Aryl hydrocarbon receptor (AHR): "pioneer member" of the basic-helix/loop/helix *per-Arnt-sim* (bHLH/PAS) family of "sensors" of foreign and endogenous signals

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Abstract

The basic-helix-loop-helix Per-Arnt-Sim (bHLH/PAS) family comprises many transcription factors, found throughout all three kingdoms of life; bHLH/PAS members "sense" innumerable intracellular and extracellular "signals" — including endogenous compounds, foreign chemicals, gas molecules, redox potential, photons (light), gravity, heat, and osmotic pressure. These signals then initiate downstream signaling pathways involved in responding to that signal. The term "PAS", abbreviation for "Per-Arnt-Sim" was first coined in 1991. Although the mouse *Arnt* gene was not identified until 1991, evidence of its co-transcriptional binding partner, aryl hydrocarbon receptor (AHR), was first reported in 1974 as a "sensor" of foreign chemicals, up-regulating cytochrome P450 family 1 (CYP1) and other enzyme activities that usually metabolize the signaling chemical. Within a few years, AHR was proposed also to participate in inflammation. The mouse [*Ah*] locus was shown (1973–1989) to be relevant to chemical carcinogenesis, mutagenesis, toxicity and teratogenesis, the mouse *Ahr* gene was cloned in 1992, and the first *Ahr(−/−)* knockout mouse line was reported in 1995. After thousands of studies from the early 1970s to present day, we now realize that AHR participates in dozens of signaling pathways involved in critical-life processes, affecting virtually every organ and cell-type in the animal, including many invertebrates.

Keywords

AHR; bHLH/PAS family of transcription factors; cancer; dose-response curve; embryonic stem cells; prostaglandins; eicosanoids; lipid mediators; mouse genetics; pro-inflammatory and post-inflammatory response; "brain-gut-microbiome"

Conflicts of interest

The author declares he has no actual, or potential, conflicts of interest.

"During the oral defense of my thesis (spring, 1964), a professor commented, 'Everyone knows that genes in the DNA are transcribed into RNA which is translated into protein. You're proposing that protein might control DNA? Why, that's heresy**!**' After a very awkward silence — my mentor Professor Howard S. Mason spoke up, 'And what's wrong with a little heresy**?**' " -----Daniel W. Nebert

1. Introduction

The first evidence for existence of aryl hydrocarbon receptor (AHR) occurred more than four decades ago. What do we know today about the AHR transcription factor, and in what critical-life processes does AHR participate?

To address these questions, we begin by describing the history of enzyme induction by foreign chemicals and inducible cytochrome P450 (CYP) monooxygenases; earliest studies were carried out in rat liver. Inbred mouse strains, unlike rats, were found to differ quite dramatically in degree of inducibility of certain P450 enzyme activities; this led to comparison of "potency" of inducers such as polycyclic aromatic hydrocarbons (PAHs) vs 2,3,7,8,-tetrachlorodibenzo-*p*-dioxin (TCDD; called "dioxin" in lay terms). Astonishingly, TCDD was found to be ~36,000 times more potent than PAHs.

A landmark study followed, comparing dose-response curves between TCDD-treated C57BL/6 (B6) and DBA/2 (D2) mice, showing that "inducible-resistant" D2 mice could be "forced" by TCDD to "turn on" their enzyme activity; due to the shape of the dose-response curve, it was concluded that a "receptor must exist that recognizes TCDD and regulates AHH acitivty." The manuscript was first rejected in 1973 by reviewers, with comments such as "heresy" and "implausible to think that a foreign chemical would bind to an intracellular receptor." Eventually, after rebuttal letters, the manuscript was accepted for publication; it appeared in 1974.

Next, AHR not only recognized foreign chemicals but was also found to be associated with inflammation — again, a hypothesis rejected by many colleagues. After the mouse *Ahr* and human *AHR* genes had been cloned and sequenced, AHR was finally identified as "a member of the PAS domain family of signal sensors." Shortly thereafter, *Ahr(−/−)* knockout mouse lines provided strong evidence of the vast importance of AHR in numerous critical-life processes independent of foreign chemical treatment. AHR is now appreciated to function during the cell cycle, cell migration, cell adhesion, and other embryonic stem (ES) cell functions; these findings are consistent with early studies that had shown AHR-dependent birth defects in PAH- and especially TCDD-treated laboratory animals. Finally, it became appreciated that AHR is involved in many signaling pathways that affect various critical-life functions in most organs, tissues and/or cell types — in both vertebrates and invertebrates.

2. History and background

2.1. Inbred mouse strain differences in enzyme induction

The earliest studies of enzyme induction by foreign chemicals — in liver of PAH-treated rats — were conducted by Allan Conney, a graduate student in the Millers' laboratory (Conney *et al.*, 1956; Conney *et al.*, 1957); as a postdoctoral fellow in the laboratory of Jim Gillette, Conney continued those studies (Conney *et al.*, 1959). Subsequently, induced "benzpyrene hydroxylase" throughout the rat gastrointestinal (GI) tract was described, following oral benzpyrene treatment; highest induced enzyme levels were found in duodenum (Wattenberg *et al.*, 1962). Thus, here was an exciting concept: a novel "signal" is introduced to the animal, or cell; the "response" is to increase enzyme(s) to metabolize that signal. This model was reminiscent of earlier studies in *E. coli*: the "signal" (addition of tryptophan to tryptophan-deficient culture medium) led to a bacterial "response" of dramatic increases in enzymes in the tryptophan-metabolizing pathway (Newton and Snell, 1965).

Following these studies by Conney and Wattenberg, the original "benzpyrene hydroxylase" name was changed to the broader term "aryl hydrocarbon hydroxylase" (AHH), because the substrate was shown to include any of several PAHs, and several PAHs were shown to be inducers having varying potency; formation of hydroxylated benzo[*a*]pyrene (*nmol/min/mg protein*) became the standard AHH assay, which was developed and applied to PAH-treated cultures of fetal hamster cells (Nebert and Gelboin, 1968a; Nebert and Gelboin, 1968b). Induced AHH activity in cell culture was shown to involve both transcription of DNA into mRNA and translation of mRNA into protein (Nebert and Gelboin, 1970). Subsequently, substantial differences in AHH inducibility between PAH-treated B6 and D2 mice were reported (Nebert and Gelboin, 1969); lack of AHH inducibility was then shown to behave usually as an autosomal recessive trait (Gielen *et al.*, 1972; Robinson *et al.*, 1974).

These genetic differences led to a model system far superior to that of PAH-treated vs untreated rats, *i.e.* an identical dose of the same chemical in genetically different mice results in striking differences, apparently based predominantly on a single gene. This single gene was subsequently found to be largely responsible for PAH-induced cancer of multiple types, mutagenesis, toxicity and birth defects [*reviewed in* (Nebert, 1989)]. In fact, PAH treatment of a pregnant mouse with a particular genotype, and then observing differences in toxicity and/or teratogenesis in utero among her offspring having different genotypes — became an especially powerful tool for studies in developmental embryology [(Nebert *et al.*, 1972; Shum *et al.*, 1979) *& reviewed in* (Nebert, 1989)].

With regard to clinical relevance, human AHH activity in placenta — comparing cigarette smokers with nonsmokers during pregnancy — revealed that cigarette smoke induces AHH activity (Welch *et al.*, 1968; Nebert *et al.*, 1969). This finding has important implications for the health of newborns from cigarette-smoking mothers.

2.2. Proof that AHH activity is a P450 monooxygenase

"Cytochrome P-450" was first detected as a "colored pigment in the cell (Strittmatter and Velick, 1957) which — when reduced with NADPH and bound to CO — shows a spectrophotometric Soret peak wavelength at 450 nm" (Omura and Sato, 1962; Omura and

Sato, 1964). Soon thereafter at the same symposium, three independent laboratories reported that "microsomal mixed-function oxidase" named for electron spin resonance properties of "microsomal Fe_x" (Mason $et\ al.$, 1965), enzymatic functions of microsomal cytochrome P-450 (Omura $et\ al.$, 1965), and particular steroid hydroxylases (Ernster and Orrenius, 1965) all appeared to be one and the same enzyme or enzyme family.

The enzyme active-site comprises a heme-iron center — with tetrahedral iron tethered to the four nitrogen atoms of the porphyrin ring, cysteine-thiolate in fifth position, and binding of H_2O or substrate (hydroxyl group, nitrogen atom, or molecular O_2) in the sixth position. The O atom transferred to the substrate is derived from atmospheric diatomic O_2 rather than H_2O (Hayaishi $et\ al.$, 1955; Mason $et\ al.$, 1955; Mason, 1957); hence, the name "monooxygenase" is more suitable for these enzymes.

Because of spectral properties similar to those of mitochondrial cytochromes, P-450 was misnamed a "cytochrome" (Omura and Sato, 1962), an inaccurate label that unfortunately has persisted to this day. A more appropriate term would have been "heme-thiolate monoogenase" (Daiber and Ullrich, 2002); however, the name "cytochrome P450" had become thoroughly entrenched — long before details of the enzyme proteins and functions had been recognized.

After PAH treatment of rats, a second form of liver microsomal cytochrome P-450, called "P-448" could be detected spectrophotometrically (Glaumann $et\ al.$, 1969; Lu $et\ al.$, 1971); another lab termed the PAH-inducible enzyme "P_1-450" (Parli and Mannering, 1970). It was thus postulated that "AHH activity" was "P-448" or "P_1-450." Therefore, a spectrophotometric assay — to study the height and location of the Soret peak — was carried out in PAH-treated fetal hamster cell cultures; indeed, upon treatment of the cell homogenate with NADPH and CO, a peak developed and was associated with increasing AHH activity as a function of time, during which the Soret peak shifted from 450 to 446 nm (Nebert, 1970). In later studies, it became clear that PAH-inducible AHH activity is associated with both two distinct enzymes, "P_1-450" and "P-448" (Atlas $et\ al.$, 1975; Atlas $et\ al.$, 1977); ultimately, these were named "CYP1A1" and "CYP1A2," respectively. The latter represents high-spin iron Fe^{3+} that causes a hypsochromic shift in the Soret peak of reduced CO-bound heme.

2.3. Genetic differences in mouse AHH induction by TCDD

In clinical studies spearheaded by Ray Suskind before 1970, workers exposed to TCDD in trichlorophenol-processing factories were shown to be at extremely high risk for chloracne and porphyria cutea tarda [$reviewed\ in$ (Zack and Suskind, 1980)]. This led Alan Poland, using chick egg liver (Poland and Glover, 1973), to show that TCDD was ~36,000 times more potent than any PAH in the induction of δ-aminolevulinic acid synthetase — a key enzyme in porphyrin synthesis.

Then came the first "**Aha!**" moment: If δ-aminolevulinic acid synthetase activity is strikingly induced by the highly potent TCDD, and heme is a product of porphyrin synthesis, and AHH represents a P450 hemoprotein, would TCDD be superior to PAHs in causing AHH induction? In particular — would the "lack of AHH induction," seen in PAH-

treated D2 mice (Nebert and Gelboin, 1969; Gielen *et al.*, 1972), be overcome by TCDD treatment? Further, would TCDD be able to increase inducible AHH activity in B6 mice to even higher levels? Following a telephone call, these questions were answered by Alan Poland visiting the Nebert laboratory, where the conclusive experiments were performed together (Poland *et al.*, 1974).

Fig. 1 illustrates dose-response curves of B6 vs D2 hepatic AHH activity as a function of TCDD dosage. Earlier studies, with PAH inducer 3-methylcholanthrene (80 mg/kg; 24 h), had shown 5- to 10-fold induction of hepatic AHH activity in B6, but no detectable increases in D2 liver AHH (Nebert and Gelboin, 1969; Gielen *et al.*, 1972); in fact, no further induction of AHH activity is found — even at 800 mg/kg, about the highest dose of 3-methylcholanthrene physically and chemically possible (*unpublished*). In contrast (Fig. 1), whereas TCDD (1 μg/kg; 24 h) elicits ~6-fold increases in hepatic AHH activity in B6 but no detectable increase in D2 mice, higher TCDD doses (10–100 μg/kg; 24 h) raise D2 hepatic AHH activity to levels similar to those of B6 mice.

The ED_{50} value denotes the "effective dose to reach 50% of maximal response." When comparing curves in Fig. 1, the ED_{50} value for D2 is shifted ~20-fold to the right, relative to that of B6 mice. The shape of such a dose-response curve is consistent with a receptor — rather than changes in enzyme itself (Poland *et al.*, 1974). Indeed, subsequent cloning and DNA sequencing studies showed that the amino-acid sequences of B6 "P_1-450" (Kimura *et al.*, 1984) and D2 "P_2-450" (Kimura and Nebert, 1986) are identical, whereas "P_3-450" (P-448) is a product of a different gene (Kimura *et al.*, 1984); the former two are now officially named "CYP1A1," whereas the latter is "CYP1A2."

Following treatment with radiolabeled TCDD, hepatic cytosol accumulation of radiolabel was found to be greatest in B6 mice, intermediate in $B6D2F_1$ mice, and least in D2 mice (Poland *et al.*, 1976) — a pattern mirroring strain sensitivity to AHH induction by TCDD, demonstrated two years earlier (Poland *et al.*, 1974). These data are consistent with a small pool of high-affinity binding-sites (estimated K_d equilibrium constant ~0.27 nM) that stereospecifically and reversibly binds TCDD. Thus, these findings strengthened the earlier hypothesis: the hepatic cytosolic TCDD-binding species is a receptor that up-regulates AHH activity. Furthermore, a proposed receptor gene mutation in D2 mice most likely causes the diminished-affinity phenotype for TCDD (Poland *et al.*, 1976). These 1974–76 studies "set the stage" for "the AHR story," paving the way for future discoveries (Okey, 2007).

2.4 Creation of mutant benzo[a]pyrene-resistant hepatoma cell lines

We would be remiss not to mention the landmark study (Hankinson, 1979) in which the mouse hepatoma line Hepa-1c1c7 was shown to exhibit inducible AHH activity — and from which benzo[*a*]pyrene-resistant clones were isolated; ultimately, the most important clones included AHR-deficient (*c2*), ARNT-deficient (*c4*) and CYP1A1-deficient (*c1* & *c37*). Mutations in the *Ahr*, *Arnt*, and *Cyp1a1* genes, respectively [*vide infra*], were shown in each case to be responsible for the phenotype (Legraverend *et al.*, 1982; Hankinson *et al.*, 1985; Kimura *et al.*, 1987b). These mutant clones, compared with the wild-type parent Hepa-1 line, became an invaluable model system for unraveling many of the mysteries of the AHR-CYP1 axis [*e.g.* (Robertson *et al.*, 1987), *among dozens of other publications*].

3.0 Early evidence for AHR involvement in inflammation

3.1 Ethanol-induced peritonitis: Ahr allelic differences

As mentioned [*vide supra*], the high- vs poor-affinity AHR, encoded by B6 *Ahr^b1^* vs D2 *Ahr^d^* alleles, respectively, were shown to be pivotal for demonstrating differences in PAH-caused cancer of several types, mutagenesis, birth defects — as well as toxicity of ovary, eye, bone marrow, atherosclerosis and oxidative stress [*reviewed in* (Nebert, 1989)]. Curiously, in 1979 differences in "ethanol sleep time" were observed in AHR "high- vs poor-affinity" mice, even among pups from the same litter.

The "ethanol sleep time" is actually a misnomer, because the mouse remains alert with eyes open; however, the anterior abdomen is warm to the touch and, upon opening the abdominal cavity, one sees an acute inflammatory response to intraperitoneal ethanol — more severe in "long-sleep" *Ahr^d^* than "short-sleep" *Ahr^b1^* mice. This intriguing finding in 1979, manuscript again rejected (as "implausible" and "heresy"), was published a decade later (Bigelow *et al.*, 1989) and ultimately presented at a Nobel Symposium (Nebert, 1994).

Planar polychlorinated biphenyls were also known to induce AHH activity (Poland and Glover, 1977). A study in chick egg liver (Rifkind and Muschick, 1983) reported that the nonsteroidal anti-inflammatory agent benoxaprofen decreases toxicity of planar 3,4,3′,4′-tetrachlorobiphenyl — suggesting involvement of "the arachidonic acid cascade." Then came another "**Aha!**" moment. AHR is associated with both planar PCB-caused toxicity in chick egg liver (Rifkind and Muschick, 1983) and ethanol-induced peritonitis in AHR poor-affinity mice (Bigelow *et al.*, 1989); thus, everything was consistent with the proposal (Nebert *et al.*, 1981) that one or more endogenous ligands of AHR might represent AHR-mediated CYP1-generated metabolites present in the arachidonic acid/eicosanoid/lipid-mediator (LM) second-messenger pathway response to inflammation. This hypothesis also makes sense, in terms of the CYP1 enzyme active-site, believed to accommodate chemicals having structural similarities in size and planar shape for both PAHs and many LMs.

3.2 Fertility, fitness and longevity: Ahr allelic differences

Besides the inflammatory response, allelic differences in *Ahr* as manifested by high- vs poor-affinity AHR — were curiously discovered to be associated with fertility, general health and longevity. Recombinant inbred (RI) lines were developed in the 1970s from progenitor C57BL/6 and C3H/He mice having *Ahr^b1^* (high-affinity) and *Ahr^b2^* (intermediate-affinity) alleles, respectively; between generations 7 and 13, individual female and male RI mice were then crossed with D2 (*Ahr^d^* allele; poor-affinity AHR) and followed for several generations. Highest levels of high-affinity AHR in both female and male offspring were found intriguingly to be associated with greater fertility, fitness, and longer life span (Nebert *et al.*, 1984). This improbable finding, difficult to explain at the time, suggested that AHR might be pivotal in one or more critical life functions.

Three decades later, these observations are now much clearer. "Disease tolerance" reflects the host's ability to lower effects of infection on host fitness; for example, an initial exposure to bacterial lipopolysaccharide (LPS) usually induces a state of refractoriness to further LPS challenge ("endotoxin tolerance"). It was shown that a first LPS exposure of mice activates

AHR and the hepatic enzyme TDO (tryptophan 2,3-dioxygenase) — which provides an active ligand for AHR, which in turn down-regulates early inflammatory gene expression. Upon LPS rechallenge, AHR participates in long-term regulation of systemic inflammation only in the presence of IDO1 (indoleamine 2,3-dioxygenase-1); moreover, AHR-complex-associated SRC (SRC proto-oncogene, non-receptor tyrosine kinase) activity enhances IDO1 phosphorylation and signaling. This resulting endotoxin-tolerant state protects mice against immunopathology of both gram-positive and -negative infections, consistent with a role for AHR in contributing to host fitness (Bessede *et al.*, 2014). Also, *Ahr(−/−)* knockout mice have been shown to be hypersensitive to LPS-induced septic shock (Ichihara *et al.*, 2007).

Bacterial pigmented virulence factors (*e.g.* naphthoquinone phthiocol of *Mycobacterium tuberculosis* and phenazines of *Pseudomonas aeruginosa*) are also known AHR ligands; upon ligand binding, AHR activation leads to virulence-factor degradation and regulated cytokine and chemokine production. The relevance of AHR to host defense was underscored by AHR-deficient mice showing heightened susceptibility to both *P. aeruginosa* and *M. tuberculosis* infections (Moura-Alves *et al.*, 2014).

Both of these recent studies (Bessede *et al.*, 2014; Moura-Alves *et al.*, 2014) are consistent with the earlier breeding studies that had been carried out in a nonsterile animal room (Nebert *et al.*, 1984). The data suggest that "resistance to chronic subclinical, low-grade infections" in a "dirty" mouse colony might have been the beneficial reason for high-affinity-AHR mice to have evolved the phenotypes of greater fertility, fitness, and longer life span.

4. *AHR* gene identified and characterized

4.1. Molecular cloning of the AHR gene

When an AHR ligand enters the cell as a "signal," it binds to cytoplasmic AHR — which is already bound to 90-kDa heat shock protein (HSP90), a 38-kDa AHR-interacting protein (AIP), and a less well-characterized "p23" protein. Because of such low intracellular concentrations of AHR, cloning of the gene had remained elusive; in fact, attempting to clone *Ahr* led first to cloning the *Arnt* gene coding for aryl hydrocarbon receptor translocator (Hoffman *et al.*, 1991). When the NH$_2$-terminal sequence of the mouse AHR protein was finally reported (Bradfield *et al.*, 1991), this led quickly to "fishing out" the B6 high-responsive *Ahr*[b1] cDNA (Burbach *et al.*, 1992; Ema *et al.*, 1992), and, subsequently the mouse *Ahr* gene (Schmidt *et al.*, 1993) and human *AHR* cDNA (Dolwick *et al.*, 1993) and gene (Le Beau *et al.*, 1994).

It could then be concluded (Burbach *et al.*, 1992) that AHR is a ligand-activated transcription factor; AHR encodes a basic region/helix-loop-helix (bHLH) protein, similar to many transcription factors that undergo dimerization for function, as well as having extensive sequence similarity to ARNT (Hoffman *et al.*, 1991) and two homologous *Drosophila* regulatory proteins "single-minded" (SIM) and "periodic" [PER; in vertebrates called "period circadian clock-1" (PER1) (Konopka and Benzer, 1971; Crews *et al.*, 1988). These data were consistent with an earlier study in mouse liver (Tukey *et al.*, 1982), demonstrating that — as TCDD-bound AHR became diminished in cytosol — it appeared in

the nucleus and was associated with increases in P_1-450 mRNA induction, *i.e.* intranuclear increases in AHR are correlated with CYP1A1 increases, reflecting induction of AHH activity.

4.2. The bHLH/PAS (per-Arnt-sim) family of signal sensors

Chemoreceptors exist in all phyla of life, comprising two types: transmembrane and cytoplasmic. Cytoplasmic chemoreceptors appear to "sense" the "signal" within the cytoplasm, including signals that enter the cell. These sensors contain diverse signal-input domains — usually located NH_2-terminally to the protein segment that defines a chemoreceptor [*i.e.* so-called "methyl-accepting" (MA) domain]; however, various other NH_2-terminal domains also exist. The most common signal-input domain is the PAS domain. It is not rare, however, for some cytoplasmic chemoreceptors to have instead, COOH-terminal domains that function in signal-sensing (Collins *et al.*, 2014).

The term "PAS" was first coined in late 1991 (Nambu *et al.*, 1991). In eubacteria, PAS domains are usually positioned near the NH_2-terminus of signaling proteins — such as sensor histidine kinases, cyclic-diGMP synthases/hydrolases, and MA chemotaxis proteins. The PAS domain family in all living organisms forms "structural clades" on the basis of two principal variables: [a] topological location (inside or outside the plasma membrane); and [b] class of small molecules to which they bind. Binding of a chemically diverse range of small-molecule metabolites is a hallmark of the PAS domain family (Fig. 2). PAS ligand-binding proteins function either as a primary cue to initiate an intracellular-signaling response, or they provide a domain having the capacity to respond to secondary physical or chemical signals — such as gas molecules, redox potential, or photons (Henry and Crosson, 2011).

Genes coding for various bHLH-PAS proteins found in eukaryotic metazoans include: vertebrate *AHR*; *ARNT*; AHR repressor (*AHRR*); *ARNTL* (ARNT-like; also called BMAL); *CLOCK* (clock circadian regulator); *NPAS2* (neuronal PAS protein-2); *MIA3* (melanoma inhibitory activity family member-3—first named in *Drosohila* as *Tgo* (tango); *EGLN1* (egl-9 family hypoxia-inducible factor; previously HIF); *SIM1* and *SIM2* (single-minded; *Sim* is a single gene in *Drosophila*); and several steroid receptor coactivators (SRCs); genes encoding bHLH-PAS proteins in *Drosophila*, but not vertebrates, include *trh* (tracheales) (Pecenova and Farkas, 2016) and *ss* (spineless) (Céspedes *et al.*, 2010), also called aristopaedia (Emmons *et al.*, 1999). Estimated numbers of bHLH/PAS sensor genes are ~30 in the human genome, twelve in *Ciona intestinalis* (sea squirt), 15 in *Drosophila*, and nine in the nematode *Caenorhabditis elegans* (Hahn *et al.*, 2006).

Cryptochromes are not to be confused with bHLH/PAS proteins or cytochromes. Cryptochromes, receptors for blue and ultraviolet UV-A light, participate in circadian rhythmicity of plants and animals; they share sequence similarity to DNA photolyases but have no photolyase activity (Lin and Todo, 2005). However, a number of plant and bacterial photosensor genes (*e.g. PLPB*, *BPLPA*) also encode PAS domain-containing receptors that exhibit ligand-binding, dimerization, and signal-transduction qualities (Pellequer *et al.*, 1998).

Similar to AHR, *Drosophila* spineless functions as a heterodimer with tango, the *Drosophila* ortholog of ARNT; moreover, spineless participates in genesis of specialized fly neurons involved in color vision. Interestingly, although spineless-tango heterodimers exhibit very similar binding specificities to AHR-ARNT heterodimers found in mammals — interaction of spineless with tango apparently does not require presence of exogenous or endogenous ligands and, as such, represents an instance in which AHR physiological function appears to be independent of ligand-binding-mediated signal cascades [*reviewed in* (Lindsey and Papoutsakis, 2012)]. AHR also does not appear to require ligand-binding prior to evoking signal-mediated pathways — in innumerable other invertebrates: the nematode *Caenorhabditis elegans* (Powell-Coffman *et al.*, 1998); the cnidarian, a sea anemone *Nematostella vectensis* (Reitzel *et al.*, 2014); the inter-tidal copepod *Tigriopus japonicus* (Kim *et al.*, 2015); the zebra mussel *Dreissena polymorphia* (Chatel *et al.*, 2015); the pearl oyster *Pinctada martesii* (Du *et al.*, 2015); and the red flour beetle *Tribolium castaneum* (Grunwald *et al.*, 2015) — just to name a few recent examples.

Although originally named "HIF1α" and "HIF1β", the latter was realized to be ARNT; thus, the former became simply "HIF" (official gene name is now *EGLN1*). Today it is known that two evolutionarily highly conserved HIF genes exist: *HIF1A* and *HIF3A* (*Hif1a* & *Hif3a* in mouse).

Although there are substantial size-differences between the bHLH-PAS family members, they all share NH_2-terminally-located bHLH and PAS domains (Fig. 2). The majority (including AHR) contain "twin-PAS segments," consisting of PAS-A and PAS-B domains. Several bHLH-PAS members also carry "Gln- and Ser-rich" motifs in COOH-terminal regions (Hahn *et al.*, 2006; Okey, 2007).

As mentioned above, bHLH proteins, like many transcription factors, undergo dimerization before becoming functionable. Thus, vertebrate AHR dimerizes with ARNT before becoming functionally active; neither AHR/AHR nor ARNT/ARNT dimers are ever functional (Wu *et al.*, 2013). Both SIM1 and SIM2 are also nuclear proteins that constitutively complex with the general bHLH/PAS partner, ARNT (Woods and Whitelaw, 2002). SIM proteins, in combination with ARNT, attenuate transcription under hypoxic conditions, by way of hypoxia-inducible response elements (HREs), which are present in promoter/enhancer regions of numerous genes.

Such cross-talk between co-expressed bHLH/PAS factors can occur through competition for ARNT — seen, for example, during SIM-mediated repression of AHR-induced transcription from "xenobiotic-response elements" (XREs; *also called* "AHREs"). Interestingly, SIM1/ARNT dimers, but not SIM2/ARNT, are able to activate transcription via the mouse *Epo* enhancer (erythropoietin gene) during normoxia. This finding implies there exists a hypoxic-switch mechanism in cells co-expressing SIM1 vs SIM2 and EGLN1 proteins during normoxia vs hypoxia; thus, up- vs down-regulation causing activation of the *EGLN1* gene occurs concomitantly with attenuation of SIM activities (Woods and Whitelaw, 2002). On the other hand, SIM1 and SIM2 do not form homodimers, and individually, neither one of them interacts with AHR (Probst *et al.*, 1997).

In animals, these bHLH transcription factors participate in numerous developmental programs — such as cell migration, cell adhesion, DNA damage control and protection from oxidative stress, environmental adaptation, organogenesis, hematopoiesis, myogenesis, neurogenesis, and sex determination — which exist in all kingdoms of life (Pecenova and Farkas, 2016). In plants, bHLH transcription factors participate in a vast array of signal-sensing; the "signals" detected include diatomic O_2, small metabolites (*e.g.* phytoalexins), and light, which control plant growth and development and plant-stress adaptation responses. For example, the signaling cascade that triggers the phytohormone jasmonate (224.3 Da) — modulates a diverse, but specific, range of aspects of plant growth, development and defense (Goossens *et al.*, 2017).

PAS domain-containing proteins take part in circadian rhythmicity and regulate responses to environmental change (Vogt and Schippers, 2015). Hence, it should come as no surprise that AHR — originally discovered as a "sensor" of 3-methylcholanthrene (268.4 Da), benzo[*a*]pyrene (252.3 Da) and TCDD (322.0-Da) molecules — should be a member of the bHLH PAS family of transcription factors.

4.3. Generation of knockout and other transgenic mouse lines

4.3.1. Generation of the Ahr knockout mouse—Following publication of the mouse AHR NH_2-terminal amino-acid sequence (Bradfield *et al.*, 1991), *Ahr(−/−)* knockout mouse lines were independently created in several laboratories (Fernandez-Salguero *et al.*, 1995; Lahvis and Bradfield, 1998; Shimizu *et al.*, 2000). Perhaps not too suprising, *Ahr(−/−)* mice display strong resistance to the exceptionally toxic effects of TCDD (Fernandez-Salguero *et al.*, 1996), protection against PAHs such as benzo[*a*]pyrene (Shimizu *et al.*, 2000), and impaired DNA-PAH adduct formation caused by CYP1 enzymes (Kondraganti *et al.*, 2003).

Far more interesting to some of us, however, was that *Ahr(−/−)* mice also exhibited: immune system impairment (Fernandez-Salguero *et al.*, 1995; Lahvis and Bradfield, 1998); lack of closure of ductus venosus in the neonate (Lahvis *et al.*, 2005); hepatic fibrosis (Fernandez-Salguero *et al.*, 1995) and liver necrosis due to formation of numerous arteriovenous (A–V) shunts (Harstad *et al.*, 2006); and impaired fertility, increased risk of embryonic death, multiple-organ dysregulation of organogenesis during in utero development, and stunted postnatal growth [*reviewed in* (Tohyama, 2014)].

More comprehensive examination has shown that AHR participates in numerous developmental signaling pathways, in many cases consistent with earlier findings in *Ahr(−/−)* mice, tissue culture, and biochemical experiments. These pathways include: cell-cycle regulation, immediate-early gene induction, cross-talk with the RB1/E2F axis, apoptosis, mitogen-activated protein-kinase cascades, and mobilization of pivotal calcium stores [*reviewed in* (Puga *et al.*, 2009)]. This information from a decade ago has now been extended to the latest discoveries in *Ahr(−/−)* mice — including AHR-mediated dysfunctions in: ectoderm transition to epithelium; development of the heart, central nervous system (CNS), inner ear, liver, pancreas, kidney, bone formation, hematopoiesis, immune cell functions, and even glucose homeostasis. These phenotypes will covered in greater detail later in this review.

4.3.2. Generation of Cyp1 knockout mice—Because *CYP1* gene induction appears to be the most sensitive to — and heavily reliant on — AHR regulation, details of these genes (*CYP1A1*, *CYP1A2*, and *CYP1B1*) are important to introduce. Whereas no human "knockout" equivalent has been found for *AHR*, *CYP1A1* or *CYP1A2*, almost 150 *CYP1B1* mutations are associated with primary congenital glaucoma [*reviewed in* (Li *et al.*, 2011)]. Metabolites in the arachidonic acid pathway, cyclooxygenases, and prostaglandins are known to be altered in glaucomatous human ciliary muscle cells, compared with normal HCM cells; these data are consistent with lipid mediator (LM)-controlled regulation of uveoscleral outflow and, hence, glaucoma formation (Husain *et al.*, 2002). Discovery of human CYP1B1 "knockouts" resulted in confirmation in the mouse, in which a defect in the ciliary body (which functions to control intraocular pressure) in *Cyp1b1(–/–)* mice is enhanced by absence of the tyrosinase gene (*Tyr*) — indicating pigmentation is important in contributing to the *Cyp1b1*-null phenotype (Libby *et al.*, 2003). These data indicate that, during embryogenesis, development of the eye's anterior chamber involves CYP1B1-mediated metabolism of a critical endogenous substrate in the LM second-messenger cascade.

CYP1 enzyme functions deserve to be mentioned. The vertebrate CYP1A1 monoxygenase metabolizes planar substrates — many of which include PAHs, certain halogenated biphenyls and dibenzofurans, β-naphthoflavone, and indole/tryptophan metabolites; CYP1A1 metabolizes only a few drugs. Constitutive CYP1A1 (mRNA or protein) expression in vertebrates is almost always nil (*i.e.* "promoter activity" only), whereas inducible CYP1A1 activity is ubiquitous, located in virtually every tissue and cell-type of the body. Inducible CYP1A1 is also seen in very early embryogenesis, even in liver of partial-hepatectomized mice (Kimura *et al.*, 1987a), and also without exposure to any foreign chemical [*reviewed in* (Nebert *et al.*, 2000; Nebert *et al.*, 2004)].

CYP1A2 metabolizes at least two dozen drugs, including caffeine and theophylline; CYP1A2 substrates include many environmental aromatic amines and PAHs. Human CYP1A2 activity is not found in embryo or kidney; AHR-dependent CYP1A2 induction occurs mostly in liver, GI tract, pancreas, nasal epithelium, brain and lung [*reviewed in* (Nebert *et al.*, 2004)]. *CYP1A2* is very likely the result of a gene-duplication event with the older gene *CYP1A1* [(Ahokas *et al.*, 1979); *reviewed in* (Nelson *et al.*, 1996)].

CYP1B1 (like CYP1A1) metabolizes many environmental chemicals — including certain PAHs and biphenyls, but in additiion metabolizes *N*-heterocyclic amines, arylamines and amino-azo dyes; metabolism can render the products carcinogenic and/or toxic, as well as detoxified, byproducts. Also similar to CYP1A1, CYP1B1 metabolizes only a few drugs. Unlike CYP1A1, however, CYP1B1 often displays substantial basal levels in endocrine tissues and certain cancers. Moreover, inducible CYP1B1 expression is about as ubiquitous as CYP1A1, with regard to expression in almost all tissues and cell-types, but especially in endocrine tissues and immune cells [*reviewed in* (Nebert *et al.*, 2004)].

Laboratory animal studies show strong correlations between induced CYP1 activities and various types of cancer [*reviewed in* (Nebert, 1989)]. However, conclusions from innumerable clinical epidemiological studies on "*CYP1* polymorphisms and cancers" are all

questionable [*reviewed in* (Nebert and Dalton, 2006)], because it is now well established that CYP1 enzymes participate not only in metabolic activation but also detoxication; "activation" vs "detoxication" varies as a function of dose of carcinogen, route-of-administration, pharmacokinetics of the chemical, target-organ specificity, time, and genotype [*reviewed in* (Nebert *et al.*, 2013a)].

Most importantly, substrates of all three CYP1 monooxygenases include an unknown number of polyunsaturated fatty acid (PUFA)-derived ω–6 and ω–3 lipid mediators (LMs). The relevance of this topic is discussed later.

4.3.3. Cyp1 gene structures and consequences of being ablated—The
CYP1A1_CYP1A2 locus, located on human Chr 15q24.1 and mouse Chr 9 spanning nucleotides 57676937–57703822, contains the two genes oriented head-to-head, sharing a bidirectional promoter. *CYP1A1* — on the reverse-strand — is 5′-ward of *CYP1A2*, with the two genes sharing a 23.3-kb (human) and 14.0-kb (mouse) bidirectional promoter between them. This "intergene-spacer region" contains multiple highly-conserved (AHREs) AHR-binding sites (Corchero *et al.*, 2001), most or all of which presumably function in regulation of one or both *CYP1A* genes. *CYP1B1*, located on the reverse-strand of human Chr 2p22.2 and mouse Chr 17 spanning nucleotides 79706953–79715041, is in a subfamily distinct from that of *CYP1A* genes, because of much less homology and therefore having evolved from the *CYP1A* locus more than 420 million years ago (Poland *et al.*, 1974; Probst *et al.*, 1997). We can estimate this evolutionary date because fish genomes carry only the *CYP1A1* and *CYP1B1* genes, but not a *CYP1A2* gene (Ahokas *et al.*, 1979); sea vertebrates are known to have diverged evolutionarily from land vertebrates ~420 million years ago (Ahokas *et al.*, 1979; Nelson *et al.*, 1996).

Studies of mouse *Ahr* gene knockouts and all three *Cyp1* single-, plus all three possible double-, and the triple-knockout (TKO) lines have been described. The single- and double-knockouts and cell-type conditional knockouts as experimental models have helped immensely in understanding the role of intestinal CYP1A1 in protection against oral benzo[*a*]pyrene [*reviewed in* (Nebert *et al.*, 2013a)].

The eight gene knockout lines (described above) are all viable and able to reproduce — although serious viability problems are seen in the *Ahr(−/−)* knockout, as mentioned earlier (Fernandez-Salguero *et al.*, 1995; Lahvis and Bradfield, 1998), and in *Cyp1a1/1a2/1b1(−/−)* TKO mice (Dragin *et al.*, 2008). Interestingly, only about one in 40 TKO F_1 newborns survived to adulthood (Dragin *et al.*, 2008); yet, astonishly, although dwarfed in size, they were able to breed and quickly became more normal-in-size and healthier in subsequent generations. Most likely, unknown epigenetic effects are at play (Nebert *et al.*, 2010). These fertile, healthy TKO mice are being shared worldwide with other laboratories.

TKO F_1 pups exhibit phenotypes that include *incomplete penetrance* for: slower weight gain and greater risk of embryolethality before gestational day-11 (GD11); increased risk of hydrocephalus, hermaphroditism and cystic ovaries; and prominent suppression of the immune response to zymosan-induced peritonitis (Dragin *et al.*, 2008). These traits were proposed (Dragin *et al.*, 2008; Nebert and Karp, 2008) to be consistent with absence of (one

or more of the three) CYP1 enzymes — leading to perturbations in critical concentrations of one or several of the >150 molecules identified in the LM second-messenger pathway [*reviewed in* (Serhan *et al.*, 2007; Nebert and Karp, 2008; Nebert *et al.*, 2013b)].

4.3.4. Generation of transgenic "knock-in" mice—Many experiments using "humanized" *CYP1* knock-in transgenic mice have been described [(Derkenne *et al.*, 2005; Dragin *et al.*, 2006; Dragin *et al.*, 2007; Jiang *et al.*, 2005; Shi *et al.*, 2008), *reviewed in* (Cheung and Gonzalez, 2008)] in which widely diverse questions were addressed. Further details are beyond the scope of this review.

4.4. Endogenous ligands of AHR

Throughout the 1970s, several colleagues argued that, if AHR recognizes only foreign chemical ligands, how could AHR also bind endogenous ligands? On the contrary, we and others argued that every endogenous receptor must have arisen during evolution primarily in order to carry out a critical life function, whereas the "capacity also to recognize one or another class of foreign chemicals" must have occurred as an "add-on" function. Indeed — in the entire field of toxicology today — the general realization and accepted rule is that uptake of the vast majority of foreign chemicals and ions into the cell occurs by way of one or another receptor, transporter, membrane moiety, or other subcellular mechanism.

From the mid-1970s to the present, therefore, many laboratories have enthusiastically searched for "the" endogenous ligand of AHR. It had first been proposed long ago (Nebert *et al.*, 1981) that one or more endogenous ligands of AHR might represent CYP1-generated metabolites of the arachidonic-acid cascade, participating in inflammation.

Probably the first intriguing report of AHH activity being induced by something other than PAHs and TCDD was hepatic cell cultures being stimulated by light (Paine, 1977). The "search for an endogenous ligand of AHR" was extended to show AHR-mediated CYP1 induction by ultraviolet photoproducts of tryptophan, tryptophan metabolites, indole-containing compounds, indioids, equilenin, heme metabolites, arachidonic acid metabolites, dietary indoles, and foodstuff containing flavonoids. Other "unusual" stimulants included inflammation (Bigelow *et al.*, 1989; Nebert, 1994), various alterations in cell culture conditions — *e.g.* changing plated cells to suspended cells, disruption of cell-cell contact, changes in cell shape, modulation by cyclic-AMP, exposure of serum to hydrodynamic shear stress, and modification of LDL levels [*elegantly reviewed in* (Nguyen and Bradfield, 2008) *& later in* (Soshilov and Denison, 2014; Hubbard *et al.*, 2015)]. Hence, because of the proposed involvement of AHR and CYP1 in inflammation, and other advances described in the preceding sections, importance of the "AHR-CYP1 axis" was realized [*reviewed in* (Robertson *et al.*, 1987; Nebert *et al.*, 2004; Nebert and Karp, 2008)].

5. AHR and cell-signaling pathways

5.1. Lipid mediator (LM) second-messenger cascade

At least since the early 1980s, it has been accepted that LM second-messengers participate in the inflammatory process; also, that certain cytochromes P450 participate in the arachidonic acid pathway which represents a substantial portion of the LM-mediated

pathway including the inflammatory response. The complexity of the LM cascade has been partially unraveled in recent years, due to advancements in high-pressure liquid chromatography, gas chromatography, tandem-mass spectrometry, and other sophisticated metabololipidomics methodologies.

Originally derived from PUFAs — the (ω–6)- and (ω–3)-fatty acid intermediates (Figs. 3 & 4A) — are now known to be further metabolized in an extensive LM second-messenger cascade having potent bioactivities and comprising >150 identified metabolites (Samuelsson, 2012). The ω–6 fatty acid-derived arachidonic acid (AA) is converted [*reviewed in* (Nebert and Karp, 2008; Campbell *et al.*, 2011; Greene *et al.*, 2011; Nebert *et al.*, 2013b; Hankinson, 2016)] to eleven known classes of LMs (Fig. 3). AA itself was shown long ago to be converted by cytochrome P450 to the EETs (Proctor *et al.*, 1987), some of which are active in inflammation (Panigrahy *et al.*, 2012). On the other hand, the two ω–3 fatty acid-derived (Fig. 3) docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) are converted to four known classes of LMs (Campbell *et al.*, 2011; Greene *et al.*, 2011; Ji *et al.*, 2011; Serhan *et al.*, 2011; Mas *et al.*, 2012).

5.1.1. LM metabolism by lipoxygenases vs P450 monooxygenases—Oxidative metabolism of LMs is carried out in multiple steps — not only by specific arachidonate lipoxygenases but also by specific P450 monooxygenases in the CYP1, CYP2, CYP3, CYP4, CYP5 and CYP8 families (Fig. 3) [*reviewed in* (Nelson *et al.*, 2004; Nebert and Karp, 2008; Nebert *et al.*, 2013b)]. Arachidonate lipoxygenases are encoded by six human genes: *ALOX5*, *ALOX12*, *ALOX12B*, *ALOX15*, *ALOX15B*, and *ALOXE3*, and seven mouse genes: *Alox5*, *Alox12*, *Alox12b*, *Alox12e*, *Alox15*, *Alox8* and *Aloxe3*. Also, cyclooxygenases PTGS1 and PTGS2 (official names "prostaglandin-endoperoxide synthetases 1 and 2" encoded by the *PTGS1* and *PTGS2* genes, respectively; commonly misnamed "COX1 and COX2") participate in the oxidative activation and inactivation of LMs. Although some P450-mediated specific reactions of bioactive LMs have been identified [(Divanovic *et al.*, 2013) *& reviewed in* (Samuelsson, 2012; Nebert *et al.*, 2013b)], the vast majority remains to be determined; this has been largely due to major technical challenges involved in identifying specific LM chemical structures, particularly with regard to their intricate stereochemistry.

Lipoxygenases (Fig. 4B) insert both atoms (Kühn *et al.*, 1987), whereas P450 monooxygenases (Fig. 4C) insert one atom (Hayaishi *et al.*, 1955; Mason *et al.*, 1955; Mason, 1957), of diatomic oxygen — into substrates to form products. Another important distinction between the two types of reactions is that, although occasionally lipoxygenases can produce epoxides (*e.g.* leukotriene A_4 formation by ALOX5), the major product is a fixed-chirality hydroperoxide; on the other hand, P450 monooxygenases can generate racemic mixtures of internal- and terminal-monohydroxy products, as well as epoxides which, following hydrolysis, often proceed to form racemic mixtures of dihydroxy products (Fig. 4C).

Ultimately, among many other functions [*reviewed in* (Nebert and Karp, 2008; Nebert *et al.*, 2013b)], AA-derived LMs more likely participate in the pro-inflammatory phase, whereas the DHA- and EPA-derived metabolomes orchestrate the resolution phase of self-limited

inflammatory responses. In addition, the location of many of these metabolic reactions is usually highly tissue- and/or cell type-specific (Nebert and Karp, 2008; Campbell *et al.*, 2011; Greene *et al.*, 2011; Nebert *et al.*, 2013b).

5.1.2. LMs and the AHR-CYP1 axis—The first convincing LM identified to act apparently as an AHR ligand was lipoxin A_4 (Schaldach *et al.*, 1999), followed later by 12(R)-hydroxy-5,8,10,14-eicosatetraenoic acid [12(R)-HETE] (Chiaro *et al.*, 2008). As a means of dissecting the challenging problems of identifying which CYP enzyme might participate in what step(s) of the LM cascade involved in acute inflammation, one possibility would be to use *Cyp1* knockout mouse lines (Dragin *et al.*, 2008) — in combination with the latest advances in metabololipidomics (Dalli and Serhan, 2012) — for separating and identifying as many unique LM metabolites as technically possible.

This approach was recently carried out, using as an endpoint, zymosan-induced peritonitis in the *Cyp1a1/1a2/1b1(−/−)* TKO mouse model, to see if an inflammatory response would alter the LM metabolite profile (Divanovic *et al.*, 2013). The names and chemical structures of the AA-, DHA- and EPA-derived LMs — which were able to be identified by the metabololipidomics instrumentation methodology used — are illustrated in Figs. 5A, 5B & 5C, respectively; also listed (enclosed in *rectangles*) are some of the critical-life responses elicited by that particular LM active metabolite (Divanovic *et al.*, 2013).

The rationale is explained in Fig. 6. When comparing TKO with wild-type mice, if one (or more) of the three CYP1 enzyme(s) participates in any metabolic step *before* LM $\textbf{\textit{B}}$, then the concentration of $\textbf{\textit{B}}$ would be decreased (due to ablation of *CYP1*). In contrast, if one (or more) CYP1 enzyme(s) is involved in any metabolic step *after* $\textbf{\textit{B}}$, then the level of $\textbf{\textit{B}}$ would be increased (due to ablation of *CYP1*), when comparing TKO with wild-type mice.

The results (Divanovic *et al.*, 2013) are summarized in Figs. 7A, 7B & 7C. When comparing TKO with wild-type mice, those steps altered — are denoted in *red* as "way up" ($P<0.05$), simply "up" or "down" ($P<0.08 >0.05$), and "up (NS)" or "down (NS)" (trend seen; $P<0.12 > 0.08$). Therefore, zymosan-induced peritonitis appears to be associated with eight substantially altered metabolic steps in TKO, compared with wild-type mice: increases in neutrophil LTB_4 levels ($P<0.05$) and decreases in PD1 were the most striking differences; six other pathways slightly affected ($P< 0.08 >0.05$) included decreased 5S-HETE, neuroprotectin D1/protectin D1, 17S-HDHA, 14S-HDHA, 18R-HEPE, 15S-HEPE, and 12S-HEPE (Divanovic *et al.*, 2013).

This initial study only screened the possibility that any (or all) of the three CYP1 monooxygenases might affect the LM metabolite profile; these data should be corroborated using larger numbers of animals. Further, the study has the limitation of failing to distinguish whether one (or more) of the three CYP1 enzymes participates in a specific step in the LM cascade; to expand on the initial study, therefore, future experiments should be carried out, using *Cyp1* single-knockout mice, compared with *Cyp1* double- and triple-knockout mice. Mouse and human enzymes in the LM metabolite pathway are not always absolutely conserved — *e.g.* the 20-hydroxy-LTB_4 conversion to 20-COOH-LTB_4 step (Fig. 7A) was found to occur in human, but not mouse, neutrophils (Divanovic *et al.*, 2013).

Hence, humanized *CYP1* knock-in mice [(Derkenne *et al.*, 2005; Dragin *et al.*, 2006; Dragin *et al.*, 2007; Jiang *et al.*, 2005; Shi *et al.*, 2008), *reviewed in* (Cheung and Gonzalez, 2008)] should also be studied. Subsequently, various other studies using *Cyp2*, *Cyp3* and *Cyp4* single-knockout mice, compared with the (replaced) human orthologous *CYP*, *CYP3*, *CYP4* gene knocked-in (Scheer and Wolf, 2014; Scheer and Wilson, 2016), should also be carried out.

Results of such studies, including examination of specific cell types — other than neutrophils and peritoneal exudate — would define contributions of specific P450 monooxygenases to the LM second-messenger response that mediates various LM-mediated critical-life processes. Possibly, these findings would ultimately provide potentially useful new therapeutic targets for treating either the pro-inflammatory or the post-inflammatory response.

5.2. AHR-signaling pathways involved in critical-life processes

An appreciation of the vast depth of AHR involvement in many critical life processes has exploded in recent years. A summary of AHR-mediated pathways is listed in Table 1. Further details comprise the remainder of this review.

AHR-signaling is now known to participate in: cell migration (Mulero-Navarro and Fernandez-Salguero, 2016), epithelial cell development (Ikuta *et al.*, 2009), cytoskeletal/ adhesion regulation (Zhang *et al.*, 2016), circadian rhythmicity (Anderson *et al.*, 2013), barrier organ physiology (Esser and Rannug, 2015), cardiovascular and respiratory physiology (Sauzeau *et al.*, 2011b), kidney development (Nanez *et al.*, 2011), inner ear cochlear development in the neonate (Safe and Luebke, 2016), bone formation (Herlin *et al.*, 2013) and osteoclastogenesis (Iqbal *et al.*, 2013; Izawa *et al.*, 2016), GI tract (Hubbard *et al.*, 2015; Schiering *et al.*, 2016), intestinal immunity (Qiu and Zhou, 2013), innate immunity (Cella and Colonna, 2015), hematopoiesis (Lindsey and Papoutsakis, 2012; Fracchiolla *et al.*, 2016), transgenerational inheritance (Baker *et al.*, 2014), reproductive organ development (Mulero-Navarro and Fernandez-Salguero, 2016), regulation of female reproduction (Hernandez-Ochoa *et al.*, 2009), prostate gland development (Schneider *et al.*, 2014), hyperlipidemia, atherogenesis, and hypertension (Xiao *et al.*, 2014), thyroid-associated eye disease (Woeller *et al.*, 2016), eye and ciliary body function (Shichi and Nebert, 1982; Volotinen *et al.*, 2009), hepatic steatosis (Mellor *et al.*, 2016), pancreatic beta-cell regulation (Sabatini and Lynn, 2015), glucose and lipid metabolism (Gooley, 2016), circadian clock and metabolic syndrome disruption (Jaeger and Tischkau, 2016), DNA damage control (Wells *et al.*, 2010), tumor prevention by regulating gut immunity and growth suppression in tumor cells (Ikuta *et al.*, 2016), and protection against oxidative stress (Wölfle *et al.*, 2014).

Is there a "common thread" connecting most — if not all — of these critical life processes? The answer is yes: LM-mediated second-messenger pathways are involved in the regulation of every one of these critical life processes.

5.2.1. AHR and the "brain-gut-microbiome"—Realizing the importance of the "brain-gut-microbione" has greatly expanded during the past decade [*reviewed in* (Luna *et al.*,

2016; Mazzoli and Pessione, 2016)]. Microbes in the GI tract are now appreciated to provide each host with innumerable beneficial functions (*e.g.* contributions to vitamin supplementation, food digestion, and defense against pathogens of all kinds). The "microbiome" interacts with the host organism's genome and physiology — by way of direct contact through surface antigens, as well as soluble molecules produced by bacterial metabolism. There is clearly bi-directional communication between the GI tract and the CNS, which also provides communication pathways between intestinal microbiota and the host's neural circuitry including the CNS. Modulation of GI tract and CNS function via the microbiome is now recognized to include such ill-defined traits as "behavior," cognitive functions, "mood," "suicidal tendencies," "obsessive-compulsive disorder," appetite, and autism spectrum disorder. Production of bioactive compounds by microbiota and their potential probiotic activities consists of neuroactive molecules — *e.g.* histamine, serotonin, catecholamines, and trace amines (Luna *et al.*, 2016; Mazzoli and Pessione, 2016).

Termed "*the holobiont*," the microbiome of each host and its organs forms a close functional entity having evolutionarily designed interactions that support nutritional intake, defense mechanisms, and reproduction (Lee *et al.*, 2016). AHR is considered as an evolutionarily highly-conserved "sensor" capable of recognizing endogenous as well as foreign chemicals/ signals — *including many ligands that are metabolites produced directly, or indirectly, by the microbiome.* Thus, AHR-signaling pathways most likely elicit substantial impact on underlying mechanisms in neurodevelopment and neurodegeneration.

The balance — between pro- and anti-inflammatory signaling — is critical for maintaining immune homeostasis under physiological conditions, as well as in responding to inflammation (activation vs suppression) in different pathological settings. Recently identified gene products found to participate in immune system regulation include: PD1 (programmed cell death-1 receptor), CTLA4 (cytotoxic T-lymphocyte antigen-4), galectins, the intracellular enzyme indoleamine 2,3-dioxygenase (IDO), CD69, AHR, and members of the growth-arrest and DNA-damage-inducible-45 (GADD45) family (Fornace, Jr. *et al.*, 1989). These models have emerged as potential targets for regulation of the activation-suppression balance in immune cell function (de la Fuente *et al.*, 2012).

In the GI tract, CYP1A1 expression depends on TOLL-like receptor-2 (TLR2) (Do *et al.*, 2012) — which recognizes bacterial surface antigens, *e.g.* lipoteichoic acid. Presence of ligands of bacterial origin for TLR2 appears to be pivotal for detoxication of oral PAHs by intestinal CYP1A1. For example, gut epithelial CYP1A1 is silenced in *Tlr2(–/–)* knockout mice, even when exposed to substantial amounts of benzo[*a*]pyrene; after dietary daily benzo[*a*]pyrene for three weeks, *Tlr2(–/–)* but not their wild-type littermates developed colonic polyps (Do *et al.*, 2012).

These data bring to mind an earlier study using an *Arnt(–/–)* conditional knockout specific for GI tract epithelium (Ito *et al.*, 2007): CYP1A1 expression (without foreign chemical treatment) was found to be strikingly elevated in virtually all non-GI-tract tissues; high CYP1A1 levels were seen — even in early-stage fetuses in pregnant *Arnt(–/–)* mice. Curiously, a dramatic induction of the metallothionein-1 gene (*Mt1*) was seen throughout the GI tract. Intriguingly, CYP1A1 over-expression was lost upon administering a synthetic

purified diet. Further, high-CYP1A1 expression was "recovered" by adding the phytochemical indole-3-carbinol to the purified diet. It is possible that these observations (Ito *et al.*, 2007) might involve TLR2 activation in GI tract, as described above (Do *et al.*, 2012).

Finally, dysregulated expression of intestinal CYP1A1 was recently found to deplete the reservoir of naturally-occurring AHR ligands, thereby generating a quasi-AHR-deficient mouse; using a *Cyp1a1* reporter model system, Schiering and coworkers identified intestinal epithelial cells as "first-line responders" to dietary AHR ligands (Schiering *et al.*, 2016). Whether CYP1A1 is constitutively expressed throughout the body, or restricted specifically to intestinal epithelial cells — the result was loss of AHR-dependent type-3 innate lymphoid cells and T-helper-17 (Th17) cells, as well as enhanced susceptibility to enteric infection. These striking effects on intestinal immune system function of excessive AHR ligand degradation, could be counter-balanced by increasing dietary AHR ligand levels. These findings indicate that intestinal epithelial cells serve as "gatekeeper" for supply of AHR ligands to the host (Schiering *et al.*, 2016), thereby emphasizing the importance of feedback-control in modulating AHR-signaling pathway activation through the brain-gut-microbiome.

There is a relevant connection between gut microbiota and **ca**spase **r**ecruitment **d**omain family member-9 (CARD9). *Card9* is a susceptibility gene for inflammatory bowel disease (IBD) and helps recovery from colitis by promoting interleukin-22 production; *Card9(−/−)* knockout mice are more susceptible to colitis. Microbiota from *Card9(−/−)* mice, as well as IBD patients cannot metabolize tryptophan efficiently into metabolites that act as AHR ligands. Colitis can be attentuated after inoculation of mice with bacteria capatable of metabolizing tryptophan or by AHR agonist treatment (Lamas *et al.*, 2016).

5.2.2. AHR-signaling in endocrine dysregulation—For at least four decades, accumulating evidence has suggested the AHR-CYP1 axis participates in one or more pathways that elicit anti-estrogenic and anti-androgenic effects, both by TCDD or PAH treatment, as well as by interference or competition with sex hormone and angiogenesis action; female and male reproductive organs — including oocytes, spermatocytes, and fertility — can be affected.

AHR ligands are suggested to be involved in endocrine disruption. A number of studies have implied AHR interactions with estrogen receptor-α and -β (ESR1, 2); however, many of the purported ligands typically induce trans-activation of estrogen-responsive genes and reporter constructs, but their potencies are usually at least 1,000-fold lower than that observed for 17β-estradiol. Moreover, many cell assays are carried out in transformed or malignant cells in culture — which are notoriously polyploid and, thus, gene-expression pathways are far from "normal". Therefore, risk assessment of estrogenic compounds on the basis of their potencies in simple reporter-gene or binding assays is likely to be inappropriate [*reviewed in* (Safe *et al.*, 2002)].

Today it is clear, for example, that AHR activation and natural cytotoxicity-triggering receptors NCR1/2/3 activation by endogenous, as yet undefined, ligands contributes to uterine non-killer (NK) cell activation/maturation and angiogenic functions during early- to

mid-gestation pregnancy. Major histocompatibility complex (MHC)-independent activation of uterine NK cells also likely provides critical contributions to human pregnancy success [*reviewed in* (Felker *et al.*, 2013)].

The doses of environmental toxicants employed in laboratory-animal experiments commonly exceed any comprehensible level found in the environment. Hence, the dose of exposure, timing, route-of-administration, and target cell-type must all be scrutinized very carefully [*reviewed in* (Safe *et al.*, 2002; Kreitinger *et al.*, 2016)].

On the other hand, a recent investigation of *Ahr(–/–)* mice without foreign chemical treatment (Huang *et al.*, 2016) revealed lowered serum testosterone levels in males and significant growth of mammary ducts with elevated ESR1 expression in the ductal epithelium. Further abnormalities included: decreased expression of *Notch1* and *Notch3* receptors and their downstream target HES1 (hairy-and-enhancer of split-1); diminished fertility with degenerative changes in the testis; germ-cell apoptosis; and decreased number of early spermatids (Huang *et al.*, 2016).

Comparing women with vs without endometriosis, intriguingly, there is an association between this uterine disorder and up-regulation of *AHR* and *ARNT* expression and all three CYP1 target genes in endometriotic tissues and stromal cells (Bulun *et al.*, 2000). This clinical study prompted an investigation in Rhesus monkeys chronically exposed to TCDD and other halogenated PAHs (Rier *et al.*, 2001). TCDD exposure and elevated serum TCDD concentrations were associated with increased serum levels of triglycerides, 1,2,3,6,7,8-hexachlorodibenzofuran, 3,3′,4,4′-tetrachlorobiphenyl (TCB), and 3,3′,4,4′,5-pentachlorobiphenyl (PnCB). Those having elevated serum levels of 3,3′,4,4′-TCB, 3,3′,4,4′,5-PnCB, and increased total serum toxic equivalents — had a high prevalence of endometriosis, and disease severity was correlated with serum concentrations of 3,3′,4,4′-TCB.

In humans, a meta-analysis of four genome-wide association studies, plus four replication studies (which included altogether totals of 25,871 endometriosis cases and 65,356 controls, and combining several ethnic groups), uncovered six statistically significant (P 5×10^{-8}) genome-wide significant loci — which included rs7521902 near *WNT4* (P = 1.8×10^{-15}) on human Chr 1p36.12 (Rahmioglu *et al.*, 2014). This association is particularly intriguing in this review because of AHR's involvement in WNT4-signaling pathways [*vide infra*].

In summary, AHR-signaling involvement in reproductive organs is associated with such diverse clinical disorders as infertility, mammary ductal epithelial cell hyperplasia, and endometriosis. The underlying cause of AHR-associated endometriosis is fascinating and will require further study; etiology remains controversial and could involve: [a] CYP1-mediated metabolic activation of unknown chemicals to toxic intermediates, or ROS formation; [b] endocrine-specific growth-factor or tumor promoter-like activity; [c] dysregulation of the immune response caused by inflammatory signals via certain environmental toxicants; or [d] epigenetic effects comprising histone modification and chromatin remodeling (Sofo *et al.*, 2015).

5.2.3. AHR-CYP1 axis and reactive oxygen species (ROS) formation—

Interestingly, administering intraperitoneal TCDD (high dose of 5 μg/kg; three consecutive days) was found to cause a dramatic, protracted oxidative stress response. Hepatic oxidized glutathione levels are sustained for at least 8 weeks; urinary levels of 8-hydroxydeoxyguanosine (a product of DNA-base oxidation and subsequent excision repair) remain ~20-fold higher than controls after 8 weeks — consistent with chronic, potentially promutagenic DNA-base damage (Shertzer *et al.*, 1998). Mitochondrial H_2O_2 production rises at 1 and 4 weeks, remaining significantly elevated at 8 weeks; enhanced H_2O_2 production is not due to either increased Mn-superoxide dismutase or decreased mitochondrial glutathione peroxidase activity (Senft *et al.*, 2002a). This was the earliest report suggesting that AHR-signaling pathways are involved in mitochondrial respiration-dependent ROS formation.

The initial hypothesis was that TCDD-induced mitochondrial ROS production most likely would be CYP1-mediated. Using *Cyp1a1(–/–)* and *Cyp1a2(–/–)* mice, alone or in combination with *Ahr(–/–)* knockout mice, however, it was concluded that both constitutive and TCDD-induced mitochondrial ROS formation is AHR-mediated — but independent of CYP1A1/2 metabolism (Senft *et al.*, 2002b).

Also noteworthy is the fact that AHR-signaling includes up-regulation of electrophile-responsive antioxidant enzymes [*reviewed in* (Nebert *et al.*, 2000)]; for example, *Nqo1* (NAD(P)H:quinone oxidoreductase-1) and *Aldh3a1* (aldehyde dehydrogenase-3A1) genes carry promoter binding-sites for both AHR and NFE2L1 [nuclear factor (erythroid-derived 2)-like 1; previous name NRF1].

Hence, there appears to be a "yin-yang equilibrium," controlled by AHR, *i.e.* both up- and down-regulation of ROS formation. This delicate balance occurs in such seemingly unrelated signaling pathways as: circadian rhythmicity (Patel *et al.*, 2014); enhanced immune response exhibiting increased levels of inflammatory cytokines and decreased *Socs2* (suppression of cytokine signaling-2) expression — that had been observed in zymosan-caused peritonitis in liver of *Cyp1a1/1a2/1b1(–/–)* triple-knockout mice (Nebert and Karp, 2008) — and infectious myocarditis (Barroso *et al.*, 2016); inflammatory plaque formation during atherosclerosis (Marinkovic *et al.*, 2013; Uno *et al.*, 2014; Pernomian and da Silva, 2015); hepatic steatosis (Gooley, 2016; Mellor *et al.*, 2016; Nault *et al.*, 2017); atopic dermatitis associated with a dysfunctional skin barrier (Nomura and Kabashima, 2016); and aging and age-associated diseases (Gasiewicz *et al.*, 2014; Patel *et al.*, 2014).

5.3. AHR-signaling pathways in embryonic stem (ES) cells

5.3.1. Early evidence of AHR-CYP1 signaling in the one-cell zygote—Why does AHR participate in such an abundance of critical-life processes in virtually every organ and cell type of the body? The first hint of "early AHR-CYP1 signaling" during embryogenesis came with sister-chromatid-exchange (SCE) studies; mouse embryo GD7.5 explant cultures in medium containing benzo[*a*]pyrene and 5-bromodeoxyuridine results in SCEs are associated with AHR-responsive, but not AHR-nonresponsive, mouse lines (Galloway *et al.*, 1980). These data provided direct evidence in 1980 that the AHR-CYP1 axis was functional at an early embryonic stage.

It was then found that AHR-CYP1 signaling is detectable in the mouse oocyte; curiously, within 12 h after fertilization — without any "foreign ligand signal" — a >100-fold increase in Cyp1a1 mRNA levels was found (Dey and Nebert, 1998), following which this disappears by the 2-cell stage (GD1.5), as well as in the blastocyst (GD3.5).

This finding, first submitted as a manuscript in 1988, was another example of being rejected from publication (as "implausible" and "heresy"). Ten years later, however, supported with additional experimental data, a proposed hypothesis became feasible for activation of AHR-CYP1 signaling in the newly formed zygote following fertilization [*reviewed in* (Nebert *et al.*, 2000)].

AHR was discovered to play pivotal roles in cell-cycle regulation and apoptosis (Fig. 8). AHR interacts directly with the RB1 protein (Ge and Elferink, 1998), and formation of the AHR-RB1 complex prevents normal progression of G_1 to S phase during the cell cycle, by blocking E2F-mediated transcription of S-phase genes (Puga *et al.*, 2000). Hence, in the presence of sufficient amounts of any unknown endogenous ligand (or undesirable foreign chemical able to bind to AHR), activated AHR would cause arrest at the G_1/S boundary (Fig. 8B). Availability of CYP1A1 monooxygenase therefore appears to be critical in the fertilized egg, because — if AHR-mediated CYP1A1 activity is able to metabolize/detoxify any harmful AHR ligand — substantial amounts of CYP1A1 activity would prevent AHR from blocking progression of the 1-cell embryo from G_1 into S phase — without which the result, of course, would be death**!** Given the details of AHR-CYP1 signaling in the second-messenger LM cascade (*vide supra*), the unknown endogenous ligand in the zygote is very likely to be one (or more) of the LM active metabolites.

5.3.2. Early studies of AHR-CYP1 axis signaling in ES cells—It therefore came as no surprise, when the AHR-CYP1 axis was reported to exist in cultured embryonic stem (ES) cells (Kuroda *et al.*, 2005). Three years later, it was shown that mouse ES cells express TCDD-inducible CYP1A1 and CYP1B1 — but not CYP1A2, nor any of three "Phase II" drug-metabolizing enzymes (NQO1, glutathione *S*-transferase-A1, or UDP glucuronosyltransferase 1A6); *Cyp1a1* gene induction was by far the most sensitive, first detected after only 3 h of culture and at "minimal effective" TCDD doses of 1 nM (Neri *et al.*, 2008). [Recall (*vide supra*) the *CYP1A1* and *CYP1B1* genes are evolutionarily much earlier than the *CYP1A2* gene.]

In an independent study, *Cyp1a1* was shown to appear earliest — following which AHR, ARNT, and glucocorticoid receptor mRNAs as well — were detected in differentiating mouse ES cells throughout the culture period (Maezawa *et al.*, 2008). The AHR-signaling pathway and sensitivity to PAH-caused toxicity were also described in human ES cells (Kee *et al.*, 2010). Moreover, inappropriate or sustained activation of AHR by TCDD during neurogenesis was found to interfere with signaling pathways that regulate neuroepithelial stem cell/neural precursor cell proliferation (Latchney *et al.*, 2011).

It then became appreciated that pluripotent stem cells can be generated from mouse somatic cells, using a combination of seven small-molecule compounds. These pluripotent stem cells were found to resemble ES cells — in terms of their gene expression profiles, epigenetic

status, and potential for differentiation and germline transmission; this model system opened the way to study "master genes" that are dispensable for cell-fate reprogramming (Hou *et al.*, 2013).

Various tryptophan derivatives — including endogenous 2-(1′H-indole-3′-carbonyl)-thiazole-4-carboxylic acid methyl-ester (ITE) — were shown to regulate transcription of the master pluripotency gene *POU5F1* (trivial name Oct4). ITE was revealed to enhance AHR binding to the *POU5F1* promoter and suppress its transcription. Lowering endogenous ITE levels in cancer cells by tryptophan deprivation, or hypoxia, leads to POU5F1 induction, which can be blocked by synthetic ITE treatment (Cheng *et al.*, 2015). Very recently, ITE-treated hepatoma-derived Huh7 cells/C3A human cells were discovered to induce CYP1A1, CYP1A2, CYP3A4 and CYP1B1 (Zhang *et al.*, 2017); these represent P450 monooxygenases in two of the six P450 families shown to participate in LM-mediated second-messenger signaling pathways (Fig. 3).

5.3.3. Crosstalk between AHR- and hypoxia-inducible factor (HIF)-signaling pathways—Before going further, it must be emphasized that during regulatory expression of numerous genes before becoming functional, AHR and HIF1A/3A both require their binding partner ARNT/ARNT2, the latter being critical for responses to intracellular hypoxia (low pO_2). Such competition/crosstalk implicates differences in AHR-signaling capacity during conditions of diminished pO_2 [*reviewed in* (Vorrink and Domann, 2014)].

The story is even more complicated than the transcriptional factors AHR and HIF competing with ARNT at AHRE-binding sites. For example, mouse *Cyp1a1* up-regulation is inhibited by hypoxia, whereas AHR activation actually *enhances* erythropoietin gene (*Epo*) up-regulation during hypoxic conditions; in this particular instance, the *Epo* regulatory region was found to contain AHREs upstream of its transcriptional start-site. Thus, *Epo* can be categorized as "an AHR-target gene" (Chan *et al.*, 1999). Because of these studies, alterations in AHR-signaling pathways must be kept in mind during any study involving hypoxia.

Using ES cell-differentiated embryoid bodies, it was found that BNIP3 (BCL2/adenovirus E1B 19-kD-interacting protein), which is a BH3-only pro-apoptotic protein, is up-regulated during cavitation in a hypoxia-dependent manner (Qi *et al.*, 2012). Silencing of the *Bnip3* gene blocks apoptosis of the core cells and delays cavitation. In contrast, *Bnip3* up-regulation is regulated by HIF1A. Ablation of either HIF1A or ARNT — partners of AHR in the complex that binds to AHRE regulatory sequences of most, if not all, AHR-target genes — inhibits *Bnip3* up-regulation, thereby blocking apoptosis and cavitation (Qi *et al.*, 2012).

Apoptosis-inducing factor (AIF) cooperates with BNIP3 to promote lumen clearance; *Bnip3* gene silencing in *Aif(−/−)* knockout embryoid bodies blocks apoptosis and cavitation. In addition, AIF regulates *Bnip3* expression by way of mitochondrial ROS production and consequent EPAS1 (previous name, HIF2A) stabilization. These exciting data demonstrate a mechanism of cavitation during morula–>blastula formation that is very much interconnected with AHR-signaling pathways, via hypoxia-induced apoptosis of the core cells mediated by HIFs, BNIP3, and AIF (Qi *et al.*, 2012).

5.3.4. AHR-signaling in cardiorespiratory functions and cardiomyogenesis—

Studies of AHR-signaling pathways and the CYP1 axis in cultured ES cells have exploded today, largely because they offer an excellent model system to study fundamental signaling pathways in undifferentiated, pluripotent cells in culture. For example, comparing *Ahr(–/–)* and *Vav3(–/–)* mouse embryonic fibroblasts, it was found that VAV3, activator of Rho/Rac GTPases, is an AHR target and, without AHR present, γ-aminobutyric acid (GABA)-ergic transmission defects occur in the ventrolateral medulla — an important cardiorespiratory brainstem region (Sauzeau *et al.*, 2011a).

Silencing *Ahr* [by short hairpin (sh)RNA interference] in mouse teratocarcinoma P19 cells allows cells to differentiate into cardiomyocytes when treated with dimethylsulfoxide. When AHR is silenced, cardiomyocyte-specific *Gata4* (*encoding GATA4-binding protein) and *Nkx2-5* (NK2 homeobox-5) gene expression become elevated, along with increases in *Gsk3b* (GSK3; glycogen synthase kinase-3β); in contrast, *Arnt*, *Cyp1a1* and *Jup* (encoding junction plakoglobin; also called β-catenin) genes are concomitantly suppressed (Zhu *et al.*, 2012). These findings suggest AHR silencing enhances P19-cell differentiation by way of AHR- and WNT-signaling pathways (†the WNT family comprises 18 genes in the human and mouse genomes). *Jup*-encoded plakoglobin plays a pivotal role in intercellular junctions; when *Jup* is suppressed by AHR — cell adherence and proliferation become dysregulated (Prochazkova *et al.*, 2013). Disruption of AHR-signaling activity thus perturbs cardiomyocyte differentiation; reasons for this perturbation include alterations in expression of homeobox transcription factors (Wang *et al.*, 2013).

If ES cells do not differentiate into cardiomyocytes, AHR-expression prevents cardiogenesis — thereby resulting in commitment to a neuronal cell fate. Any undesirable early expression of AHR in specific cells of the developing early embryo must therefore be repressed, in order to maintain ES cell mitotic progression and to prevent premature loss of pluripotency (Ko *et al.*, 2016).

5.3.5. AHR-signaling in neurogenesis and neuronal cell development—

As mentioned earlier, active bHLH/PAS heterodimers comprise a ubiquitous signal-regulated subunit (*e.g.* HIF1A/3A, AHR) or a tissue-specific subunit (*e.g.* neuronal PAS-domain proteins NPAS1/3/4, SIM1/2), paired with a general partner protein, ARNT or ARNT2. High ARNT/ARNT2 ratios in P19 embryonic carcinoma cells and ES cells — upon neuronal differentiation — become dramatically reverted to high ARNT2/ARNT ratios, whereas *Arnt* and *Arnt2* mRNA half-lives do not change during neuronal differentiation; the alteration to predominant ARNT2 expression in neurons is proposed to allow specific functions of neuronal bHLH/PAS factors to occur, and/or to evade neuronal bHLH/PAS factors from interfering with AHR/ARNT-signaling functions (Hao *et al.*, 2013).

5.3.6. AHR-signaling in early development of hematopoiesis—

HIF1A is known to help hematopoietic stem cells adapt to stress. In *Arnt(–/–)* conditional knockout mice,

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*GATA is a family of transcription factors that activate or repress erythroid development; they bind to DNA sites having the consensus sequence 5′-[AT]GATA[AG]-3′ within regulatory regions of GATA-responsive genes.
†SP factors represent a family of nine specificity-protein/krüppel-like factor (SP/KLF) transcriptional factors that bind to GC and GT boxes; these are common recurring motifs in promoters, as well as distal regulatory elements of mammalian genes.

ARNT was discovered to be essential for adult and fetal hematopoietic stem-cell viability and homeostasis, but ARNT deficiency is directly HIF-dependent (Krock *et al.*, 2015). Hence, HIF activity likely regulates hematopoietic stem cell homeostasis by way of these pro-survival factors.

Overexpression of RNA-binding protein MSI2 (musashi-2) is known to induce multiple pro-self-renewal phenotypes — including 17-fold increases in short-term blood-progenitor repopulating cells and a net 23-fold ex vivo expansion of long-term repopulating hematopoietic stem cells. Recently it was shown that MSI2 directly attenuates AHR-signaling, via posttranscriptional down-regulation of a canonical AHR-pathway in cord-blood hematopoietic stem cells (Rentas *et al.*, 2016).

5.3.7. AHR-signaling in osteogenesis and bone formation—As mentioned earlier, AHR-mediated pathways are involved during bone formation (Herlin *et al.*, 2013) and osteoclastogenesis (Iqbal *et al.*, 2013). During studies of mouse ES cell culture, between GD0.5 and 3.5 during the period of panmesoderm development, TCDD treatment disrupts expression of genes in the transforming growth factors-β (TGFB1/2/3), bone morphogenetic proteins-2, -4 (BMP2/4), and WNT3A/5A (wingless-type MMTV integration site members-3a and -5a)-signaling pathways; AHR-signaling specifically suppresses secretion of BMP4, WNT3A, and WNT5A. Comparing *Ahr(+/+)* wild-type and *Ahr(−/−)* knockout ES cells treated with TCDD, the mitochondrial copy number was found to be enhanced — suggesting mitochondrial stress (ROS formation) and remodeling might occur during this period of panmesoderm development (Wang *et al.*, 2016).

5.3.8. Early embryonic AHR gene-signaling pathways fundamental to development—As the zygote develops, cells that first are totipotent become pluripotent stem cells, differentiating into endoderm, ectoderm and mesoderm — which then proceed to form specific cell-types that include *e.g.* intestinal stem cells, epithelial stem cells, and mesenchymal stem cells, respectively. As mentioned earlier, AHR-CYP1 axis signaling during the cell cycle will clearly elicit effects at any and all of these steps (Fig. 8). For example, inappropriate or sustained activation of AHR was found to interfere with signaling pathways that regulate neuroepithelial stem cell/neural precursor cell proliferation (Latchney *et al.*, 2011).

In mouse ES cells, interactions between[*]POU3/4 (OCT3/4), NANOG, SOX2 and polycomb group proteins (PcG) binding to *Ahr* gene promoter region were discovered to repress *Ahr* expression. Removal of repressive signals in *Ahr* promoter-region chromatin leads to release of pluripotency factors and PcG proteins, and binding of[†]SP factors — which result in

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[*]The acronym **POU** is derived from names of three transcription factors: pituitary-specific **P**IT1; octamer proteins **O**CT1/2, and from *Caenorhabditis elegans* the neural **U**NC-86. The octamer sequence is ATGCAAAT. There are six POU classes of genes in the mammalian genome. POU3 and POU4 domains are present evolutionarily "as far back" as the common ancestor of sponges and eumetazoans. **NANOG** protein is a protein evolutionarily having a highly conserved homeodomain motif localized to the nucleus and facilitates DNA-binding. **SOX2** is one of 19 members of the SOX family [SRY (sex-determining region Y)-box-1 family of transcription factors known to play pivotal roles in many stages of mammalian development]. **Polycomb group (PcG) proteins** are epigenetic regulators of transcription essential for stem-cell identity, differentiation, and disease states — functioning within multiprotein complexes, called polycomb repressive complexes (PRCs); PRCs modify histones (and other proteins) and silence target genes.

establishing open chromatin; this, in turn, engages RNA polymerase-2 to drive full-length RNA transcript elongation. These data indicate that AHR-signaling is required for ES cells to become activated quickly, as needed, during embryonic development (Ko *et al.*, 2014).

5.3.9. AHR-signaling pathways during DNA synthesis and repair—As emphasized earlier, to maintain mitotic progression and pluripotency, ES cells must maintain low or negligible *Ahr* expression. Repression of AHR-signaling pathways cannot, however, be a completely one-way street. AHR-signaling pathways are often vital and therefore must fluctuate reversibly between expression and repression — with some fraction of cells escaping repression, as needed, to become activated at any one time. Cells that escape *Ahr* repression (*i.e.* activated AHR) lead to higher levels of POU5F1 and SOX2 (Fig. 8A, *lower left*), which then result in activation) of *Mid1* (midline-1). Subsequently cells show extended mitotic traverse times, *i.e.* disruption of the MID1-PP2A-CDC25B/26-CDK1 signaling pathway (Fig. 8A) that regulates mitosis (Ko *et al.*, 2016).

Control of the eukaryotic cell cycle includes passage through "checkpoints" from one phase to the next — requiring a coordinated set of proteins that monitor cell growth and DNA integrity. Obviously, uncontrolled cell division, or propagation of damaged DNA, can lead to genomic instability and disease states — including cancer.

The G_1/S checkpoint (Fig. 8) controls progression of cells into **S** phase (DNA synthesis). As mentioned earlier, during G_1 (Fig. 8B), hypo-phosphorylated RB1 blocks E2F action by binding to E2F (E2F includes a family of eight proteins). RB1 phosphorylation, by[*]cyclin-dependent kinase-2 (CDK2) in late G_1, causes dissociation of RB1, allowing E2F-mediated transcription of **S**-phase-promoting genes to proceed. Also described earlier, formation of an AHR-RB1 binding complex prevents normal progression of G_1 to **S** phase by stalling E2F-mediated transcription of **S**-phase genes [*reviewed in* (Nebert *et al.*, 2000)]. Hence, in the presence of sufficient amounts of unknown endogenous ligand — or planar foreign chemical such as TCDD, PAHs or halogenated biphenyls — that bind to AHR, activated AHR would cause arrest at the G_1/S boundary; this would be lethal to the 1-cell zygote or early embryo.

DNA damage (Fig. 8A, *upper right*) activates response pathways through ATM/ATR (gene products of "ataxia telangiectasia-mutated"/"ataxia telangiectasia and RAD3-related") and checkpoint-1/2 (CHEK1/2) kinases to suppress CDC25A, thereby preventing CDK4/6 and CDK2 activity; this results in cell-cycle arrest at the **G1/S** boundary, followed by either DNA repair or, at end of the G_2 phase, cell death (apoptosis). DNA damage also activates multiple kinases that phosphorylate tumor protein-53 (TP53). MDM2 and MDM4 — acting apparently as non-redundant components of a complex (Shadfan *et al.*, 2012) — are known to be major essential negative regulators of[*]TP53 in normal cells that suppress TP53 activity. Under conditions of cellular stress, the[†]MDM2-MDM4 complex must be

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[*]CDKs comprise a family of five mammalian protein kinases, known for participation in regulating the cell cycle; they also participate in transcription regulation, mRNA-processing, and differentiation during neurogenesis. CDKs are so highly conserved that yeast cells can divide normally when the human homolog is replaced with the yeast *Cdk* gene product.

[*]The original term "p53" is encoded by the human *TP53* gene, called "transformation protein-53", and by the mouse *Trp53* gene, called "transformation-related protein-53".

[†]The official term "MDM2" has been variously named MDM2 proto-oncogene, E3 ubiquitin protein ligase, and transformed mouse 3T3 cell double-minute-2. "MDMX" is the previous name for MDM4; the latter is now the official nomenclature.

suppressed so that TP53 is able to respond to that stress. Phosphorylation of TP53 thus stimulates PAK1-6 (p21) to prevent CDK2 action, which arrests the cell at the **G1/S** boundary, thereby allowing binding of TP53 to DNA.

Protein phosphatase 2A (PP2A) is known to phosphorylate more than 300 substrates involved in the cell cycle (Fig. 8) — controlling virtually all major pathways and cell-cycle checkpoints (Wlodarchak and Xing, 2016). CDC25B/26 (cell division cycle-25B, -26; there are 23 highly conserved *CDC* genes in vertebrates) is a complex that acts, along with many other regulators of the core cell cycle machinery, in signaling pathways and transcriptional programs controlling cell fate choices — *i.e.* when to proceed into apoptosis vs when to switch between differentiation and proliferation (Agius *et al.*, 2015).

7. Concluding remarks

> "When reaching the great mountaintop, we can behold all mountains in a single glance." -----Du Fu, prominent Chinese poet (712–770 AD) of the Tang dynasty.

From the mountain top as described in this review, looking back over six decades of scientific history and first evidence of AHR in the early 1970s, what have we learned? AHR is now realized to be among the earliest-discovered "pioneer" members of the bHLH/PAS family, now numbering about 30 in the human and mouse genomes; the only earlier one was the *Drosophila* "periodic" (*per*) locus (Konopka and Benzer, 1971). We now know that bHLH/PAS proteins are sensors of both foreign and endogenous "signals" to which the organism, or individual cell, responds. Although the name "PAS" (abbreviation for per-Arnt-sim) for this family was not coined until the 1990s — evidence for AHR, the binding partner of ARNT, was reported almost two decades earlier than ARNT. In that landmark publication (Poland *et al.*, 1974), AHR was proposed to be a sensor of TCDD and PAHs, and an up-regulator of CYP1A1-mediated metabolism of PAHs.

How we propose bHLH/PAS members to operate is shown in Fig. 9A. A particular (foreign or endogenous) signal is "sensed" by the inactive sensor bHLH/PAS protein, causing activation of the sensor, which then stimulates/activates its primary target; this results in regulatory expression of downstream targets in the pathway — resulting in the "response" or "responses." At any of the downstream steps, it is likely one or more moieties will inactivate the sensor which, in turn, represses the no-longer activated primary target. The inactive sensor then awaits the next signal — before becoming activated again.

Fig. 9B illustrates specifically how the AHR-CYP1 axis operates in signal-sensor functions. In this instance, an endogenous ligand (EL) such as particular tryptophan metabolites, TCDD, or planar foreign chemical (*e.g.* PAHs or halogenated biphenyls) binds to AHR, causing it to become activated (**AHR***)**;** this in turn up-regulates CYP1 enzymes and other non-CYP1 targets, resulting in downstream events leading to the response(s) — which, in this case, includes metabolism (degradation) of the EL (or ELs) as well as many foreign chemicals (metabolic activation or detoxication). Eventually, a downstream moiety, or signal, will inactivate AHR* to AHR, resulting in down-regulation of CYP1 and non-CYP1 targets. Then, inactivated AHR awaits the next signal before becoming activated.

First, foreign chemicals were postulated to be "recognized by" (bound to) AHR (Poland *et al.*, 1974), thereby up-regulating CYP1 in various organs, cell types, and as a function of age (of the embryo or postpartum animal). Within the next six years, it was proposed (Nebert *et al.*, 1981), and then demonstrated (Rifkind and Muschick, 1983; Bigelow *et al.*, 1989), that AHR is likely to be involved also in "recognition" (binding) of endogenous compounds involved in the inflammatory process. In this case, up-regulated CYP1-mediated metabolism (most likely, of LMs) are converted either from active to inactive products, or vice versa. It is also clear that activated AHR must bind to ARNT before becoming the transcriptionally-active heterodimer (Burbach *et al.*, 1992).

These two details (AHR binding of foreign chemicals, as well as endogenous compounds) appear to be common to all vertebrates. Intriguingly, AHR-signaling has also been found in the fly, worm, sea anemone, crustaceans, and even the beetle [*vide supra*]. With regard to spineless, the *Drosophila* homolog of AHR — although spineless-tango heterodimers exhibit similar transcriptional properties to those of mammalian AHR-ARNT heterodimers, *there appears to be* no ligand-binding by spineless before spineless-tango heterodimer interaction [*reviewed in* (Lindsey and Papoutsakis, 2012)]. Hence, the implication is that perhaps all invertebrate AHR homologs have no ligand-binding properties.

To the knowledge of this author, however, binding to an endogenous ligand has not been ruled out in any invertebrate study of AHR-signaling pathways. Although *CYP1* genes evolutionarily are not present in invertebrates, we believe that up-regulation mediated by the AHR homolog of other enzymes remains a possibility among invertebrates.

Furthermore, little is currently known, evolutionarily, as to how extensive LM second-messenger signaling cascades might be in invertebrates. In crustaceans, PUFAs and prostaglandins have been detected. In one intriguing relevant study of the black tiger shrimp (*Penaeus monodon*) genome (Wimuttisuk *et al.*, 2013), TBLASTX analysis found homologues of ten mammalian prostanoid biosynthesis genes: *PLA2* (cytosolic phospholipase A_2), *HPGDS* (hematopoietic prostaglandin D synthase), *PGDS* (glutathione-dependent prostaglandin D synthase), *PGTES2* and *PTGES3* (prostaglandin E synthases, 2 and 3), *PGE2* (prostaglandin E**2**), *AKR1C3* (prostaglandin F synthase), *PGF2A* (prostaglandin $F_{2\alpha}$), *TBXAS1* (thromboxane A synthase, also known as CYP5A1), and *PTGS1* (cyclooxygenase-1). Thus, for invertebrates, it must be emphasized that additional research will be required — before any further conclusions can be reached about the possibility of any AHR-CYP or AHR-enzyme-homolog signaling pathways (*illustrated in general for bHLH/PAS sensors in* Fig. 9A).

In summary, the exciting unraveling of all the secrets of AHR, the AHR-CYP1 axis, and AHR-signaling pathways (Table 2) — over more than the past four decades — has been nothing short of incredible. Perhaps this is especially so, because of several of these quantum leaps, along the way, in which the data were considered "heresy" at the time, when first presented to the scientific community.

Acknowledgments

Abbreviations

AA
arachidonic acid

AHH
aryl hydrocarbon hydoxylase

AHR
aryl hydrocarbon receptor

ARNT
aryl hydrocarbon receptor translocator

B6
C57BL/6J mice

bHLH
basic-helix-loop-helix domain in proteins

Chr
chromosome

CNS
central nervous system

CYP
cytochrome P450

CYP1 family of enzymes
CYP1A1, CYP1A2, and CYP1B1

D2
DBA/2J mice

DHA
docosahexaenoic acid

DHETEs
dihydroxyeicosatrienoic acids

EETs

epoxyeicosatrienoic acids

EPA
eicosapentaenoic acid

GI
gastrointestinal

HETEs
hydroxyeicosatetraenoic acids

HIF
hypoxia-inducible factor

HpETEs
hydroperoxyeicosatetraenoic acids

LM
lipid mediator

LPS
lipopolysaccharide

LTB$_4$
leukotriene B$_4$

NQO1, NAD(P)H
quinone oxidoreductase-1

PAH
polycyclic aromatic hydrocarbon

PAS
per-Arnt-sim

Per
periodic locus (*Drosophila*)

PUFA
polyunsaturated fatty acid

Sim
single-minded locus (*Drosophila*)

TCDD
2,3,7,8,-tetrachlorodibenzo-*p*-dioxin

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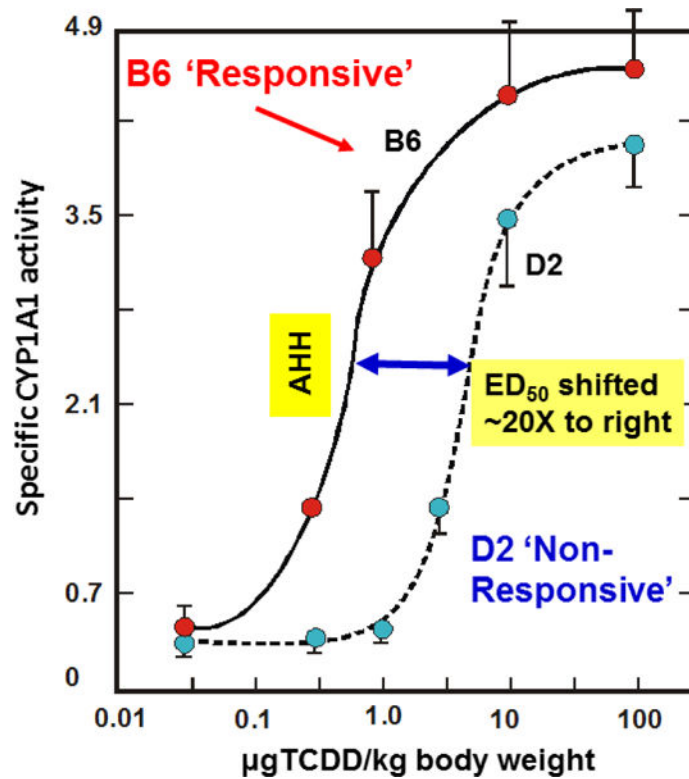
Fig. 1.
Dose-response curve of induced AHH activity (the CYP1A1 monooxygenase), as a function of intraperitoneal TCDD administered 48 h earlier — comparing B6 (C57BL/6 mouse with high-affinity AHR) with D2 (DBA/2 mouse with poor-affinity AHR). *Units* on Y-axis denote "nmol of 3-hydroxybenzo[*a*]pyrene formed per min per mg liver microsomal protein" [*redrawn and modified from data in* (Poland *et al.*, 1974)].
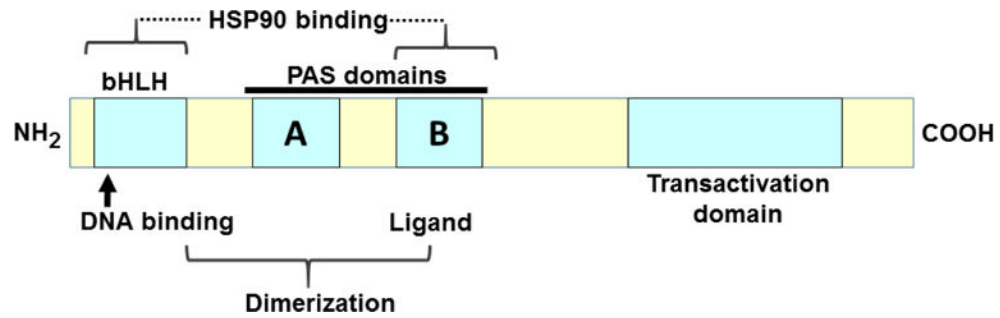
Fig. 2.
Schematic representation of bHLH/PAS sensor proteins, in which specific functions are localized to particular regions, or modules. Ligands bind to the PAS-B domain; binding to HSP90 includes both the bHLH and PAS-B domains; dimerization with ARNT involves both the bHLH domain and PAS domains; DNA-binding and the nuclear localization signal reside in the basic region of the bHLH domain; the trans-activation function comprises a large segment toward the carboxy (COOH)-terminus. The carboxyl half of AHR protein displays the greatest amino-acid variation across the animal kingdom, whereas bHLH/PAS domains are highly conserved [*redrawn and modified from* (Okey, 2007) *and references therein*].
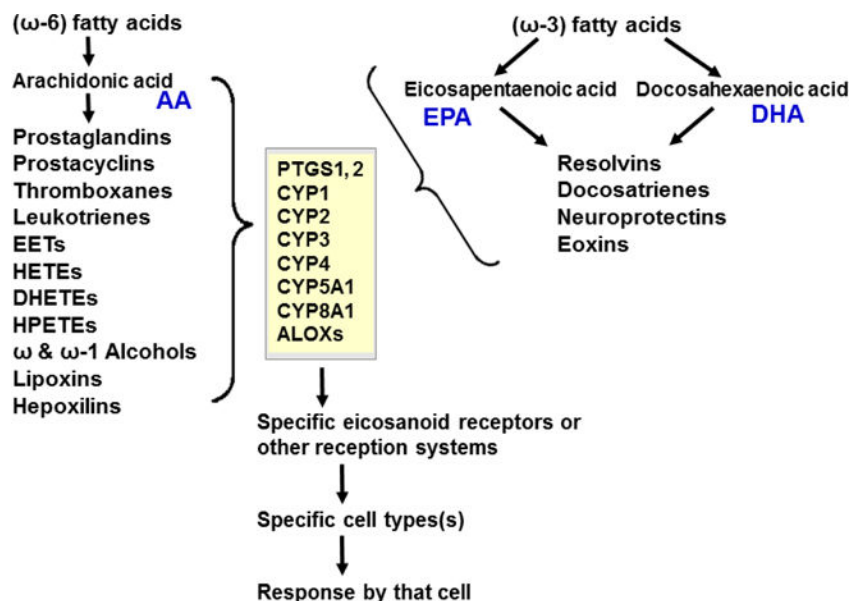
Fig. 3.

LM second-messenger pathways, derived from (ω–6)- and (ω–3)-fatty acids. The former gives rise to eleven classes of LMs derived from arachidonic acid (AA). The latter gives rise to four classes of LMs derived from eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). Enzymes involved in the synthesis and degradation of the more than 150 total LMs in the 15 classes include two cyclooxygenases, members from six families of CYP monooxygenases, plus arachidonate lipooxygenases (ALOXs; six in human; seven in mouse). The "active" LMs are then "sensed by" (bind to) appropriate receptors in various organs and cell types, leading downstream to virtually every critical-life response in the organism [*modified from* (Nebert *et al.*, 2013b)].
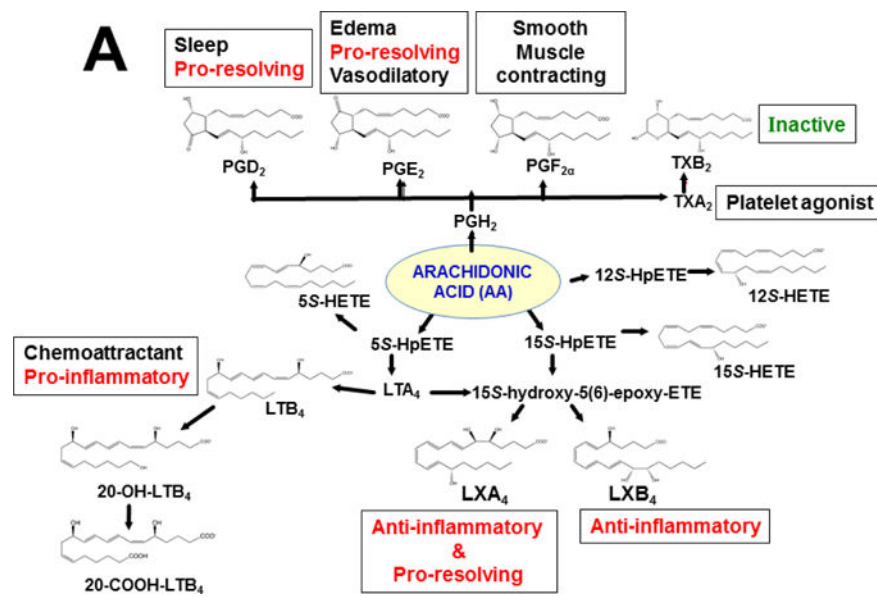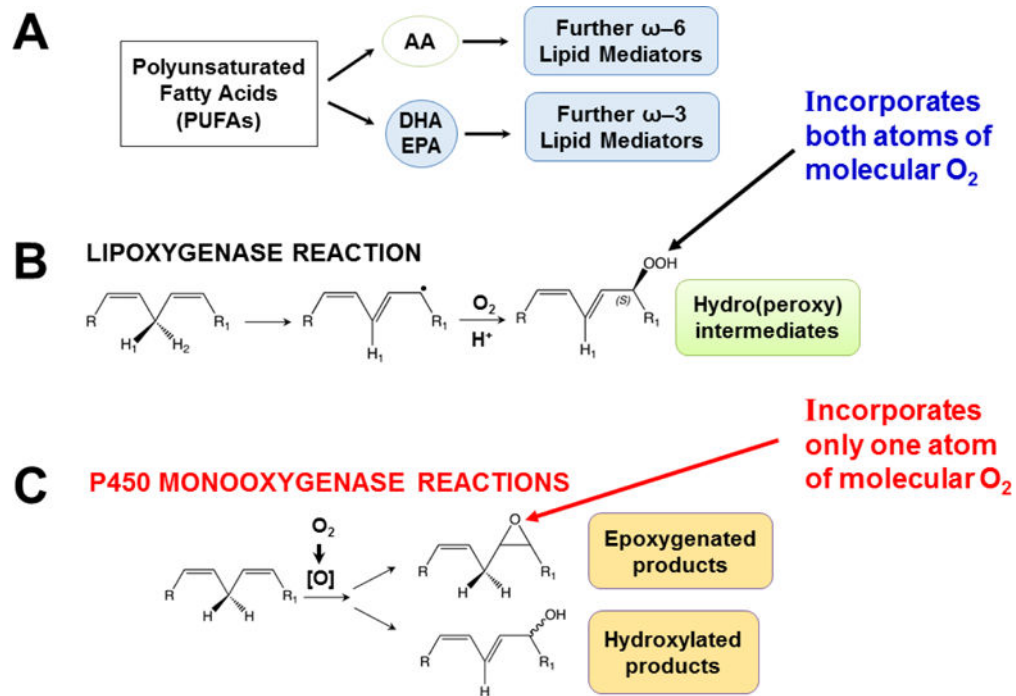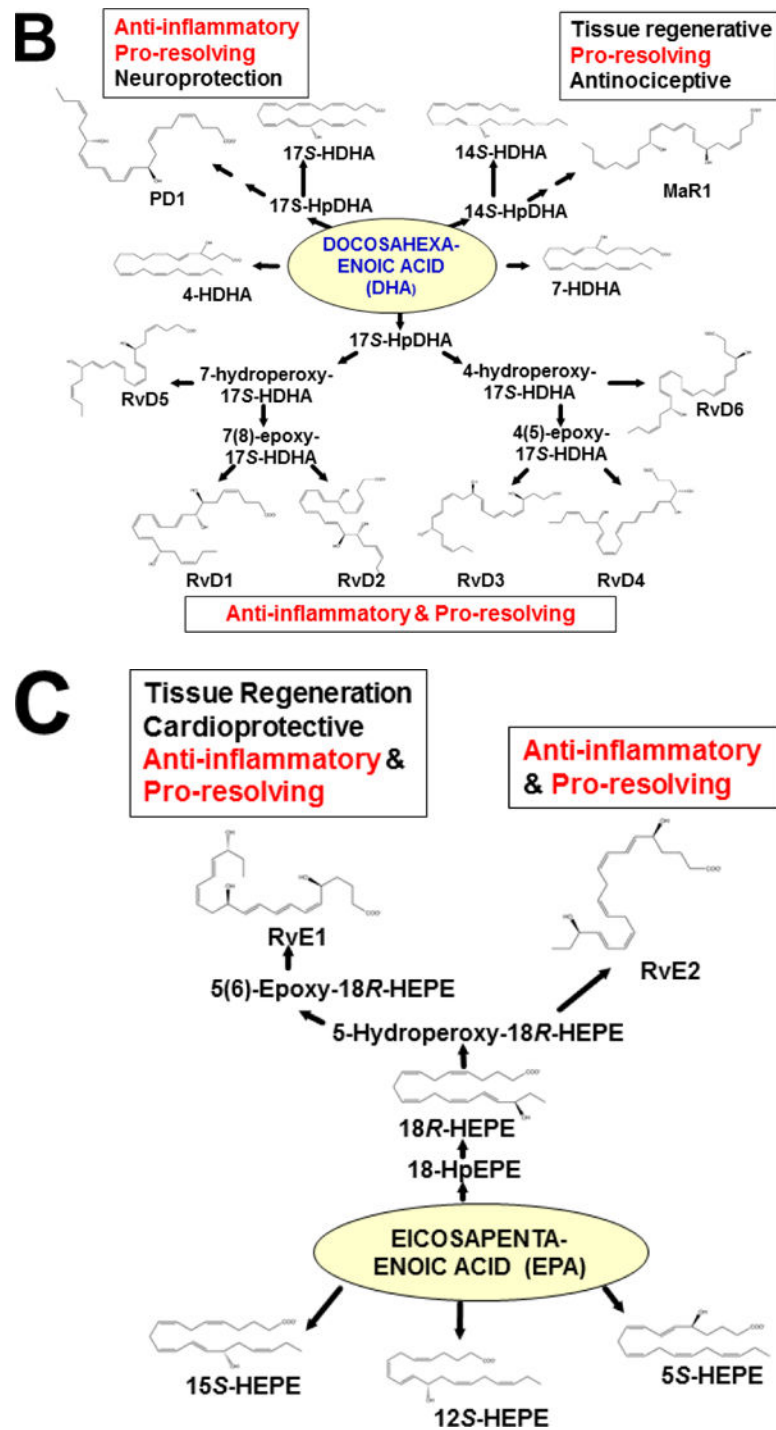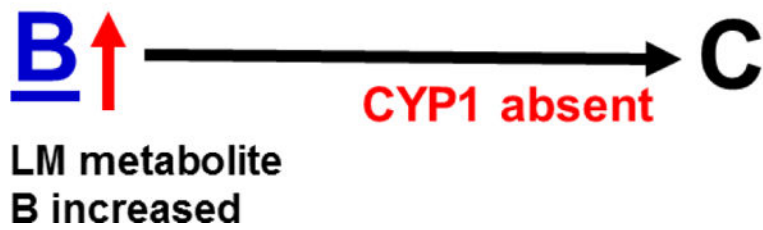
Fig. 4.
Polyunsaturated fatty acids (PUFAs), lipoxygenase vs monooxygenase mechanisms of reactions, and LM biosynthesis pathways. *A*, scheme depicting the origin of ω–6 and ω–3 LMs. *B*, diagram showing mechanism for the lipoxygenase reaction. *C*, diagram showing mechanisms for the P450-monooxygenase reactions.
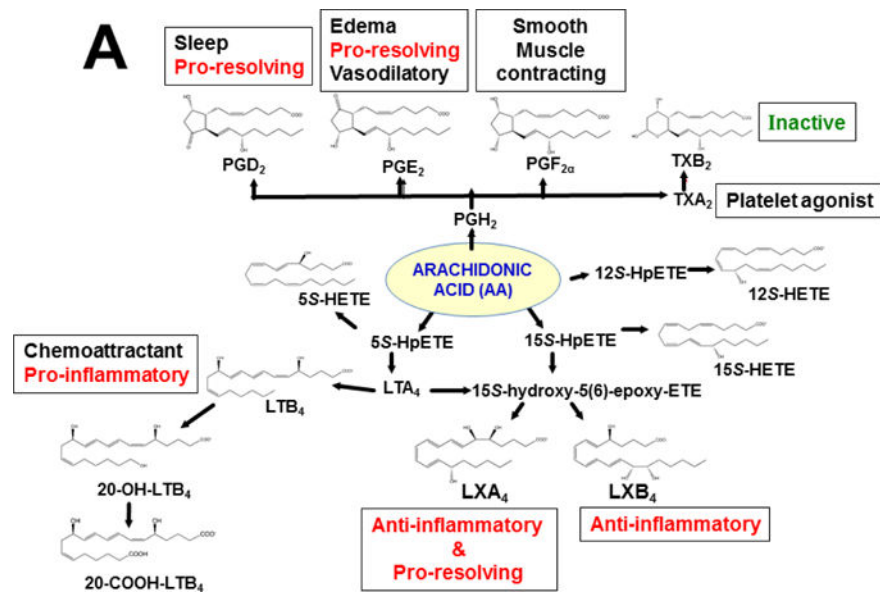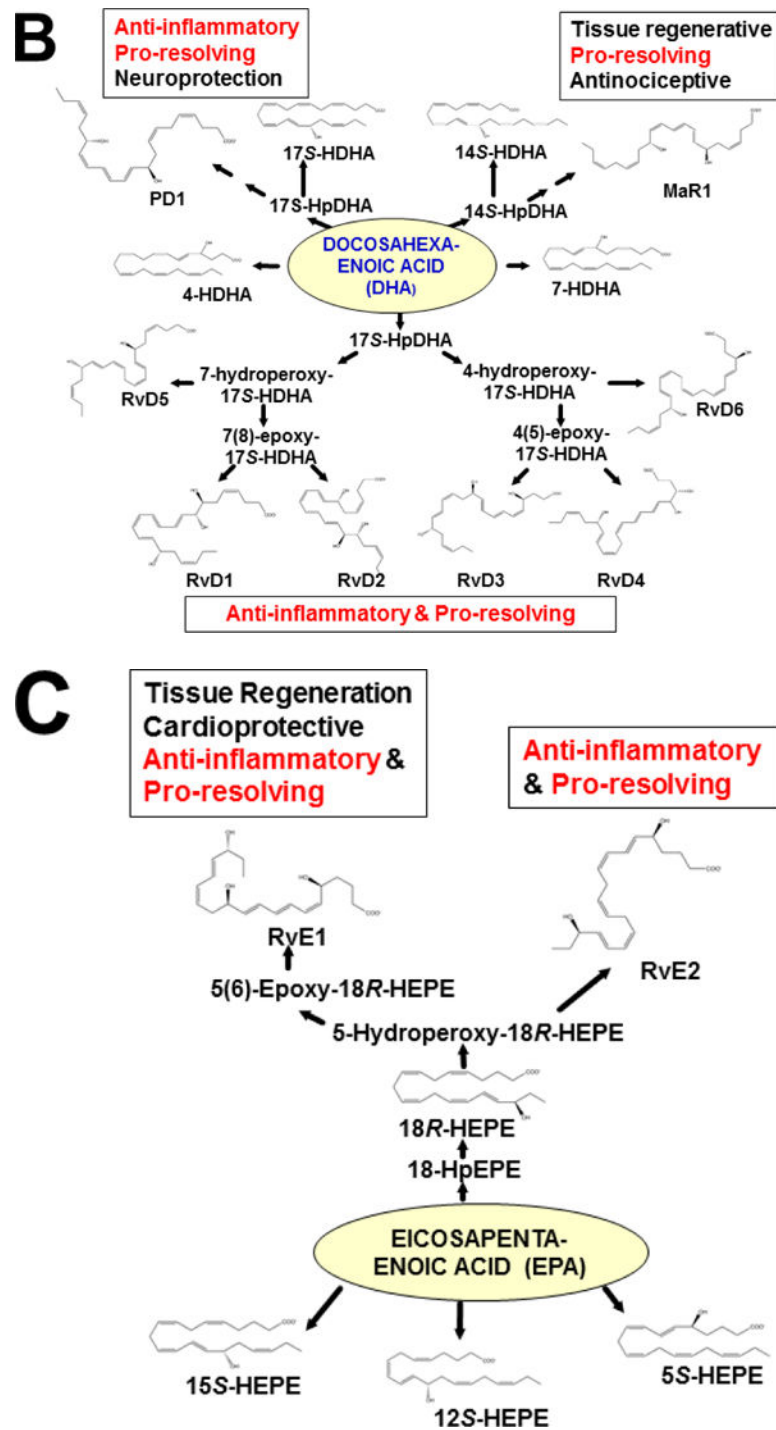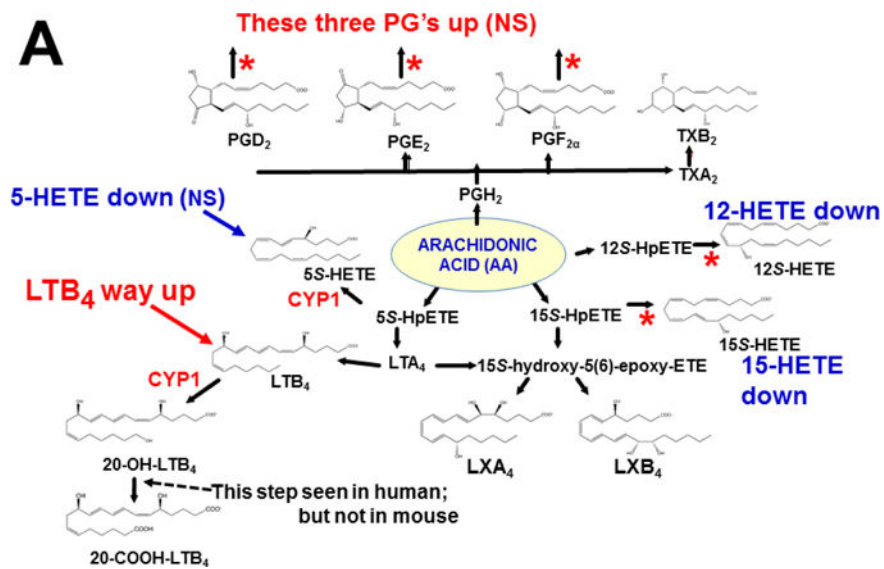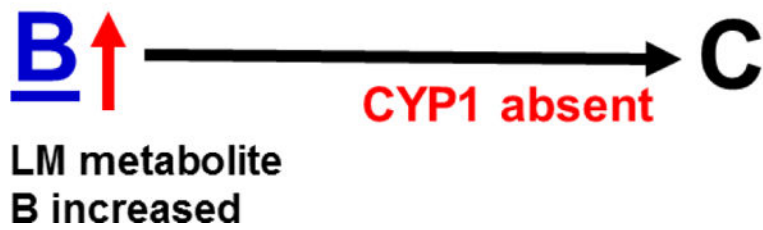
Fig. 5.

LM metabolites that were able to be identified and quantified by the multiple-reaction monitoring and liquid chromatography-UV coupled with tandem mass spectrometry-based LM metabololipidomics system used (Divanovic *et al.*, 2013). The three LM metabolomes include: *A*, arachidonic acid (AA)-derived, *B*, docosahexaenoic acid (DHA)-derived, and *C*, eicosapentaenoic acid (EPA)-derived LMs. For various metabolites in the pathways, some of

the established critical-life processes elicited by specific LMs are depicted in the *rectangular boxes*. Processes described in *red font* denote pro-inflammatory and pro-resolving inflammatory functions, *i.e.* initiation and resolution of inflammation, respectively.
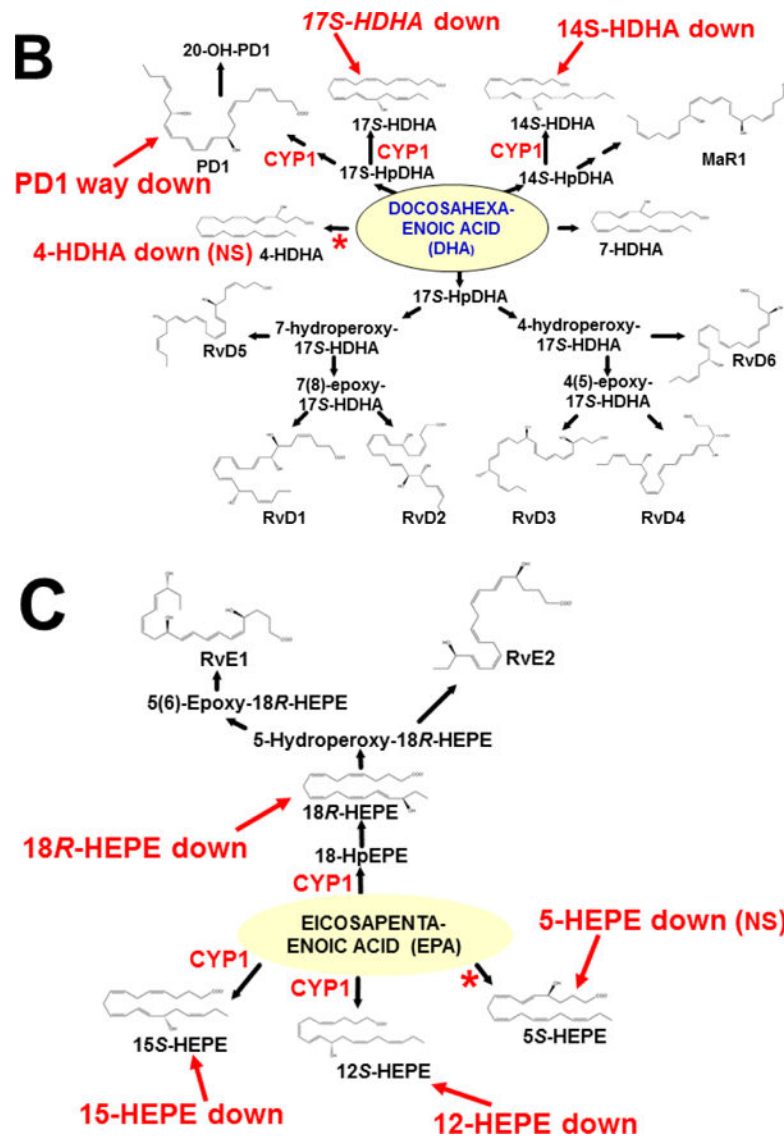
Fig. 6.
Hypothesis designed for the study of peritoneal exudate of zymosan-treated mice (Divanovic *et al.*, 2013). **A**, **B** and **C** denote metabolites in the LM second-messenger cascade that can be identified and quantified by the multiple-reaction monitoring and liquid chromatography-UV coupled with tandem mass spectrometry-based LM metabololipidomics system (Divanovic *et al.*, 2013). If CYP1A1, CYP1A2 and CYP1B1 monooxygenases are all involved, just two involved, or just one of the three enzymes involved — in a metabolic step between A and B, then metabolite B levels will be decreased in TKO mice, compared with wild-type (WT) mice. If three, two or one of the CYP1 enzymes are(is) involved in a metabolic step between B and C, then metabolite B levels will be increased in TKO mice, compared with WT mice.
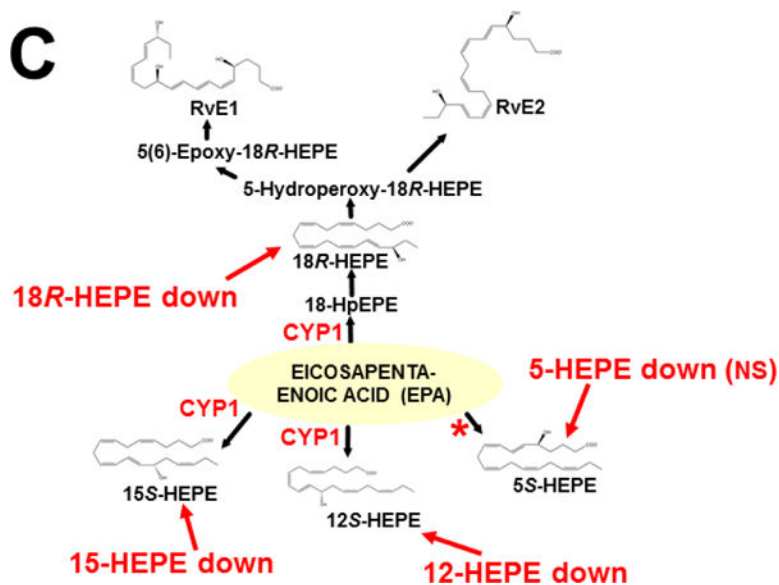
Fig 7.
Identification of metabolic steps at which CYP1 enzymes are proposed to participate (Divanovic *et al.*, 2013). *A*, AA-derived lipoxins, prostaglandins, thromboxanes, and leukotrienes. *B*, DHA-derived resolvins, protectins and maresins. *C*, EPA-derived resolvins. CYP1 labels are placed in accordance with the findings in the study, combined with what is known about CYP-mediated LM metabolism: "way up" or "way down" = $P<0.05$; "up" or "down" = $P<0.08 > 0.05$; and ***** denotes "Trend," = $P<0.12 > 0.08$. Positions of the (*bolded red*) CYP1 labels and the *bolded red asterisks* depict the proposed steps, as determined in the study (Divanovic *et al.*, 2013). In cases where there are two or more steps between the identifiable LM metabolite, the precise step at which CYP1 acts, and which one (or two or three) of the three CYP1 enzymes participates — will require further experiments.
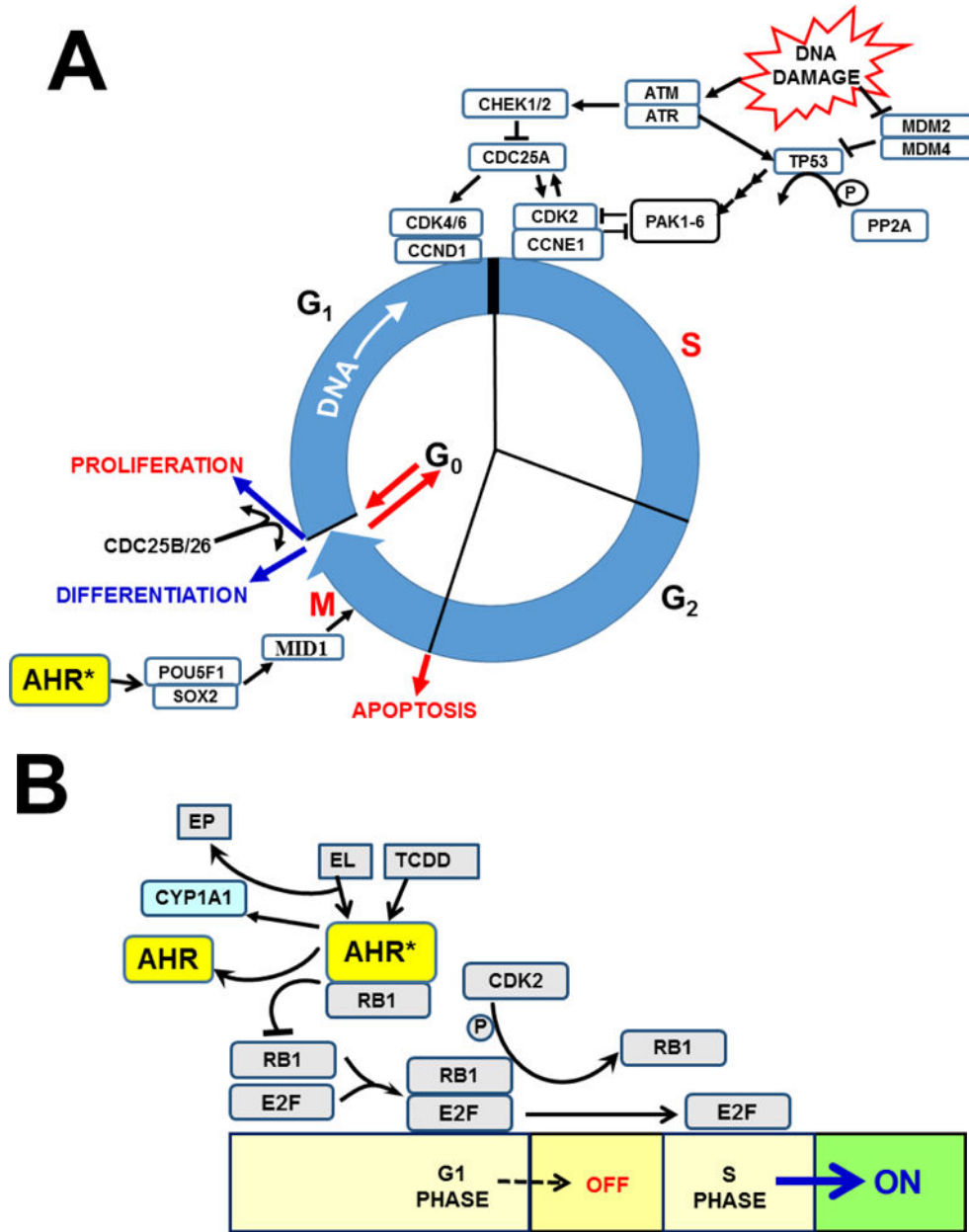
Fig. 8.
Established effects of AHR and the AHR-CYP1 axis on cell cycle functions. *Arrows* denote
"activation or stimulation" whereas *lines with a foot* indicate "repression or inhibition." *A*,
blue circle denotes the cell cycle ($G_1 \to S \to G_2 \to M \to$ return to the G_1 phase). The
G_1/S cell cycle checkpoint controls commitment of eukaryotic cells to transition through the
G_1 phase to enter into the DNA synthesis S phase. The G_2/M checkpoint precedes the cell's
entrance into mitosis, with locations indicated when cells have specific options (*e.g.* G_0 for
the inactive, or resting, phase; versus processes such as apoptosis, differentiation, and
proliferation). Two cell cycle kinase complexes — CDK4/6 (cyclin-dependent kinase 4 &
6)-CCND1 (cyclin D1), and CDK2-CCNE1 (cyclin E1) — participate as part of a dynamic
transcription complex, to move the cell from G_1 to S phase (described further in panel *8B*).

DNA damage (*upper right*) activates ATM and ATR (serine-threonine kinases), and also represses MDM2 and MDM4 (MDM proto-oncogenes 2 & 4), thereby releasing their inhibition of TP53 in concert with activation of TP53 by ATM/ATR. Phosphorylation (**P** denotes inorganic **PO$_4$**) of TP53 by PP2A (protein phosphatase-2A), via several steps, activates PAK1–6 (p21 (RAC1; CDKN1A)-activated kinases-1 through -6), which results in repression of CDK2. Whereas CDC25A (cell division cycle-25A) activates both CDK4/6 and CDK2, CDC25A is suppressed by CHEK1/2. At the **M/G$_1$** boundary (*lower left*), the CDC25B/26-CDK1 complex induces either differentiation or proliferation. Activated **AHR*** stimulates the complex of POU5F1 (POU class-5 homeobox-1; formerly OCT4) and SOX2 (SRY-box-2), which in turn up-regulates MID1 (midline-1), thus allowing MID1 to activate mitosis. For the sake of avoiding clutter, many additional factors participating in the cell cycle are not shown. The two cell cycle kinase complexes — CDK4/6-CCND1 (cyclin D1) and CDK2-CCNE1 (cyclin E1)-CDK2 — work in concert to relieve inhibition of the dynamic transcription complex (shown in panel **B**) that includes RB1 and E2F. **B**, in **G$_1$**-phase uncommitted cells, hypo-phosphorylated RB1 binds to the E2F-transcription complex, whereas phosphorylation of RB1 by CDK2 releases RB1, thus allowing E2F-mediated **S**-phase genes, a requirement for DNA synthesis, to be turned on. At *top*, TCDD (or other planar foreign chemical) or endogenous ligand (EL) binds to AHR, which activates it to **AHR*;** this allows it to complex with RB1 which in turn prevents RB1 binding to E2F. AHR*-mediated up-regulation of CYP1 results in metabolic degradation of EL to the endogenous product EP. This removes EL from AHR*, inactivates AHR which releases RB1, making it then able to bind to E2F and promote **G$_1$–>S** progression. [*Portions of panel A were helped by* https://www.cellsignal.com/common/content/content.jsp?id=pathways-cc-g1s.]
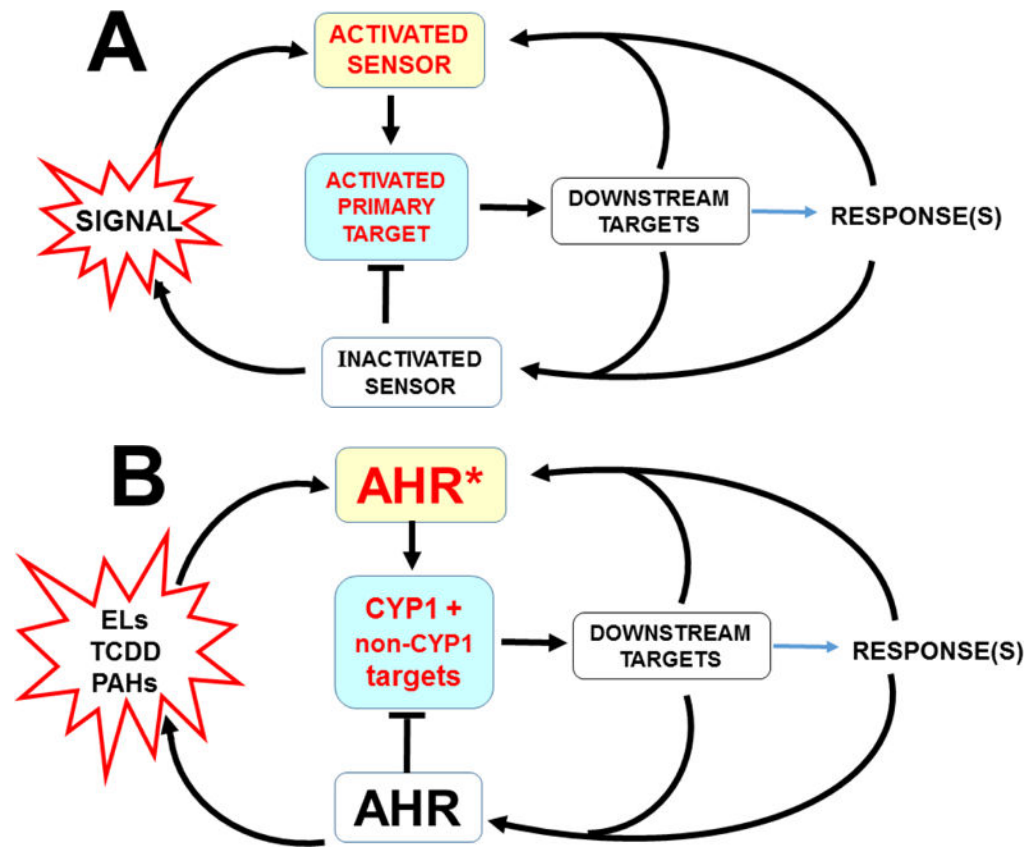
Fig. 9.
Overall scheme postulated to operate for some, perhaps all, of the bHLH/PAS sensors. *A*, generalized diagram. *B*, specific scheme for the AHR-CYP1 axis. ELs, endogenous ligands. See text for details.

Table 1

Summary of organs, systems, cell functions, and developmental biology in which AHR-signaling is involved.

Location	AHR-signaling pathway involvement
Central nervous system	Development of brain and nervous system; Neurogenesis; Neuronal cell development; Cardiorespiratory brainstem development in ventrolateral medulla; "Brain-gut-microbiome"
Eye	Ciliary body formation and function; Thyroid-associated eye disease
Gastrointestinal tract	Development of GI tract; Rectal prolapse during aging; "Brain-gut-microbiome"
Heart	Development of heart organ; Cardiovascular physiology; Atherogenesis; Cardiomyogenesis; Cardiorespiratory function
Hematological system	Development of blood cell-forming system; Hematopoiesis; Activation or suppression of erythroid development
Immune system	Immune system development; The immune response; Innate immunity; Pro-inflammatory response; Anti-inflammatory response; Immunomodulatory effects
Inner ear	Development of the cochlea
Kidney	Development of the kidney; Hypertension
Liver	Development of liver organ; Hyperlipidemia; Glucose and lipid metabolism; Hepatic steatosis
Musculoskeletal system	Transmesoderm–>osteoblast transition; Bone formation; Osteoclastogenesis
Pancreas	Development of pancreas; Beta-cell regulation; Pancreatic fibrosis
Endocrine system	Serum lowered testosterone levels; Infertility; Mammary gland duct cell epithelial hyperplasia; Degenerative changes in testis; Gerrm-cell apoptosis; Endometriosis
Reproductive system	Development of male and female sex organs; Spermatogenesis; Fertility
Respiratory tract	Development of respiratory tract; Disruption of GABA-ergic transmission defects; Cardiorespiratory function
Vascular system	Angiogenesis; Atherosclerotic plaque formation
Skin	Barrier physiology; Atopic dermatitis
Cellular functions	Cell migration; Cell adhesion; Circadian rhythmicity
DNA changes	DNA synthesis; DNA repair; DNA-adduct formation; Mutagenesis
Oxidative stress	Mitochondrial ROS formation; Anti-oxidant protection against ROS formation; Mitochondrial H_2O_2 production; Crosstalk with hypoxia and HIF-signaling pathways; Transforming growth factor-signaling pathways; MID1-PP2A-CDC25B-CDK1 signalig pathway regulating mitosis
Tumor cells	Growth suppression; Tumor initiation; Tumor promotion
ES cell basic functions	Ectoderm–>epithelium transition; Cell adhesion; Cell-cycle regulation; Apoptosis; Cavitation during morula–>blastula formation; Activator of Rho/Rac GTPases; WNT-signaling pathways; Homeobox-signaling pathways
Other basic functions	Transgenerational inheritance; Epigenetic effects; Chromatin remodeling; Histone modification; Aging-related and degenerative diseases

Table 2

Timeline of proposed key discoveries, as the exciting "AHR-signaling story" developed.

Year	Discovery
1956	"Benzpyrene hydroxylase" activity induced by PAHs in rat liver
1962	Benzpyrene hydroxylase activity induced throughout rat GI tract by oral benzpyrene
1968	"AHH activity" assay designed; AHH induction by PAHs in fetal hamster cell cultures
1969	Differences in AHH inducibility by PAHs between inbred strains of mice
1970	PAH-induced AHH activity represents a cytochrome P450 (P_1-450)
1972	PAH-induced AHH activity in B6 × D2 crosses exhibits predominantly Mendelian inheritance
1972	In utero AHH induction in fetuses by treatment of mother with PAHs
1973–1989	Importance of AHR-mediated CYP1 shown for cancer, mutagenesis, toxicity, and teratogenesis
1974	Evidence of AHR, based on dose-response curve: AHH induction as a function of TCDD concentration in B6 vs D2 inbred mouse strains
1976	Radiolabeled TCDD binding assay detects cytosolic AHR
1979	Development of benzo[*a*]pyrene-resistant clones in Hepa-1 cells: c1 & c37, c2, and c4
1980	AHR-mediated CYP1A1-induced sister-chromatid-exchange detected in GD7.5 embryo
1981, 1989	AHR-mediated P_1-450 induction proposed to be involved in inflammatory process
1982	Intranuclear appearance of radiolabeled TCDD (AHR) asspcoated with P_1-450 induction
1988, 1998	AHR-mediated CYP1A1 induction demonstrated in GD0.5 one-cell zygote
1991	Mouse *Arnt* gene cloned (human *ARNT* gene shortly thereafter)
1991	The term "bHLH/PAS" was first coined
1992	Mouse *Ahr* gene cloned (human *AHR* gene shortly thereafter)
1995	Creation of first of several *Ahr(–/–)* knockout mouse lines
1996	Standardized gene nomenclature for CYP genes in all species becomes well established
2005	AHR-CYP1 axis demonstrated in cultured ES cells
2008, 2013	Underscoring the importance of AHR-CYP1 axis in LM second-messenger pathways involving innumerable critical life events — in virtually every organ, tissue and cell-type