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Analysis of immune gene expression modulated by benzo[a]pyrene in head kidney of olive flounder (*Paralichthys olivaceus*)

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ABSTRACT

Poly aromatic hydrocarbons (PAHs) are known to cause functional disorder of fish immune responses. Alteration of inflammatory cytokines and other immune gene expressions by PAHs in immune organs may play a pivotal role in immunotoxicity. Thus this study aimed to elucidate the immunotoxic mechanism of PAH using benzo[a]pyrene (BaP) by analyzing the gene expression of cytokines (IL-1β, TNFα, IL-6, IL-8, IFNγ, Mx), apoptosis (FasL, SOD) and other immune related substances (Lysozyme, IgM) in head kidney and macrophage in olive flounder. In Q-PCR analysis, proinflammatory cytokine (IL-1β, IL-6, IL-8, TNFα) gene expressions were significantly upregulated by BaP while Mx and IgM gene expressions were significantly downregulated in head kidney by a longer exposure to BaP in vivo and in vitro. Lysozyme gene expression was initially upregulated but later downregulated in head kidney in vivo and in vitro. Inhibition test revealed that TNFα gene expression was upregulated by BaP via the AHR pathway as blocked by ANF while IL-6 and IFNγ gene expressions were upregulated by a calcium dependent pathway (i.e. NFAT) as blocked by EGTA. In primary macrophage cells, only IL-8 gene expression was significantly upregulated among proinflammatory cytokines while IFNγ, lysozyme and IgM gene expressions were downregulated by BaP. FasL and SOD expressions were not altered in head kidney cells but significantly upregulated in macrophage cells, indicating apoptosis and oxidative stress. These results indicate that exposure to BaP causes the downregulation of immune response by triggering the death of macrophage cells, the reduction of effectors like IgM and lysozyme, and the decrease of macrophage cell activity.

1. Introduction

Poly aromatic hydrocarbons (PAHs) are known to have adverse effects on marine organisms by suppressing immune responses and increasing susceptibility to pathogens (Catsiki et al., 2003; Zaghden et al., 2007). PAHs are known to change both specific and non-specific immunity in fish (Faisal and Huggett, 1993; White et al., 1994; Carlson et al., 2004a,b). According to Reynaud and Deschaux (2006) who reviewed the immunotoxicity of PAHs on the fish immune system, PAHs may affect lymphocyte proliferation, serum lysozyme level and activity, respiratory burst activity, and NK cell activity, inducing apoptosis of immune cells.

Immunotoxicity of PAHs in fish was also studied at the molecular level. For example, Nakayama et al. (2008) have reported that down-regulation of immunoglobulin light chain and up-regulation of interleukin (IL)-8 were detected by microarray analysis after exposure to heavy oil containing PAHs for 4 days in kidney of olive flounder. More recently it was reported that alkyl PAH retene increased production of Th2 related CD4 co-receptor transcripts including CD4, T cell receptor, tumor necrosis factor (TNF)α in spleen and increased B cell density in spleen and head kidney (Hogan et al., 2003). Song et al. (2012) have also reported that heavy oil pollution caused the downregulation of IgM gene expression in olive flounder. Despite these reports, the molecular mechanisms of PAH induced immunotoxicity still have not been studied enough in fish.

In mammals PAHs are known to induce immunotoxicity related gene expressions. It has been reported that short term exposure of human macrophages to PAHs caused upregulation of proinflammatory cytokines including IL-1β, IL-8, TNFα, and chemokines (Lecureur et al., 2005; N'Diaye et al., 2006; Podechard et al., 2008) while chronic exposure to PAHs leads to PAH related DNA adducts and P53-related apoptosis (Grevenynghe et al., 2004; Sparfel et al., 2006). Sparfel et al. (2010) demonstrated that exposure of human primary macrophages to BaP resulted in differentially expressed 96 and 1100 genes including chemokine receptor CXCR5, the G protein-coupled receptor 35 (GPR35), TNFα, IL-8 and IL-1β in microarray analysis followed by validation using RT-qPCR.

These altered gene expressions by PAHs are known to be induced by two major pathways i.e., AHR and NFAT pathways. Binding of PAH such as tetrachlorodibenzo-*p*-dioxin (TCDD) or benzo[a]pyrene (BaP) leads to the translocation of aryl hydrocarbon receptor (AHR) to the nucleus and the AHR dimerizes with AhR nuclear translocator (arnt). Arnt induces

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target gene expression by binding to specific xenobiotic response elements (XREs) in promoter regions (Petrulis and Perdew, 2002; Backlund and Ingelman-Sundberg, 2005). The existence of AhR has been identified in fish (Ferraris et al., 2005).

It was also demonstrated that PAHs activate a nuclear transcription factor of activated T cells (NFAT) by increasing intracellular Ca^{2+} concentration (Tannheimer et al., 1997; Crabtree and Olson, 2002; Le Ferrec et al., 2002). The resulting elevated levels of cytosolic Ca^{2+} activate NFAT (Crabtree and Olson, 2002), stimulating some cytokines highly sensitive to intracellular levels of Ca^{2+} (Latinis et al., 1997; Hogan et al., 2003). NFAT plays a key role in the activation of many early immune responses by increasing the transcription of multiple mediators in response to the intracellular Ca^{2+} level after interacting with AP-1 to co-regulate expression of interleukins, TNFα, IFNγ, GM-CSF, Fas ligand, CD25, and Cox-2 as well as activating T and B cells in humans and mammals (Macian, 2005).

In fish, several studies have also documented the role of AhR and NFAT in eliciting toxicological effects. However the exact mechanism of action still remains to be elucidated. Thus, in this study, we analyzed the immunotoxicity of BaP in flounder by assessing the expression of cytokines (IL-1β, TNFα, IL-6, IL-8, IFNγ, Mx), apoptosis (FasL, SOD) and other immune related (Lysozyme, IgM) genes using Q-PCR analysis. We have chosen the flounder because it lives in the ocean floor and is at the very top of the marine food chain. Immunotoxicity of BaP via AhR and a calcium dependent pathway was also assessed by analyzing the modification of gene expression induced by BaP using an AhR antagonist (ANF) and a calcium chelator (EGTA).

2. Materials and methods

2.1. Chemicals

Benzo[a]pyrene (BaP), dimethyl sulfoxide (DMSO) and α-naphthoflavone (ANF) were purchased from Sigma-Aldrich (USA). Ethylene glycol tetraacetic acid (EGTA) was purchased from USB Corporation (USA). BaP was prepared as stock solution in DMSO. The final concentration of solvent did not exceed 0.2% (v/v); controls received the same dose of solvent as treated counterparts.

2.2. Fish

Olive flounder (*Paralichthys olivaceus*; mass around 100 g) were kept at the Marine Biology Center for Research and Education at Gangnung-Wonju National University (Gangnung, Korea) in circular 200 L tanks supplied with seawater at an ambient temperature of approximately 15 ± 1.2 °C with 12/12 h illumination and fed with a commercial pellet diet (Suhyupfeed, Korea). Fish were fed twice daily (9:00 and 17:00 h).

2.3. In vivo analysis of gene expression

Eight olive flounder (100 g) in each group were anesthetized using MS222 (Woogene B&G, Korea) and intraperitoneally injected with 200 μL of BaP solution dissolved in DMSO at the final concentration of 10 mg/kg body mass. Control fish were injected with DMSO only. At two days and 7 days post-injection, fish was anesthetized and sacrificed by cutting the spinal cord. Head kidney was aseptically removed, immediately frozen in liquid nitrogen and total RNA extracted using Trizol reagent (Invitrogen, USA).

2.4. In vitro analysis of gene expression

Four healthy flounder (100 g) were anesthetized and killed by cutting the spinal cord. Head kidney was aseptically removed and gently passed through a sterile mesh (BD, USA) in a petri dish containing L-15 medium (Welgene, Korea) containing 10% FBS and 100 U penicillin and 100 μg streptomycin/mL (Gibco). The head kidney cells were washed by centrifugation at 800 *g* at 4 °C for 10 min and adjusted to 2×10^6 cells/mL. One mL of head kidney cell was added into a well of a 24-well plate and incubated with 0.01, 0.1, 1, and 10 μM of BaP dissolved in DMSO or only with DMSO for 6 h at 20 °C for the dose response. For the time course head kidney cells were incubated with 0.1 or 1 μM of BaP dissolved in DMSO or only with DMSO for 3, 6, 24, and 48 h at 20 °C. The doses (0.1 and 1 μM) for the time course experiment was chosen from our previous data where the highest level of CYP1A1 and Scinderin like gene expression was observed by 1 μM of BaP and a suboptimal dose was 0.1 μM of BaP in a dose response experiment (Hur and Hong, 2012).

To analyze the mechanism of BaP induced gene expression, head kidney cells were incubated in the presence of 1 μM BaP with or without 1 μM ANF (Sigma, USA), an AHR antagonist, or 4 μM EGTA (Sigma, USA), a calcium chelator, for 6 h. This condition was proved to block AHR dependent CYP1A1 and NFAT dependent Scinderin like gene expression in olive flounder (Hur and Hong, 2012).

Immune gene expression was also analyzed in head kidney macrophage cells after BaP exposure in vitro. To isolate macrophage cells, 5 mL of head kidney cells from 3 fish in L-15 medium containing 0.1% FBS were added into 3 of 25 cm^2 flasks at 2×10^7 cells/mL and incubated at 20 °C. Unattached cells were washed away twice using the same medium at 2 h and 24 h. The medium was replaced with fresh L-15 medium containing 10% FBS and macrophage cells were incubated with 0.1 μM of BaP or DMSO only for 12, 24, and 48 h at 20 °C. Total RNA was extracted from each flask using TRIsure (Bioline, UK).

2.5. Real-time PCR assay

Total RNA was reverse transcribed to cDNA as described by Laing et al. (1996). Briefly, 3 μg of RNA in 13 μL DEPC-water was incubated with 1 μl of oligo $(dT)_{12-18}$ (500 μg/mL, Promega) for 10 min at 70 °C. Then, 1 μL of Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Promega), 4 μL 5× first strand buffer (Promega), and 1 μL of 10 mM dinucleoside triphosphate (dNTP) mix (Promega) were added and the mixture incubated at 42 °C for 1.5 h. The reaction was terminated by heating to 94 °C for 15 min and 30 μL of DEPC water was added to make up the volume to 50 μL.

Gene expression was analyzed by quantitative real-time PCR (Q-PCR) using the ABI 7500 HT real-time thermocycler (Applied Biosystems, USA). Gene-specific primers were chosen using Primer Express software (Applied Biosystems) and listed in Table 1. At least one primer was designed to cross an intron not to amplify genomic DNA. The Q-PCR reaction was performed in a 20 μL reaction containing 10 μL of SYBR Green Real time PCR Master Mix (TaKaRa, Japan), 0.4 mM of each forward and reverse primers, 500 nM ROX reference dye II and 2 μL of cDNA in duplicate using the following protocol: 60 s at 95 °C; the template was amplified for 40 cycles of denaturation for 15 s at 95 °C, annealing and extension for 1 min at 60 °C. The linearity of the dissociation curve was analyzed using the ABI 7500 HT software and the mean cycle time of the linear part of the curve was represented cycle time (Ct). For the in vivo study all target gene expressions were analyzed with EF1α in the same plate and normalized to EF1α (GenBank accession no. AB017183) using the following equation: $\Delta Ct_{GENE} = Ct_{GENE} - Ct_{EF1\alpha}$. The fold change of target gene expression relative to DMSO control was calculated using the following equation: fold change $= 2^{\Delta\Delta CtGENE}$, $\Delta\Delta Ct_{GENE} = \Delta Ct_{GENE}$ of the control $- \Delta Ct_{GENE}$ of each sample. Values are mean fold change ± SD.

For in vitro analysis, common references containing an equal molar amount of purified PCR products was used for quantification throughout. Serially diluted references were used for absolute quantification analysis. After normalization to the expression level of EF1α, fold change was calculated by dividing the ratio to EF1α by DMSO treatment sample in each time point.