SUPPRESSION OF ARYL HYDROCARBON RECEPTOR AGONIST (PCB-126)-INDUCED CYP1A1 AND UDPGT EXPRESSION BY XENOESTROGENIC NONYLPHENOL IN PRIMARY CULTURE OF SALMON HEPATOCYTES

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Introduction

Polychlorinated biphenyls (PCBs) are ubiquitous contaminants in the environment and in biological systems including fish, wildlife, and human adipose tissue, breast milk, and serum. The biological effects and toxicological properties of coplanar PCB congeners are related to their chlorination pattern and structural similarity to polychlorinated dibenzodioxins (PCDDs)[1]. 3,3,4,4,5-pentachlorobiphenyl (PCB-126) is a coplanar PCB congener substituted in the non-*ortho*-position. PCBs belonging to the non-ortho-substituted group are the most toxic congeners with high affinity to the aryl hydrocarbon receptor (AhR)[1,2,3]. The AhR is a ligand dependent transcription factor that contains basic helix-loop-helix–PER-ARNT-SIM (BHLH-PAS) domains and its ligand-activated transcription are often regulated in cells to meet the need for cellular homeostasis. Upon ligand-binding, the AhR translocates to the cell nucleus, where it dimerizes with AhR-nuclear translocator (ARNT) and activate the transcription of a battery of target genes including phase-I cytochrome P450 members (CYP1A1 and CYP1A2) and phase-II members (uridine-diphosphate glucuronosyltransferase: UDPGT and glutathione S-transferase: GST) through the xenobiotic response elements (XREs)[4]. Thus, AhR controls a battery of genes involved in the biotransformation of xenobiotics.

Cytochrome P450 gene families play a central role in the oxidative metabolism or biotransformation of a wide range of foreign compounds (xenobiotics) including environmental pollutants, drugs, and endogenous compounds (steroids, bile acids, fatty acids and prostaglandins). Several factors (biotic and abiotic) are known to influence the hepatic P450 system in fish. The modulation of P450s by gender, reproductive status, steroid hormone and hormone mimics is well documented[5-7]. In fish species, both *in vivo* and *in vitro* studies have described that exposure to AhR agonists could be associated with several reproductive disturbances[8-12], thus demonstrating an interaction (cross-talk) between these two signaling systems. However, the mechanism of cross-talk signaling effect is not well studied in fish and is subject of continuos discussion and research.

Recently, we reported the transcriptional interference between the AhR and estrogen receptor (ER) gene signaling pathways and showed a bi-directional modulation of PCB-77 (AhR-agonist) induced CYP1A1 expression and nonylphenol (NP) (ER agonist) induced gene responses in primary culture of rainbow trout (*Oncorhynchus mykiss*) hepatocytes[13]. Suppression of biotransformation pathways by NP may alter xenobiotic metabolism leading to the production of toxic reactive molecules, altering pharmacokinetics and diminishing the clearance rate of individual chemicals from the organism. By virtue of its estrogenic activities, NP has the potential to interact with xenobiotic- and drug-metabolizing system, including members of the CYP1A and UDPGT enzymes. In toxicological studies, biochemical investigation of metabolizing enzyme induction and inhibition is of special interest. Therefore, the present study was designed to investigate the anti-AhR signaling effect of nonylphenol (NP), a documented estrogen mimic in fish *in vitro* and in vivo systems, using primary culture of Atlantic salmon hepatocytes. These effects were studied by quantifying changes in PCB-126 induced transcriptome for a suite of gene responses (AhR, ARNT, CYP1A1, UDPGT) belonging to the Ah-gene battery and enzyme activity levels. Only two (CYP1A1 and UDPGT) of the studied AhR gene battery will be shown in this extended abstract. We hypothesize that exposure of salmon hepatocytes to NP and PCB-126 will show a concentration-specific modulation of AhR and its gene-signaling pathway.

Materials and Methods

Salmon primary hapatocytes were isolated by two-step collagenase perfusion method as previously described by Mortensen et al.[13]. Juvenile Atlantic salmon (*Salmo salar*) of approximately 400-500 g were kept at the animal holding facilities at the Biology Department, NTNU. Fish were supplied with continuously running saltwater at a constant temperature of 10^{o}C. Prior to liver perfusion all glassware and instruments were autoclaved before use. Solutions were filtration sterilized by using 0.22 µm Millipore filter (Millipore AS, Oslo, Norway). A cell viability value of > 90% was a criteria for further use of the cells and cells were plated on 35 mm Primaria culture plates (Becton Dickinson Labware, USA) at the recommended density for monolayer cells of 5 x 10^{6} cells in 3 ml DMEM medium (without phenol red) containing 2.5% (v/v) FBS, 0.3 g/L glutamine, and 1% (v/v) Penicillin-Streptomycin-Neomycin Solution. The cells were cultured at 10^{o}C in a sterile incubator without additional O_2/CO_2 for 48 hr prior to chemical exposure. The cells were exposed (6 dishes for each exposure group) to 0.01% DMSO (control), NP (5 or 10 µM) and PCB126 (0.001 or 0.05 µM)

singly and also in combination. Media were replaced once after 24 h with fresh media containing the appropriate chemical and at the same concentrations. Cells (3 plates for each exposure group) for enzyme assays were harvested after 48 h exposure and stored in -80 C. Cells from the remaining 3 plates of each exposure were lysed in E.Z.N.A lysis buffer for total RNA isolation according manufacturers protocol (Omega Bio-Tek, Doraville, GA, USA)).

Total RNA was isolated and complementary DNA (cDNA) was generated from all samples using poly-T primers from iScript cDNA Synthesis Kit (BioRad). CYP1A1 and UDPGT gene expression profiles were performed using quantitative (real-time) PCR with 200 nM each of the following primers pairs in 5´-3´ directions; CYP1A1 (76 bp): GAGTTTGGGCAGGTGGTG (forward) TGGTGCGGTTTGGTAGGT (reverse) and UDPGT (113 bp): ATAAGGACCGTCCCATCGAG (forward), ATCCAGTTGAGGTCGTGAGC (reverse). Gene expression patterns were evaluated using Mx3000P REAL-TIME PCR SYSTEM (Stratagene, La Jolla, CA, USA). The real-time PCR program included an enzyme activation step at 95°C (10 min) and 40 cycles of 95°C (30 sec), 60ºC (30 sec) and 72°C (30 sec). We included controls lacking cDNA template to determine the specificity of target cDNA amplification. Cycle threshold (*Ct*) values obtained were converted into ng/µl total RNA using standard plots of *Ct*-values versus log ng/µl. The standard plots were generated for each target sequence using known amounts of plasmid containing the amplicon of interest. Data obtained for target cDNA amplification were averaged and expressed as percentage of the control value.

Results and Discussion

The effects of NP on PCB-126 induced AhR-dependent gene expressions were analyzed by real-time PCR with gene specific primers. Exposure to PCB-126 alone caused a concentration-dependent induction of CYP1A1 and UDPGT gene expression (Fig. 1A and B, respectively). Exposure of hepatocytes to 10 µM NP caused significant induction of CYP1A1 and UDPGT gene expression, compared to control or 5 µM NP (Figure 1A and B, respectively). The combined exposure of cells to 0.001 and 0.05 µM PCB-126 and NP concentrations caused significant reduction in PCB-126 induced CYP1A1 gene expression (moreso with 10 µM NP; Figure 1A). However, the exposure of cells to 10 µM NP in combination with 0.001 µM PCB-126 potentiated PCB-126 induced UDPGT expression compared with individual chemical exposures, while combined exposure with 5 µM NP did not alter the expression pattern of UDPGT gene (Figure 1B). On the contrary, 5 and 10 µM in combination with 0.05 µM PCB-126 resulted to significant decrease in PCB-126 induced UDPGT expression (Figure 1B).

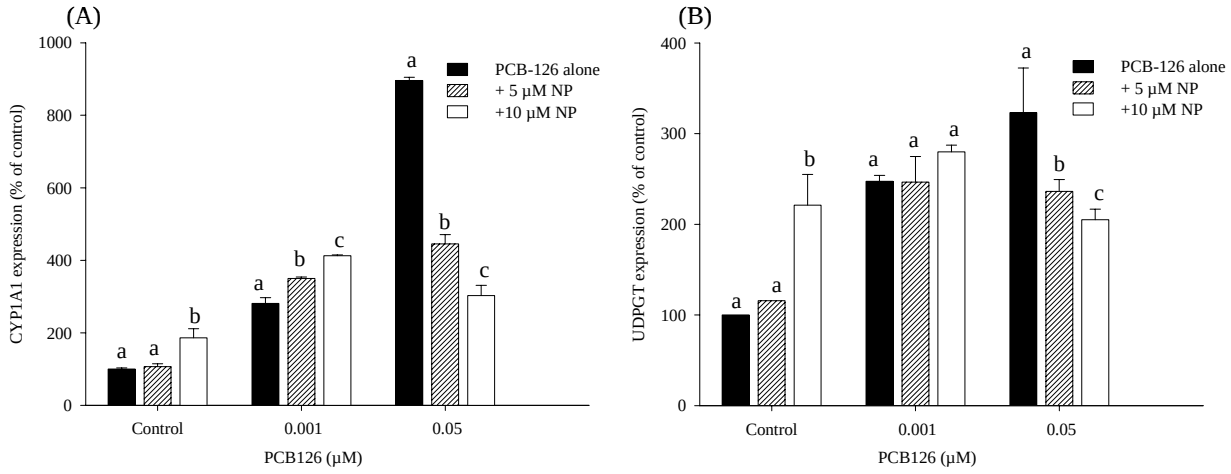

Figure 1. Modulation of CYP1A1 (A) and UDPGT (B) mRNA levels in primary culture of salmon hepatocytes treated for 48 h with PCB-126 at 0.001 and 0.05 µM, singly and also in combination with NP at 5 and 10 µM. Messenger RNA (mRNA) transcription levels were quantified using quantitative PCR with specific primer pairs. Data are given as mean (n=3) ± standard error of mean (SEM). Different letters denote statistical significant differences between individual PCB126 concentration and in combination with different NP concentrations, using ANOVA followed by Dunnett's test ($p<0.05$)

The expressions of CYP1A1 and UDPGT in fish have shown to be modulated by sex steroids, as a number of studies have demonstrated possible relationship between plasma E2 fluctuations and CYP1A-mediated 7-ethoxyresorufin O-deethylase (EROD) activity levels during reproductive cycle[5,6]. The decreasing EROD activity with increasing E2 levels was explained by inhibitory action of the steroid on CYP1A activity. However, the observations that E2 also causes variation on CYP proteins and mRNA expression suggest a possible transcriptional modulation. CYP enzymes belonging to the 1A1 and 1A2 subfamily are considered to be of significant toxicological importance since they bioactivate or form reactive metabolites from polycyclic aromatic hydrocarbons (PAHs), aromatic and heterocyclic amines, azobenzene derivatives and planar polyhalogenated biphenyls[4]. They also form products for phase-II reactions that involve the conjugation of metabolized xenobiotics to endogenous substances such as glucuronic acid through the UDPGT enzyme activity or glutathione (glutathione S-transferase), thereby rendering it more hydrophilic, facilitating excretion via bile or urine[14].

The effect of NP reported in the present study are in accordance with previous studies showing that other estrogenic compounds such as estradiol-17ß (E2) and ethynylestradiol (EE2) significantly suppressed hepatic CYP1A1 mRNA levels, EROD activity and CYP1A1 protein in fish *in vivo* and *in vitro* experiments[8,15]. We propose the following hypotheses in explaining the CYP1A1 and UDPGT down-regulation by NP; firstly, that NP can bind to these proteins[16], and through this binding may directly or indirectly inhibit the regulation of their transcription[13] most probably through competitive interaction. This proposal is supported by the observation in this study that the down-regulation of PCB-126 induced CYP1A1 and UDPGT expressions by NP paralleled the down-regulation of AhR and ARNT expressions, and secondly, that the inhibitory action of NP could be mediated, at least in part, through the hepatic estrogen receptor (ER) where the ER-NP complex can interfere with these genes directly or alternatively interacting with the AhR (see above), and indirectly regulate the expression of these gene through binding to the XRE[8]. The second proposal is also supported by our observation that the combined exposure of PCB-126 and NP concentrations caused different effects on ERα and ERß isoforms and these effects were dependent on PCB-126 and NP concentration. In addition, NP may control the recruitment of ER and possibly other co-activators, besides activating the detoxification pathway. This argument is supported by recent study showing that E2 can exert its effects by activating the AhR/ARNT heterodimer, which is able to interact with the unliganded ER, leading to induction of estrogenic pathway[17]. The modulation of CYP1A1 system by NP, E2, and BNF was recently shown to parallel the AhR repressor (AhRR) gene expression[18]. In this regard, the associations between basic-helix-loop-helix-PAS (Per-AhR/Arnt-Sim homology sequence) of transcription factor in forming heterodimers, AhR/ARNT or AhRR/ARNT, and subsequent binding to the XRE sequences in the promoter regions of the target genes to regulate their expression need to be studied in more detail using other estrogenic and organic contaminants.

Since the P450 system metabolizes both endogenous and exogenous substances, interactions between foreign chemicals and physiological processes are possible. In this respect, the relationship between induction of biotransformation enzymes in fish liver and altered steroid metabolism *in vitro* and *in vivo* deserves more attention. In several investigations a relationship between elevated P450 activities and disturbed physiological endocrine functions, essential for successful reproduction has been found. Although no direct links between the induction of P450 and impaired reproductive functions have yet been established, it is nevertheless important that the mechanism by which potential P450 inducers may affect sexual development and fertility is elucidated. Therefore, the modulation of phase-I and II gene expressions by NP reported in this study may alter pharmacokinetics and diminish the clearance rate of xenobiotics from the organism. Hence, the balance between metabolizing enzyme induction and inhibition is of special interest in biochemical investigation of toxicological studies.

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