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Effects of 4-nonylphenol on hepatic gene expression of peroxisome proliferator-activated receptors and cytochrome P450 isoforms (CYP1A1 and CYP3A4) in juvenile sole (*Solea solea*)

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HIGHLIGHTS

- Waterborne 4-NP exposure increased hepatic PPARα and RXRα mRNA levels in *S. solea*.
- The PPARα but not the RXRα mRNA expression was up-regulated by E2.
- PPARβ gene expression was not modulated neither by E2 nor by 4-NP.
- 4NP-induced PPARα mRNA levels coincide with lower expression of CYP1A1 and CYP3A4.

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ABSTRACT

The objective of the present study was to investigate the modulatory effects of the xenoestrogen 4-nonylphenol (4-NP) on hepatic peroxisome proliferator-activated receptor (PPAR) α and β gene expression patterns in relation to the detoxification pathways mediated by cytochrome P450 isoforms (CYP1A1 and CYP3A4). Waterborne 4-NP-induced effects were compared with those of 10^{-8} M 17β-estradiol (E2) by using *in vivo* dose–response experiments carried out with juvenile sole (*Solea solea*). Compared to the controls, significantly higher levels of PPARα mRNAs were found in fish treated with E2 or 4-NP (10^{-6} M) 3 d after exposure; the highest dose of 4-NP also caused up-regulation of retinoid X receptor α (RXRα) transcript levels. On the contrary, PPARβ gene expression was not modulated by E2 or 4-NP. Our data show that 4-NP-induced PPARα mRNA levels coincide with suppression of CYP1A1 and CYP3A4 expression similarly to E2. The results from these *in vivo* studies suggest the presence of cross-talk between nuclear receptor-mediated signaling pathways and PPARα that may result in modulation of CYP450 isoforms expression following 4-NP treatment in sole liver.

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1. Introduction

4-Nonylphenol (4-NP) is a well known estrogenic pollutant that derives from microbial degradation of alkylphenolpolyethoxylates (APnEOs) resulting in persistent accumulation in the aquatic ecosystem components (Soverchia et al., 2005; Lacorte et al., 2006). The occurrence data of 4-NP ranges from 0.64 to 180 μg L^{-1} for surface waters (Naylor et al., 1992; Blackburn and Waldock, 1995), and from 180 to 330 μg L^{-1} for sewage effluents and downstream wastewater treatment plants (Hill and Janz, 2003). Ying et al. (2003) estimated that 4-NP has a half-life of 5 d for seawater and 5.8 d for marine sediment under oxic conditions. In this regard, laboratory and field experiments have demonstrated that 4-NP can bio-accumulate in fish showing a bioconcentration factor (BCF) that varies from 3 to 1400 (Ekelund et al., 1990; Huang et al., 2007). Recent studies on metabolic fate of these alkylphenols in living organisms suggest that 4-NP was glucuronidated by liver microsomes in mammals (Doerge et al., 2002). It has been also reported that 4-NP was extensively metabolized to the glucuronide conjugate in rainbow trout (Thibaut et al., 1998a,b). In addition, the kinetics of excretion of alkylphenol-glucuronides into the bile is closely related to the length of alkyl chain (Daidoji et al., 2003). Anyhow, liver proves the major organ of accumulation, biotransformation and degradation of environmental pollutants (Soverchia et al., 2005; Matos et al., 2007).

Many studies have investigated the toxicity of 4-NP, mainly by demonstrating estrogen-like effects, on a wide variety of animals including fish (Palermo et al., 2008, 2012a; Pomatto et al., 2011). It has been well established for teleost fish that endocrine-disrupting chemicals (EDCs), like 4-NP, have toxicological effects that can affect multiple pathways (Isidori et al., 2010; Palermo et al., 2012b), among which aryl hydrocarbon receptor (AhR) and peroxisome proliferator-activated receptor (PPAR) – mediated pathways seem to be some of the most relevant (Fang et al., 2012). In fact, the AhR pathway mainly regulates the transcription of genes that

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encode several metabolic enzymes such as the hepatic cytochrome P450 (CYP450) enzymes which are the major responsible for phase-I xenobiotic-metabolism (Cheng and Klaassen, 2008). In addition, among hepatic CYP450 enzymes, members of CYP1A and CYP3A subfamilies are implicated in the oxidative metabolism of many endogenous compounds, including steroids (Nebert et al., 1991). Low levels of CYP1A subfamily gene expression have been found in fish; moreover, these expression patterns can be increased by AhR agonists (Hasselberg et al., 2004). On the contrary, the CYP3A isoforms are the major CYP450 subfamily products synthesized by liver and gastrointestinal tract in fish (Lee et al., 2001; Hegelund and Celander, 2003). Because of their key role in the metabolism of xenobiotics, modulation of hepatic CYPs expression markedly affects potential toxicity mechanisms of EDCs (Williams et al., 1998a).

PPARs are members of the nuclear hormone receptor superfamily and are involved in controlling key cellular functions, including lipid metabolism, inflammation, and cell differentiation (Latruffe et al., 2001; Tan et al., 2001). The PPAR family includes at least three isoforms identified as PPARα, PPARβ/δ, and PPARγ in mammals (Berger and Moller, 2002). PPARα is expressed primarily in tissues with a high degree of fatty acid oxidation, including liver, heart, skeletal muscle, brain, and intestine, but slightly or not at all in other tissues. In contrast, PPARγ is largely expressed in adipose tissue, whereas PPARβ (also called PPARδ) is ubiquitously distributed (Berger and Moller, 2002). Several recent studies have shown the primary involvement of PPAR isoforms α and β in the regulation of important biological processes, including lipidic and glucidic metabolism (Wu et al., 2001; Wang et al., 2008). Interestingly, PPARs heterodimerize with the retinoid X receptor (RXR), to fully activate gene transcription (Mangelsdorf and Evans, 1995; Chandra et al., 2008). Although differences in genomic structure, fish PPAR isoforms are homologous to their mammalian counterparts (Leaver et al., 2005, 2007). In addition, it has been demonstrated that fish PPARα and β show an activation profile in response to a large set of ligands similar to that of the mammalian PPARs (Leaver et al., 2005). The ligand-binding domain of PPARs is unusually large, and consequently, these receptors are relatively promiscuous, being activated by a number of natural and synthetic ligands of different chemical structure (O'Sullivan, 2007). In this context, PPARs were found to interact with tributyltin oxide (TBTO) (Colliar et al., 2011) and phthalate mono-esters (Bility et al., 2004) suggesting PPAR-dependent effects in response to pollutants. Several studies have suggested that peroxisome proliferation could be used as biomarker of exposure to environmental contaminants (Cajaraville et al., 2003; Desvergne et al., 2009). Therefore fish species that can be exposed to several contaminants via waters may be particularly predisposed to PPAR disruption. In the present work, we have used bottom feeding fish (i.e. *Solea solea*) as study models because they are at higher risk of exposure to chemical that accumulate in sediments through direct physical contact with the sediment (Baker et al., 2009) or interstitial water (Hallare et al., 2011).

The aim of the present study was to investigate the modulatory effects of environmentally relevant concentrations of 4-NP on hepatic PPAR (isoforms) α and β gene expression patterns in relation to RXRα, CYP3A4 and CYP1A1 mRNA levels in juvenile sole (*S. solea*).

2. Materials and methods

2.1. Fish and treatment

Juvenile sole (*S. solea*) (mean weight and length: 20.3 ± 4.8 g and 13.1 ± 1.2 cm) were purchased from an Italian fish farm, Orbetello Pesca Lagunare s.r.l. (Orbetello, GR, Italy) and kept in 100-L glass aquaria filled with 80 L of seawater (temperature 15–16 °C, dissolved oxygen 6.7–8.0 mg L^{-1}, salinity 34–36 g L^{-1}, pH 7.0–8.0, natural photoperiod) at Centro Universitario di Ricerca per lo Sviluppo e la Gestione delle Risorse dell'Ambiente Marino e Costiero (UNICRAM), University of Camerino in San Benedetto del Tronto (AP, Italy). Fish were not fed during the experimental procedure. The experiment was performed under static conditions, and the experimental water was not changed during the 3-d exposure period on the basis of Palermo et al. (2012a) and Meucci and Arukwe (2006). Fish were divided into four groups (each consisting of seven individuals): one served as negative control, one as positive control (E2 10^{-8} M; Sigma, St. Louis, MO, USA) and the others were exposed to different concentrations (10^{-8} and 10^{-6} M) of 4-NP (CAS number: 104-40-5, Sigma–Aldrich Chemicals, St. Louis, MO, USA). Chemicals were dissolved in ethanol, while the control group received carrier solvent alone. 4-NP concentrations were chosen for this study because they are environmentally relevant (Petrovic and Barcelo, 2000; Hill and Janz, 2003); in addition, these doses of 4-NP, including that of E2, caused estrogenic effects (i.e. vitellogenin synthesis and estrogen receptor mRNA up-regulation) in juvenile sole (Palermo et al., 2009, 2012a). Three replicates were set up for each experimental group. For the sampling, each animal was anaesthetized with 3-aminobenzoic acid ethyl ester (Sigma; 100 mg L^{-1}) within 5 min after capture and liver tissues were harvested, immediately frozen in liquid nitrogen and stored at −80 °C for molecular biology analyses. Animal manipulation was performed according to the recommendations of the University Ethical Committee, to the European Union directive (2010/63/EU) and under the supervision of the authorized investigators.

2.2. RNA extraction and real time PCR

Total RNA was extracted from 100 mg of liver samples using Trizol Reagent (Invitrogen, Milan, Italy) according to the manufacturer's instructions. DNase digestion (2U, 30 min, 37 °C; Ambion, Austin, TX) was performed to eliminate genomic DNA contamination. RNA concentration and purity were assessed spectrophotometrically at absorbance of 260/280 nm, and the integrity was confirmed by electrophoresis through 1% agarose gels stained with ethidium bromide. The complementary DNA (cDNA) was synthesized from 2 μg of total RNA in 20 μL of total volume reaction using random hexamers (50 ng μL^{-1}) and 200 U of SuperScript™ III RT according to manufacturer's instruction (Invitrogen Life Technologies, Milan, Italy). SYBR green-based real-time PCR (q-PCR) was used to evaluate expression profiles of PPARα, PPARβ, RXRα, CYP1A1 and CYP3A4 (Ribecco et al., 2011) target genes and the acidic ribosomal protein (ARP) (Palermo et al., 2011, 2012b; Piccinetti et al., 2011) as an internal standard. The expression of individual gene targets was analyzed using the Mx3000P Real-time PCR system (Stratagene, La Jolla, CA, USA). Thermo-cycling for all reactions was for 15 min 95 °C, followed by 40 cycles of 10 s at 95 °C and 40 s at 58 °C. Fluorescence was monitored at the end of every cycle. Results were calculated using the relative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) and means of mRNA levels are expressed with respect to control fish ± standard error of the mean (SEM).

3. Statistical analysis

Data were first examined for their fit to a normal distribution and homogeneity of variance using Kolmogorov–Smirnov and Levene median tests. A one-way analysis of variance (ANOVA) was used to compare results between groups, followed by the Tukey post hoc test. Differences between means were considered

significant when $P < 0.05$. All statistical analyses were performed using R (R Development Core Team, 2008).

4. Results

4.1. Hepatic PPARα, PPARβ, RXRα mRNA levels

Compared to the controls, significantly ($P < 0.05$) higher levels of PPARα mRNAs were found in fish treated with E2 or 4-NP (10^{-6} M) (Fig. 1A); the highest dose (10^{-6} M) of 4-NP also increased hepatic RXRα mRNA transcripts with respect to control fish (Fig. 2). On the contrary, PPARβ gene expression was not modulated by E2 or 4-NP (Fig. 1B).

4.2. Hepatic CYP1A1 and CYP3A4 mRNA levels

Changes in *S. solea* hepatic mRNA levels following exposure to 4-NP were investigated for two members of the P450 family, CYP1A1 and CYP3A4 respectively. Hepatic CYP1A1 mRNA levels were decreased by E2 and the highest dose of 4-NP (10^{-6} M) whereas the lower dose of 4-NP (10^{-8} M) did not affect the CYP1A1 expression profile (Fig. 3A). CYP3A4 mRNA levels were decreased by E2 (10^{-8} M) and by 4-NP at both doses (10^{-8} and 10^{-6} M) (Fig. 3B).

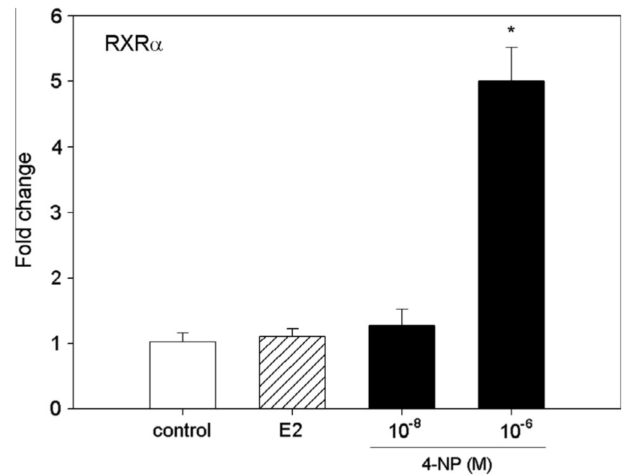

Fig. 2. Expression of hepatic retinoid X receptor α (RXRα) in juvenile sole (*Solea solea*) exposed to various concentrations (10^{-8} or 10^{-6} M) of 4-nonylphenol (4-NP) or to 10^{-8} M 17β-estradiol (E2) for 3-d. Values were normalized against acidic ribosomal protein (ARP) as a housekeeping gene and represent relative mean mRNA expression value ± S.E.M ($n = 7$) to that of control. Statistically significant differences when compared with control, $^{*}P < 0.05$ level.

5. Discussion

The present study first examined the effects of 4-NP waterborne exposure on PPARα, PPARβ and RXRα transcript abundance in a

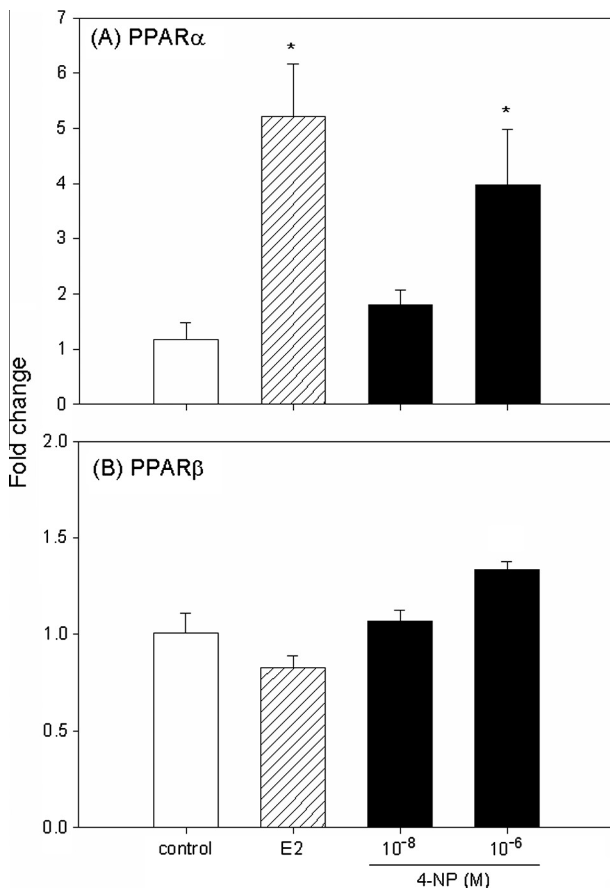

Fig. 1. Expression of hepatic peroxisome proliferator-activated receptor (PPAR) α (A) and β (B) in juvenile sole (*Solea solea*) exposed to various concentrations (10^{-8} or 10^{-6} M) of 4-nonylphenol (4-NP) or to 10^{-8} M 17β-estradiol (E2) for 3-d. Values were normalized against acidic ribosomal protein (ARP) as a housekeeping gene and represent relative mean mRNA expression value ± S.E.M ($n = 7$) to that of control. Statistically significant differences when compared with control, $^{*}P < 0.05$ level.

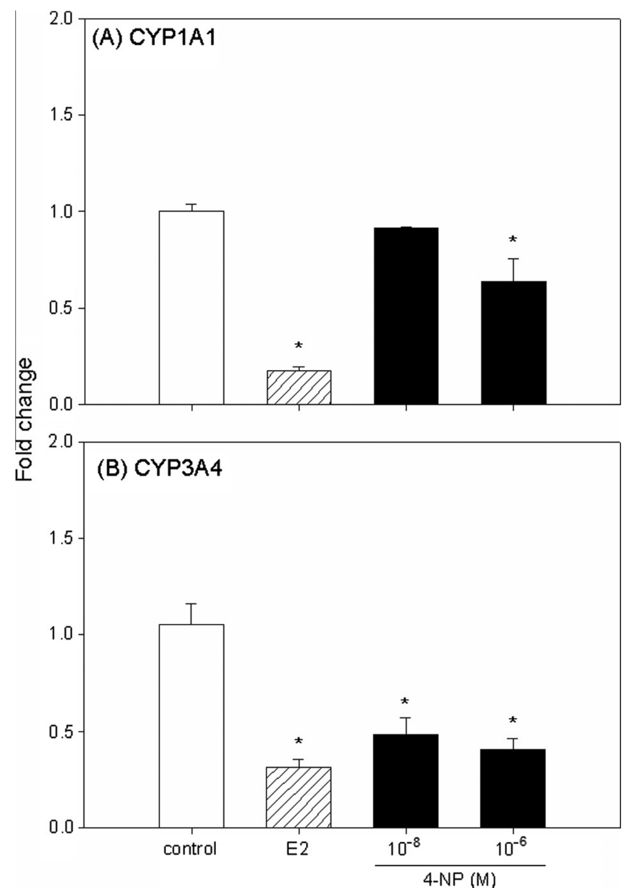

Fig. 3. Expression of hepatic cytochromes P450 1A1 (CYP1A1) (A) and P450 3A4 (CYP3A4) (B) in juvenile sole (*Solea solea*) exposed to various concentrations (10^{-8} or 10^{-6} M) of 4-nonylphenol (4-NP) or to 10^{-8} M 17β-estradiol (E2) for 3-d. Values were normalized against acidic ribosomal protein (ARP) as a housekeeping gene and represent relative mean mRNA expression value ± S.E.M ($n = 7$) to that of control. Statistically significant differences when compared with control, $^{*}P < 0.05$ level.

marine fish species (i.e. *S. solea*) which is considered a sentinel organism for monitoring environmental pollution (Ribecco et al., 2011; Palermo et al., 2012a,b). The obtained results indicate 4-NP potential in modulating hepatic PPAR gene expression. In fact, our data show that both E2 and 4-NP (10^{-6} M) increased hepatic PPARα mRNA levels; on the contrary, mRNA expression of PPARβ was not significantly affected by any of these treatments. Several studies have demonstrated the estrogen (i.e. E2) – mediated effect on PPARs expression and activity (Djouadi et al., 1998; Ma et al., 1998; Keller et al., 2000; Ibabe et al., 2005), but little is known about the involvement of 4-NP in the modulation of genes encoding for PPARα and PPARβ. It was found that E2 is a potent transcriptional activator of the PPARs isoforms in skeletal muscle in Sprague-Dawley rats (Campbell et al., 2003). However, E2 failed to induce PPARα expression in zebrafish hepatocyte cultures confirming that estrogens show tissue and concentration-dependent effects (Ibabe et al., 2005). It has also been suggested that the estrogen-dependent production of PPAR activating metabolites could result in peroxisome proliferation (Ma et al., 1998). Moreover, ERs are able to activate PPARα target genes through interacting with peroxisome proliferator responsive elements (PPREs) (Djouadi et al., 1998) which consist of a direct repeat of the consensus sequence AGGTCA spaced by 1 or 2 bp [direct repeat 1 or 2 (DR1 or DR2)] (Desvergne and Wahli, 1999) (Supplementary material (SM), Fig. SM-1). Interestingly, the signal cross-talk between ER and PPAR has been suggested to be bidirectional (Keller et al., 1995; Wang and Kilgore, 2002; Jeong and Yoon, 2007). An alternative DR1 element has been found in the human PPARα promoter suggesting that PPARα itself modulates its own expression (Pineda Torra et al., 2002). In this regard, induction of PPARα gene expression by PPARα agonists has been demonstrated in different rodent cell lines (Sterchele et al., 1996; Zhou et al., 1998; Valmaseda et al., 1999). The induction of PPARα mRNA levels by 4-NP is in line with a previous report that found strong activation of PPARα gene expression in zebrafish exposed to 10^{-7} M of either 4-NP or E2 (Baker et al., 2009).

Nuclear receptor PPARs need to heterodimerize with the X receptor for 9-cis-retinoic acid (RXR) for binding to PPREs and for modulating transcription of target genes (Marcus et al., 1993). The results of the present study revealed an up-regulation of RXRα gene expression following exposure to the highest dose of 4-NP. Interestingly, the observed increase in RXRα transcript abundance is consistent with concomitant 4-NP-induced increases in PPARα mRNA levels. Recent reports have demonstrated that RXRα regulates the response to contaminated sediment exposure (Janosek et al., 2006) and its up-regulation in expression was found to be associated with increases of PPARα and PPARβ mRNA levels (Ribecco et al., 2011). In this regard, male turbot sampled from polluted areas of Southern California coastal regions exhibited higher increase in RXR expression than control fish (Baker et al., 2009). RXRα has been shown to be up-regulated by both E2 and 4-NP in zebrafish (Baker et al., 2009). The lack of E2-induced RXRα mRNA levels could be ascribed to the different chemicals concentrations and duration of exposure adopted in the our study with respect to the work of Baker et al. (2009). Among EDCs, bisphenol A (BPA), a well-known xenoestrogen, was found to induce up-regulation of RXRα mRNA expression in murine embryos leading to disruption of xenobiotic metabolism and retinoid signaling (Nishizawa et al., 2005). Interestingly, the authors suggest that BPA may exert this effect by altering the estrogen-dependent signal transducing system (i.e. working as an antiestrogen compound). In this regard, it has been demonstrated that BPA functions in ER subtype specific way, thus showing dual actions as an estrogen agonist and antagonist (Hiroi et al., 1999). E2 has also no effect on the expression of RXRs in human endometrial stromal cells *in vitro* (Kumarendran et al., 1994). Taken together, these findings highlight that the modes of actions of xenoestrogens are more complex than expected further suggesting that 4-NP is able to act through an alternative pathway to that employed in the estrogen-activated mechanism of action.

Together with PPAR isoform expression changes, we have investigated the effects of 4-NP waterborne exposure on the detoxification pathways by evaluating the mRNA levels of CYP1A1 and CYP3A4. Treatment with 4-NP (10^{-6} M) or E2 down-regulated both CYP1A1 and CYP3A4 mRNA expression. Similarly to our results, Williams et al. (1998b) found low levels of CYP1A mRNA in atlantic tomcod (*Migrogadus tomcod*) females captured at river sampling sites in which high concentrations of E2 were measured. An inhibiting effect of E2 on CYP1A mRNA was also found in studies on cultured trout hepatocytes (Navas and Segner, 2000). *In vivo* studies demonstrated that 3-d treatment with 4-NP resulted in a decrease in CYP1A1 mRNA expression in juvenile salmon (Meucci and Arukwe, 2006). Both 4-NP and E2, via *i.p.* injection, lowered CYP1A1 basal levels in the marine fish *Gobius niger* (Maradonna et al., 2004). In addition, E2 exposure resulted in decreased CYP3A at both mRNA and protein levels in juvenile rainbow trout (Buhler et al., 2000). Interestingly, Meucci and Arukwe (2006) speculated that CYP3A mRNA levels may be modulated through dose-dependent interaction of 4-NP with pregnane X-receptor (PXR) and/or other receptor–coactivators/repressors. In mammals, CYP3A protein expression was found to be decreased after dietary 4-NP exposure at the doses of 25 and 2000 ppm (Laurenzana et al., 2002). However, comprehensive mechanisms responsible for the down-regulation of fish CYP3A isoforms mRNA levels following exposure to 4-NP have not yet been proposed.

Nuclear receptors involved in the regulation of CYP450 isoforms include PPARs, aryl hydrocarbon receptor (AhR), constitutive androstane receptor (CAR) and PXR (Baldwin et al., 2005). PPARα regulates CYP4A gene transcription in mouse that are treated with peroxisome proliferators (Johnson et al., 1996). In juvenile Atlantic salmon, a potential xenoestrogens-mediated regulation of hepatic CYP1A expression via AhR has been suggested by Meucci and Arukwe (2006). Also in zebrafish, the regulation of CYP3A expression seems to be mediated by the AhR-signaling pathway (Prasch et al., 2003; Tseng et al., 2005). It was suggested that the ER-E2 complex can affect CYP1A1 gene transcription either directly or indirectly by interacting with the AhR pathway (Navas and Segner, 2000). In fish, CYP1A expression was found to be induced by environmental contaminants through activation of AhR (Timme-Laragy et al., 2007; Jonsson et al., 2007a,b). Particularly for xenobiotics such as 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs), which bind to the AhR, antiestrogenic activities have been observed in both mammals (Navas and Segner, 1998) and teleosts (Anderson et al., 1996). Several evidences indicate that the AhR-mediated antiestrogenicity involves interactions with estrogen-dependent genes, including down-regulation of ER expression (Navas and Segner, 2000). On the other hand, ERs have been found to induce ubiquitination processes of aryl hydrocarbon receptor nuclear translocator (ARNT) that is required for AhR signaling pathways (Lim et al., 2011). For teleost fish, down-regulation of CYP1A1 by estrogens and xenoestrogens seems also to be mediated through the activation of the AhR repressor (Maradonna et al., 2004). On the basis of the observed results, we suggest that the inhibitory effect of 4-NP on CYP450 isoforms may be related, at least in part, to the stimulatory effects of short-term 4-NP exposure on PPARα mRNA expression. In mammals, Shaban et al. (2004) have demonstrated that treatment with PPARα ligands (e.g. clofibric acid) results in down-regulation of AhR protein expression and CYP1A1/A2 protein and mRNA levels. These findings are probably related to the competition between PPARα and AhR for a common pool of co-activators such as SRC-1 and p300 which have been found

to be involved in co-activation of PPARα (Zhu et al., 1996; Dowell et al., 1997) or AhR (Kobayashi et al., 1997; Ke et al., 2001).

5.1. Conclusion

In conclusion the present study demonstrates that 3-d exposure of juvenile sole to environmentally relevant concentration of 4-NP clearly modulates PPARα and RXRα expression in sole liver at transcriptional level. Another interesting finding of our work is that 4-NP-induced PPARα mRNA levels coincide with down-regulation of both CYP1A1 and CYP3A4 gene expression values. Collectively, our results suggest the presence of cross-talk between nuclear receptor (e.g. ER, AhR)-mediated signaling pathways and PPARα that may result in modulation of CYP450 isoforms expression. However the molecular mechanism by which 4-NP induces PPARα expression remains to be elucidated.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chemosphere.2013.06.058.

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