Advances on Toxicological Mechanism of AhR Pathway and Early Biomonitoring of Persistent Organic Pollutants POPs in Aquatic Animals

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Abstract With the development of industry and agriculture, the case of cancer is incresing gradually in the last thirty years. Considerable part of the cases are caused by persistent organic pollutants POPs and some of them belong to the environmental endocrine disruptors. POPs are ubiquitous in the environment, especilly in the aquatic ecosystem which has aroused the extensive attention of the world. The mechanism of POPs toxicology is very complicated, but it is mainly mediated by the aryl hydrocarbon receptor AhR pathway in aquatic animals. The overall goal of this review paper is to highlight the toxicological mechanism of AhR pathway that may contribute to a more holistic understanding of each AhR pathway gene behavior in the toxicological process, as well as the early biomonitoring methods of POPs in aquatic animals. Finally, we propose some perspectives for future toxicological mechhanism research of interest.

Keywords persistent organic pollutants aquatic animals toxicological mechanism AhR pathway biomonitoring

Persistent organic pollutants POPs are organic compounds of natural or anthropogenic origin that resist photolytic, chemical, and biological degradation. They are low water solubility and high lipid solubility, resulting in bioaccumulation in adipose tissues of living organisms Mehmetli and Koumanova, 2007 . They are not only toxic, but also prone to long-range transport. Most of them can be classified into three groups: 1 Industrial chemical product such as polychlorinated biphenyls PCBs ; 2 Combustion and by-products such as polychlorinated dibenzo-p-dioxins and dibenzofurans PCDD/Fs , and 2,3,7,8-tetrachlorodibenzo-p-dioxin 2,2,7,8-TCDD ; 3 and Pesticides such as dichlorodiphenyl-trichloroethane DDT , dihedron, and toxaphene.

POPs can enter aquatic ecosystem in effluent, atmospheric deposition, runoff, and groundwater. They are not only very harmful to the health of aquatic animals but also that of humans. Many results show that POPs may impair most systems of human. For example, PCBs and OH-PCBs may damage brain Kimura-Kuroda et al., 2007 . Polycyclic aromatic hydrocarbons PAHs damage the immunity system Davila et al., 1995 and the reproductive system Den Besten et al., 1990 , and cause DNA damage Lemiere et al., 2005 . PCB, PAH and other POPs are the most important risk factor to cause breast cancer Gammon et al., 2004 , lung cancer Okona-Mensah et al., 2005 and prostate cancer Ritchie et al., 2005 . So the widespread occurrence of POPs has attracted considerable attention. In order to avoid or reduce the damage

that caused by POPs, we should comprehensively understand the toxicological mechanism of POPs, as well as related monitoring methods.

1 The regulation mechanism of POPs toxicology

A series of experiments have revealed that aryl hydrocarbon receptor AhR pathway plays a pivotal role in the mediation of POPs toxicology in aquatic animals. AhR pathway genes e.g. AhR, AHRR have been found in many aquatic animals, and are detectable in many tissues. Their structure and function have been studied in aquatic animals, including killfish, rainbow trout, zebrafish, harbour seal, medaka, red seabream etc.. A large body of experiments have revealed that the mechanisms of the AhR-dependent CYP1A1 gene induction see Fig. 1 . There are four major steps of toxicology regulation, including formation of cytosolic complex, translocation of AhR, heterodimerization of AhR, and induction of CYP1A.
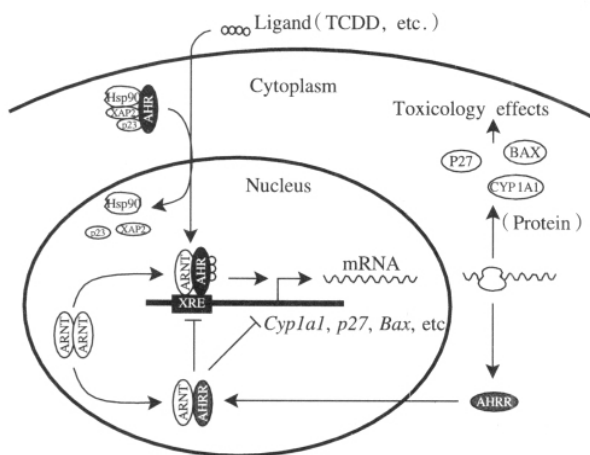

Fig.1 Regulation mechanism of AhR pathway
（Adapted from Mimura and Fujii-Kuriyama, 2003）

1.1 Formation of cytosolic complex

In absence of ligands, AhR is associated with a cytoplasmic protein complex with two molecules of heat shock protein 90 Hsp90 Perdew, 1988 , immunophilin-like protein XAP2 also referred to as AIP or ARA9 Carver and Bradfield, 1997 , and a 23-kDa co-chaperone protein p23 Kazlauskas *et al.*, 1999 . Hsp90 is an essential component of the

AhR-signaling pathway, and loss of Hsp90 most likely results in an improperly folded or destabilized receptor protein Hsp90, one subunit of the AhR complex, appears to direct proper folding and maintenance of the high affinity ligand binding conformation of the AhR in some species Soshilov *et al.*, 2006 .

Though the other two members of cytosolic complex are not essential for the AhR signaling, but they play an important role in stabilizing the cytosolic complex. The capability of XAP2, also known as AhR interacting protein Ma and Whitlock Jr, 1997 to modulate AhR function has been studied extensively in cell culture systems. XAP2 is capable of stabilizing the AhR, as well as enhancing cytoplasmic localization of the receptor. And it binds to both the AhR and Hsp90 in the receptor complex, and is also capable of binding independently to both Hsp90 and the AhR Petrulis and Perdew, 2002 , but it is not a limiting component in AhR regulation Hollingshead *et al.*, 2006 . Mechanistically, p23 appears to promote AhR/ARNT/DRE complex formation in an Hsp90-dependent manner by assisting with the heterodimerization of the AhR and ARNT. Further research of Cox and Miller Iii 2004 show that p23 can inhibit Hsp90 ATPase activity, thereby stabilizing ATP-Hsp90-client protein complexes. However, p23 does not interact directly with either the AhR or ARNT Kazlauskas *et al.*, 1999 .

1.2 Translocation of AhR

Upon binding to a ligand TCDD or other POPs , the AhR complex translocates into the nucleus and the AhR dissociates from Hsp90 complex to form a heterodimer with its partner molecule, ARNT Poland and Knutson, 1982 . AhR has been extensively studied as a mediator of toxicity of a diverse group of xenobiotics, including PCDD/Fs, PCBs, and PAHs etc. Ma, 2001 . AhR is a ligand-activated transcription factor and a member of the basic helix-loop-helix/PER-ARNT-SIM family of DNA-binding proteins Chen and

ZHOU Hai-long *et al*. Advances on Toxicology Mechanism of AhR Pathway and Early Biomonitoring of Persistent
Organic Pollutants POPs in Aquatic Animals

1   11

Perdew, 1994 , and it contains three well -conserved domains involved in substrate binding. The first domain located in the N -terminal region of the molecule, consists of the bHLH domain found in many transcription factors e.g. MyoD, c -myc, and Max Olson and Klein, 1994; Murre *et al*., 1989; Kadesch, 1992 . The second domain is very similar to the Drosophila circadian rhythm gene per and the Drosophila single -minded protein sim and, therefore, is referred to as the PAS domain Hoffman *et al*., 1991; Takahashi, 1992 . The third domain, located at the C -terminal end of the molecule, is glutamine Q -rich. The ligand -binding function apparently resides in the PAS region of AhR Dolwick *et al*., 1993 .

In addition, a large body of literature has implicated AhR in multiple signal transduction pathways. AhR is known to interact with signaling pathways that are mediated by estrogen receptor and other hormone receptors, hypoxia, nuclear factor B, and retinoblastoma protein Carlson and Perdew, 2002 . Furthermore, AhR complexes may affect cellular signaling through interactions with various other regulatory and signaling proteins, including PAS heterodimerization partners, chaperone and immunophilin -like proteins, protein kinases and phosphatases Carlson and Perdew, 2002 . So we can conclude that AhR not only play a crucial role in the POPs toxicology but also has an important physiological function in aquatic animals.

1.3 Heterodimerization of AhR

When AhR binds to ligand, it is translocated to the nucleus and dissociates from the Hsp90 complex to form a heterodimer with ARNT. The AhR/ARNT heterodimer binds to the xenobiotic response elements XRE sequence in the promoter regions of target genes encoding drug-metabolizing enzymes, including CYP1A1, quinone reductase, etc., and alters their expression Kikuchi *et al*., 2003 . ARNT belongs to the bHLH -PAS family. Binding with AhR, ARNT also interacts with SIM1 Single Minded 1 , SIM2

Single Minded 2 , HIF1α hypoxia -inducible factor 1α , CHF1 Cardiovascular helix -loop -helix factor 1 and EPAS1 Endothelial PAS domain protein 1 to regulate neurogenesis, the hypoxia response, cardiovascular development and pathological angiogenesis Mimura and Fujii -Kuriyama, 2003; Swanson, 2002; Taylor and Zhulin, 1999 . Therefore, ARNT may serve as a central player in regulating these diverse signaling pathways. AHRR is an AhR related protein, and represses the transcription activity of AhR by competing with AhR by heterodimerization with ARNT and subsequently binding to XRE sequence Mimura *et al*., 1999 . These results indicate that AhR and AHRR form a regulatory feedback loop Mimura and Fujii - Kuriyama, 2003 . Recently, Evans *et al*. 2008 propose a mechanism of AHRR action involving "transrepression"of AhR signalling through protein - protein interactions rather than by inhibition of the formation or DNA binding of the AhR-ARNT complex. In the future, targeted knock -down of one or both AHRR proteins by application of morpholino oligonucleotides can be used to further characterize these duplicate zebrafish AHRRs and to elucidate their potential roles in development and in the developmental toxicity of chemicals such as TCDD.

Now, although it remains to be studied how AhR and AHRR are involving in the other TCDD - induced biological effects such as teratogenesis and immunosuppression than induction of XMEs, it is well known that these adverse biological effects are caused by untimely activation of gene expression by ligand -activated AhR and AHRR in the biological processes.

1.4 Induction of CYP1A

The ligand -AhR -ARNT heterodimer interacts with AhR response elements AhREs; also known as XREs or DREs to activate or repress gene expression from target genes Hahn *et al*., 2005; Hahn *et al*., 2006 . The best characterized targets of the AhR pathway are Cytochrome P4501A CYP1A genes,

which are strongly induced by TCDD and PAHs Whitlock, 1999 . They have a broad affinity for polycyclic, aromatic hydrocarbons, as well as aromatic amines, and some endogenous substrates Gonzalez and Kimura, 2003; Teraoka *et al.*, 2003 . And they play a central role in biotransformation, detoxication and elimination of various structurally diverse xenobiotics Monostory and Pascussi, 2008 . The induction of CYP1A family member expression is regulated by a heterodimer composed of the AhR and ARNT Fujii-Kuriyama and Mimura, 2005 . In contrast, the expression of CYP2, 3, and 4 family members is regulated by the nuclear receptors CAR Constitutive androstane receptor , PXR Pregnenolone X receptor , and PPAR Peroxisome proliferator activated receptor , respectively Waxman, 1999 .

The induction of CYP 1A is an important step in the response to POPs. Some researches have identified several consensus response elements, there are eight potential xenobiotic response elements XREs in the promoter region of the european flounder CYP 1A gene, but not all of these sequences are necessarily for activation, just only four out of eight different XREs are functional in the regulation of CYP 1A. The activity of these response elements enhances the evidence for considerable diversity in vertebrate CYP1A regulation Lewis *et al.*, 2004 . In a word, we can conclude that AhR pathway plays a pivotal role in the regulation of POPs toxicology in aquatic animals.

2　The regulation mechanism of toxicology in zebrafish development

Zebrafish is a very perfect model aquatic animal. At present, the AhR pathway of zebrafish has been extensively studied. Thus, it is very important for us to comprehend the AhR pathway toxicological mechanism of POPs in zebrafish.

Proper regulation of AhR is needed for normal vertebrate cardiovascular development. In zebrafish, there are two AhR genes, zfAhR1 and zfAhR2. ZfAhR2 binds TCDD with high affinity and

transcriptional active which plays a major role in mediating the developmental toxicity of TCDD, whereas zfAhR1 lacks the ability to bind TCDD and activate transcription, and it's function is not known. A new zebrafish AhR designated AhR1B, which shares 34% amino acid sequence identity with AhR1 AHMA . The AhR1B gene resides on chromosome 22, adjacent to AhR2, whereas the AhR1A gene is located on chromosome 16. AhR1B is expressed in embryos as early as 24 hours post-fertilization and increases through the next 2 days, but expression is not inducible by TCDD. So we can conclude that AhR1B may play a physiological role in embryonic development, whereas AhR2 mediates the response to xenobiotics Karchner *et al.*, 2005 .

When AhR was hyperactivated by TCDD during the process of zebrafish embryo development, it altered heart morphology and function, culminating in death. Within 1 to 2 hours of exposure, TCDD shows rapidly induced expression, in 42 genes which have function in xenobiotic metabolism, proliferation, heart contractility and pathways that can regulate heart development. Furthermore, these expression changes preceded signs of cardiovascular toxicity, including decreased stroke volume, peripheral blood flow, and a halt in heart growth, these which are good candidates for AhR target genes Carney *et al.*, 2006 , and so these genes can be used to monitor the pollution condition of early aquatic ecosystem.

It is well known that TCDD is a potent developmental toxicant in most vertebrates, but several frog species are insensitive to TCDD, especially during early life stages. Some experiments with frog suggest that TCDD insensitivity results largely from rapid elimination. But recent study confirms that rapid elimination of TCDD is unlikely to contribute to TCDD insensitivity during development of the cardiovascular system Philips *et al.*, 2006 . Another research of AhRs from X. laevis demonstrates that these proteins bind TCDD with 25 ~50 -fold lower affinity than AhRs from more sensitive species

ZHOU Hai-long *et al*. Advances on Toxicology Mechanism of AhR Pathway and Early Biomonitoring of Persistent
Organic Pollutants POPs in Aquatic Animals

1   13

Lavine *et al*., 2005 , a property likely that the structural and functional diversity of AhR proteins may confer species-and strain-specific differences in the sensitivity to toxic AhR ligands Hahn *et al*., 2005 .

3 Biomontoring of POPs in aquatic animals

Toxicological effects of POPs in aquatic ecosystem lead to the deterioration of water quality and adversely impact on aquatic animal and human health. The highly lipophilic nature of these pollutants may enter fish through the diet or by water -borne exposure Wong *et al*., 2001 . In order to prevent and reduce the harm of POPs for the health of aquatic animals and people, it is very important to explore some idealism biomarkers for early biomonitoring. At present, many investigations have been done, briefly including the following two aspects:

3.1 Biochemical biomarker

Now, many biochemical biomarkers have been studied, such as ethoxyresorufin-O-deethylase EROD which has the catalytic function of the CYP1A, microsomal oxygenase subfamily which is a popular biomarker for exposure to xenobiotics, polyhalogenated aromatic hydrocarbons PHAHs in particular. It has been widely used to assess the pollution condition of aquatic environment both *in vivo* and *in vitro* Kuiper *et al*., 2004 . EROD is also can be used to determine the quantities of POPs in fish muscle Havelkova *et al*., 2007 . A model to assess pollutant exposure via food supply has been developed for the sentinel organism. Mussels were fed for 4 weeks with Benzo a pyrene B a P - contaminated feed, and the result suggests that B a P hydroxylase BPH , acetylthiocholine esterase AChE , DT-diaphorase DTD and catalase CAT activity as suitable biomarkers of PAH exposure for these sentinel species Akcha *et al*., 2000 . The carboxylesterase activity, metallothionein, total haemolymph protein Hamm *et al*., 2003 and vitellogenin Zhan *et al*., 2007 were the most useful

biochemistry biomarkers.

3.2 Molecular biomarker

Previous researches indicate that AhR plays an important role in the regulation pathway of POPs toxicology. Thus the induction of the CYP1A1 gene of fish has been used as a sensitive, early warning method in the monitoring of contamination in aquatic ecosystems Wong *et al*., 2001 . Expression of CYP1A1 gene and its related enzyme activity have long been used as a biomarker for AhR activation and a warning of dioxin -like toxicity Hu *et al*., 2007 . At the same time, we should pay more attention to other factors in real station. For example, on certain conditions, seasonal factors might affect the biomarker e.g., antioxidant and peroxisomal enzymes responses, to a greater extent than pollution variations Orbea *et al*., 2002 .

In addition, micronucleus MN frequency and DNA single strand break were used as biomarkers of genotoxicity. Mussels were also analyzed for PAH and heavy metals Hg and Cd Bolognesi *et al*., 2004 . MN response can be used as a sensitive indicator of exposure to relatively low levels of genotoxicants and that MN response in ussel gill cells can be a stable biomarker of genotoxicity Siu *et al*., 2004 . Moreover DNA adduct is also a molecular biomarker of exposure to PAHs, and is now well established in ecotoxicology. DNA adduct level in aquatic organisms has been found to produce a better correlation with PAH exposure than PAH concentration in organisms Shaw and Connell, 2001 , because relatively high levels of DNA adducts can be produced by some non -carcinogenic PAHs, while other non -carcinogenic compounds do not produce detectable adducts. In addition, it has been shown that all carcinogenic PAHs investigated produce DNA adducts and that a relationship exists between relative adduct formation and carcinogenic potency.

We can conclude that the molecular biomarkers are more effective than biochemical biomarkers, and

we should pay more attentions on exploring more useful molecular biomarkers to monitor the status of POPs contamination in the future.

4　Summary

POPs have become ubiquitous and aroused the attentions in the world　Swain, 1988 .Over the last decades, numerous studies have been investigated on the formation, distribution, accumulation, bioamplification through the food chain, toxicity, toxicology, genotoxicity, biomonitoring and embryo toxicity of POPs. At present, we can find that most of the aquatic animals all conformed to the same toxicology mechanism, namely the AhR pathway. It mainly consists of four major steps, including formation of cytosolic complex, translocation of AhR, heterodimerization of AhR, and induction of Cyp1a. In addition, there are some other species are insensitive to the TCDD toxicity, especially at the early life stage, for example, African clawed frog, which factor causes the difference between different animal species　It is still an unresolved issue.

Furthermore, the embryo of aquatic animal is the most critical stage in the total process of development and growth of aquatic animal, and it can be used as one kind of biosensors to monitor the pollutant status of aquatic ecosystem. On the other hand, the research of molecular mechanism of POPs embryo toxicology can elucidate the mechanism of some cancer, tumours and teratogenesis. But previous researches just focus on one or few genes in the AhR pathway. Actually, it is universally acknowledged that there are a series of related genes involving in the regulation of POPs toxicology. Then, you may ask that which and how many genes are involved in the regulation, and what's the relationship between them　With the development of modern molecular biology and biotechnology, we believe that these issues will be resolved gradually in the future. For example, we can use differential display technique and microarray technology to explore it, and it can provide a more quickly and

effectively method to monitor the POPs pollution in aquatic ecosystem as early as possible.

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