEvent
Aop:294 - Increased reactive oxygen and nitrogen species (RONS) leading to increased risk of breast cancer	KeyEvent
Aop:494 - AhR activation leading to liver fibrosis	KeyEvent

Biological Context

Level of Biological Organization

Cellular

Domain of Applicability

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens		NCBI
Vertebrates	Vertebrates		NCBI

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Key Event Description

The inflammatory response is the cornerstone of the body’s defense mechanism against bacterial and viral pathogens, as well as physical-, chemical- and environmental-mediated tissue and organ damage. Leucocyte recruitment at the site of pathogen evasion or sterile tissue injury is a critical adaptation for the preservation of tissue integrity. Neutrophils are the cell population that acutely responds to the alterations of inflammatory micro-environment. Neutrophil infiltration takes place within 6-8 hours from the initiation of the inflammatory process and is followed by the recruitment of other cell populations, like monocytes, lymphocytes, and eosinophils, which either promote or drive the resolution of inflammation. Leukocyte infiltration into sites of infection or sterile inflammation is a tightly regulated process that follows a sequence of adhesive events, termed as leukocyte adhesion cascade. One can broadly generalize that most leukocytes follow a similar multi-step cascade in the peripheral (non-lymphoid) vasculature with some exceptions. Accordingly, an updated adhesion cascade in postcapillary venules involves free-flowing leukocytes initial attachment or tethering and slow velocity rolling (step 1),stable adhesion (arrest) on endothelial cells (step 2), leukocyte flattening (step 3), and subsequent crawling on the vascular endothelium, transendothelial cell migration (TEM) between (paracellular route) or through (transcellular) the vascular endothelium (step 4), and uropod elongation to complete transmigration of postcapillary venules (step 5). The initial attachment and rolling steps are initiated by interactions of endothelial E- and P-selectins and their counterreceptors on leukocytes L-selectin (Leick et al., 2014).

Each of these steps is necessary for effective leukocyte recruitment; these steps are not phases of inflammation, but represent the sequence of events from the perspective of each leukocyte. At any given moment they all happen in parallel, involving different leukocytes in the same microvessels.

From the initial selectin-dependent leukocyte tethering to endothelial cells to the final migration of leukocytes into the sub-endothelium, this process depends on the interplay between leukocyte receptors and endothelial cell counter-receptors, as well as on the presence of endogenous inhibitors of leukocyte adhesion enabling the targeted recruitment of leukocytes to inflamed tissues.

To enable the infiltration of leukocytes at the site of inflammation, a series of alterations in endothelial cells and leukocytes takes place:

- regulation of the expression of adhesion molecules in leukocytes
- increased secretion of chemokines by endothelial cells
- increased expression of adhesion molecules in the luminal surface of endothelial cells

(Kourtzelis and Mitroulis, 2015) (Robbins and Cotran: Pathologic Basis of Disease 2010).

After recruitment, activation includes phenotype modification with morphologic alterations, changes in marker proteins

(MHC, adhesion molecules, co-stimulatory signal), expression of mediators, enzymes, and pro-inflammatory proteins/lipids. Recruited monocytes recruited mature into macrophages with phagocytic activity and elaboration of a myriad of mediators of inflammation. The macrophage can replicate within tissues or die, including by apoptosis.

How it is Measured or Detected

in vivo imaging:

- Flow cytometry (FC/FACS),
- immunhistochemistry
- two photon-intravital microscopy (TP-IVM) (van Grinsven et al., 2017)
- Spinning Disk Confocal Microscopy-IVM (Jenne et al., 2011)
- Histology, increased cell numbers and altered composition

In vitro

- transwell Migration Assay (Justus et al., 2014)
- T-Lymphocyte & Innate Immune Cell Activation Assays
- Leukocyte Surface Markers (Monoclonal Antibodies to Leukocyte Surface Markers)
- Markers of leukocyte activation – protease release, ROS/RNS, NADPH oxidase (NOX), defense response - expression of anti-oxidants.
- organs-on-a-chip (Bnam et al., 2016; Ribas et al., 2017; Wufuer et al. 2016)

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Event: 1501: Increased, extracellular matrix deposition

Short Name: Increased extracellular matrix deposition

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:383 - Inhibition of Angiotensin-converting enzyme 2 leading to liver fibrosis	KeyEvent
Aop:414 - Aryl hydrocarbon receptor activation leading to lung fibrosis through TGF-β dependent fibrosis toxicity pathway	KeyEvent
Aop:415 - Aryl hydrocarbon receptor activation leading to lung fibrosis through IL-6 toxicity pathway	KeyEvent
Aop:494 - AhR activation leading to liver fibrosis	KeyEvent

Biological Context

Level of Biological Organization

Tissue

Key Event Description

ECM is a macromolecular structure that provides physical support to tissues and is essential for organ function. The composition of ECM is tissue specific and consists mainly of fibrous proteins, glycoproteins, and proteoglycans. The ECM in lung is compartmentalised to basement membrane and the interstitial space. Fibroblasts found in the interstitial space are the main sources of ECM in lung (White, 2015). Altered composition of ECM is observed in several lung diseases of inflammatory origin in humans including chronic obstructive pulmonary disease, asthma and idiopathic lung fibrosis. The composition and architecture of the ECM determines 1) the open sites of attachment that are available to cells, 2) the mechanical properties of the ECM and 3) the mechanical loading (breathing) experienced by the cells. Thus, changes in the ECM composition during the exaggerated wound healing process determines if an organism commits to fibrotic process or completes the wound healing (Blaauboer et al., 2014).

Evidence for its perturbation in the context of pulmonary fibrosis:

In lung fibrosis, an exaggerated amount of ECM is distributed in the alveolar parenchyma in a non-heterogenous manner, leading to lower spirometry readings implying occlusion of alveolar regions and reduced gas exchange. Collagen 1 and Collagen III are suggested to be the main components of the ECM in the thickened alveolar septa in fibrosis with other constituents such as fibronectin, elastin and tenacin C (Zhang et al., 1994; Hinz, 2006; Kuhn & McDonald, 1991; Crabb et al., 2006; Bensadoun et al., 1996; Klingberg et al., 2012; McKleroy et al., 2013). It is suggested that ECM composition dramatically changes during the fibrotic process. The early fibrotic process is characterised by collagen III deposition and collagen 1 predominates the later stages of the fibrosis. Excessive collagen production by myofibroblasts is necessary for the development of fibrosis (scarred tissue), with established areas of scar formation containing almost exclusively Type I collagen (Bateman et al., 1981; McKleroy et al., 2013; Zhang et al., 1994). Studies have demonstrated that while total collagen increases in IPF, there is also a shift toward the less elastic type I collagen, which contributes to the stiffness of the scar tissue within the lung (Nimni, 1983; Rozin et al., 2005; McKleroy et al., 2013).

The fibrotic ECM contains characteristic accumulation of fibroblasts and myofibroblasts, which are the major contributors of ECM synthesised. The proliferation of fibroblasts and their differentiation into myofibroblasts is, in turn, guided by the composition and structure of the ECM. For example, studies have demonstrated that cytokines secreted in response to inflammation are capable of activating fibroblasts, and that these changes could cause alterations in the fibroblasts that lead to excessive proliferation and ECM deposition (Sivakumar et al., 2012; Wynn, 2011).

How it is Measured or Detected

qRT-PCR, Immunosorbant assays, and immunohistochemistry:

The qRT-PCR, ELISA, and immunohistochemistry are routinely used to assess the levels of protein and mRNA levels. The various genes and proteins that are assessed include, collagen I, collagen III, elastin and tenacin C. Histological staining with stains such as Masson Trichrome, Picro-sirius red are used to identify the tissue/cellular distribution of collagen, which can be quantified using morphometric analysis both in vivo and in vitro. The assays are routinely used and are quantitative.

Sircol Collagen Assay for collagen quantification:

The Serius dye has been used for many decades to detect collagen in histology samples. The Serius Red F3BA selectively binds to collagen and the signal can be read at 540 nm (Chen & Raghunath, 2009; Nikota et al., 2017).

Hydroxyproline assay:

Hydroxyproline is a non-proteinogenic amino acid formed by the prolyl-4-hydroxylase. Hydroxyproline is only found in collagen and thus, it serves as a direct measure of the amount of collagen present in cells or tissues. Colorimetric methods are readily available and have been extensively used to quantify collagen using this assay (Chen & Raghunath, 2009; Nikota et al., 2017).

Ex vivo and in vitro models of ECM deposition:

No models currently exist which allow for in vitro assessment of ECM deposition. Using single, or co-cultures containing fibroblasts, the production of soluble ECM components can be assessed after exposure to a stressor of interest using either ELISA or qRT-PCR experiments as a proxy.

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List of Adverse Outcomes in this AOP

Event: 344: N/A, Liver fibrosis

Short Name: N/A, Liver fibrosis

Key Event Component

Process	Object	Action
liver fibrosis	liver	occurrence

AOPs Including This Key Event

AOP ID and Name	Event Type
Aop:38 - Protein Alkylation leading to Liver Fibrosis	AdverseOutcome
Aop:144 - Endocytic lysosomal uptake leading to liver fibrosis	AdverseOutcome
Aop:383 - Inhibition of Angiotensin-converting enzyme 2 leading to liver fibrosis	AdverseOutcome
Aop:494 - AhR activation leading to liver fibrosis	AdverseOutcome

Biological Context

Level of Biological Organization

Level of Biological Organization

Organ

Organ term

Organ term

liver

Domain of Applicability

Taxonomic Applicability

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

Life Stage Applicability

Life Stage	Evidence
All life stages	

Sex Applicability

Sex	Evidence
Unspecific	

Human: Bataller and Brenner, 2005;Merck Manual, 2015; Blachier et al., 2013.

Rat, mouse: Liedtke et al., 2013

Key Event Description

Liver fibrosis results from perpetuation of the normal wound healing response, as a result of repeated cycles of hepatocyte injury and repair and is a dynamic process, characterised by an excessive deposition of ECM (extracellular matrix) proteins including glycoproteins, collagens, and proteoglycans. It is usually secondary to hepatic injury and inflammation, and progresses at different rates depending on the aetiology of liver disease and is also influenced by environmental and genetic factors. If fibrosis continues, it disrupts the normal architecture of the liver, altering the normal function of the organ and ultimately leading to liver damage. Cirrhosis represents the final stage of fibrosis. It is characterised by fibrous septa which divide the parenchyma into regenerative nodules which leads to vascular modifications and portal hypertension with its complications of variceal bleeding, hepatic encephalopathy, ascites, and hepatorenal syndrome. In addition, this condition is largely associated with hepatocellular carcinoma with a further increase in the relative mortality rate (Bataller and Brenner, 2005; Merck Manual,2015)

Liver fibrosis is an important health issue with clear regulatory relevance. The burden of disease attributable to liver fibrosis is quite high; progressive hepatic fibrosis, ultimately leading to cirrhosis, is a significant contributor to global health burden (Lim and Kim, 2008). In the European Union, 0.1 % of the population is affected by cirrhosis, the most advanced stage of liver fibrosis with full architectural disturbances (Blachier et al., 2013). Besides the epidemiological relevance, liver fibrosis also imposes a considerable economic burden on society. Indeed, the only curative therapy for chronic liver failure is liver transplantation. More than 5.500 orthotopic liver transplantations are currently performed in Europe on a yearly basis, costing up to €100.000 the first year and €10.000 yearly thereafter (Van Agthoven et al., 2001).

How it is Measured or Detected

Liver biopsy is an important part of the evaluation of patients with a variety of liver diseases. Besides establishing the diagnosis, the biopsy is often used to assess the severity of the disease. Until recently it has been assumed that fibrosis is an irreversible process, so most grading and staging systems have relatively few stages and are not very sensitive for describing changes in fibrosis. In all systems, the stages are determined by both the quantity and location of the fibrosis, with the formation of septa and nodules as major factors in the transition from one stage to the next. The absolute amount of fibrous tissue is variable within each stage, and there is considerable overlap between stages. Commonly used systems are the Knodell score with 4 stages - no fibrosis (score 0) to fibrous portal expansion (score 2) to bridging fibrosis (score 3) and Cirrhosis (score 4) – and the more sensitive Ishak fibrosis score with six stages - from no fibrosis (stage 0) over increasing fibrous expansion on portal areas (stages 1-2), bridging fibrosis (stages 3-4), and nodules (stage 5) to cirrhosis (stage 6) (Goodman, 2007). Liver biopsy is an invasive test with many possible complications and the potential for sampling error. Noninvasive tests become increasingly precise in identifying the amount of liver fibrosis through computer-assisted image analysis. Standard liver tests are of limited value in assessing the degree of fibrosis. Direct serologic markers of fibrosis include those associated with matrix deposition — e.g.procollagen type III amino-terminal

peptide (P3NP), type I and IV collagens, laminin, hyaluronic acid, and chondrex. P3NP is the most widely studied marker of hepatic fibrosis. Other direct markers of fibrosis are those associated with matrix degradation, ie, matrix metalloproteinases 2 and 3 (MMP-2, MMP- 3) and tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1, TIMP-2).These tests are not commercially available, and the components are not readily available in most clinical laboratories. Some indirect markers that combine several parameters are available but not very reliable. Conventional imaging studies (ultrasonography and computed tomography) are not sensitive for fibrosis. Hepatic elastography, a method for estimating liver stiffness, is a recent development in the noninvasive measurement of hepatic fibrosis. Currently, elastography can be accomplished by ultrasound or magnetic resonance. Liver biopsy is still needed if laboratory testing and imaging studies are inconclusive (Carey, 2010; Germani et al., 2011) .

Regulatory Significance of the AO

From the OECD - GUIDANCE DOCUMENT ON DEVELOPING AND ASSESSING ADVERSE OUTCOME PATHWAYS - Series on Testing and Assessment 18: "...an adverse effect that is of regulatory interest (e.g. repeated dose liver fibrosis)"

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Appendix 2

List of Key Event Relationships in the AOP

List of Adjacent Key Event Relationships

Relationship: 19: Activation, AhR leads to Up Regulation, CYP1A1

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to hepatic steatosis	adjacent	High	
AhR activation leading to liver fibrosis	adjacent	High	High

Relationship: 2887: Up Regulation, CYP1A1 leads to Increase, ROS

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to liver fibrosis	adjacent	High	High

Relationship: 3219: Activation, AhR leads to Increased, Liver Steatosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to liver fibrosis	adjacent	High	Moderate

Relationship: 3218: Increased, Liver Steatosis leads to Cell injury/death

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to liver fibrosis	adjacent	Moderate	Moderate

Relationship: 68: Cell injury/death leads to Activation, Stellate cells

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Protein Alkylation leading to Liver Fibrosis	non-adjacent	High	
AhR activation leading to liver fibrosis	adjacent	Moderate	Moderate

Evidence Supporting Applicability of this Relationship

Taxonomic Applicability

Term	Scientific Term	Evidence	Links
human	Homo sapiens	High	NCBI
Rattus norvegicus	Rattus norvegicus	High	NCBI
Mus musculus	Mus musculus	High	NCBI

Human: [7][18] Rat: [12] Mouse: [4]

Key Event Relationship Description

Damaged hepatocytes can lead to activation of hepatic stellate cells (HSCs) through the release of ROS, cytokines and chemokines. Engulfment of apoptotic bodies from hepatocytes results in HSC activation and induces NOX (NADPH oxidases) expression in HSCs. DNA from apoptotic hepatocytes induces toll-like receptor 9 (TLR9)-dependent changes of HSCs that are consistent with late stages of HSC differentiation (activation), with up-regulation of collagen production and inhibition of platelet derived growth factor (PDGF)-mediated chemotaxis to retain HSCs at sites of cellular apoptosis. The release of latent TGF-beta complex into the micro-environment by damaged hepatocytes is likely to be one of the first signals for adjacent HSCs leading to their activation.

[1] [2] [3] [4] [5] [6] [7] [8] [9] [10]

Damaged hepatocytes also influence liver sinusoidal endothelial cell (LSECs), which make an integral part of the hepatic reticulo-endothelial system and have a role in HSC activation. LSECs are morphologically identified by their fenestrations, which are transcytoplasmic canals arranged in sieve plates. In healthy liver, hepatocytes and HSCs maintain this phenotype of LSECs through release of vascular endothelial growth factor (VEGF). Differentiated (i.e. fenestrated) LSECs prevent HSC activation and promote reversal of activated HSC to quiescence, but LSEC lose this effect when they are de-differentiated due to liver injury. Preclinical studies have demonstrated that LSECs undergo defenestration as an early event that not only precedes liver fibrosis, but may also be permissive for it. Changes in LSEC differentiation might be an integral part of the development of fibrosis. Furthermore, in fibrosis LSECs become highly pro-inflammatory and secrete an array of cytokines and chemokines [11] [12] [13] [14] [15]

This relationship is classified as indirect as HSCs activation is partly mediated by TGF-β1 and LSECs.

Evidence Supporting this KER

Biological Plausibility

There is a functional relationship between KE 1 and KE 4 consistent with established biological knowledge.[1] [2] [3] [4] [5] [6] [7] [8] [9] [10]

Empirical Evidence

There is temporal concordance as HSC activation follows hepatic injury and there is experimental evidence for this KER. Canbay et al. could show that Fas-mediated hepatocyte injury is mechanistically linked to liver fibrogenesis. Markers of HSC activation were significantly reduced when apoptosis was prevented in Fas-deficient bile duct ligated mice. These findings (reduction of inflammation, markers of HSC activation, and collagen I expression) could be repeated by pharmacological inhibition of liver cell apoptosis using a pan-caspase inhibitor. Coulouarn et al found in a co-culture model that hepatocyte - HSC crosstalk engenders a permissive inflammatory microenvironment. [16] [17] [18]

Uncertainties and Inconsistencies

There are no inconsistencies

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Relationship: 2890: Cell injury/death leads to Leukocyte recruitment/activation

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to liver fibrosis	adjacent	Moderate	Moderate

Relationship: 2891: Leukocyte recruitment/activation leads to Increased extracellular matrix deposition

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to liver fibrosis	adjacent	Moderate	Moderate

Relationship: 2892: Activation, Stellate cells leads to Increased extracellular matrix deposition

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
AhR activation leading to liver fibrosis	adjacent	High	High

Relationship: 2325: Increased extracellular matrix deposition leads to N/A, Liver fibrosis

AOPs Referencing Relationship

AOP Name	Adjacency	Weight of Evidence	Quantitative Understanding
Inhibition of Angiotensin-converting enzyme 2 leading to liver fibrosis	adjacent	Moderate	Not Specified
AhR activation leading to liver fibrosis	adjacent	High	High