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Fast Genomic Biomarker Responses of Retene and Pyrene in Liver of Juvenile Rainbow Trout, *Oncorhynchus mykiss*

Kati Räsänen · Tiina Arsiola · Aimo Oikari

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Abstract We studied the transcriptive effects of two PAHs, retene (RET) and pyrene (PYR), in three equimolar sublethal concentrations (0.9–10 μg/L) in the liver of juvenile rainbow trout, *Oncorhynchus mykiss*. After 24 h of in vivo exposure, expressions of selected genes (CYP1A, Hsp30, Hsp70, Grp78, Sep15, GP1) were analyzed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). These PAHs changed the studied gene transcriptions differently, but not significantly, except for CYP1A, which was induced only by RET. RET induced CYP1A gene expression even at low, environmentally realistic concentrations in the liver of juvenile rainbow trout.

Keywords CYP1A · Environmental chemicals · Toxicity · Transcription · *Oncorhynchus mykiss*

Aquatic environments act as sinks of numerous chemicals, many of which are toxic to aquatic species, including ubiquitous polycyclic aromatic hydrocarbons (PAHs). While PAHs are taken up by fishes, some of them can disturb development, immune system, reproduction, growth and survival, and may also cause blue sac disease (Gravato and Santos 2002; Vehniäinen et al. 2003; Häkkinen et al. 2004; Colavecchia et al. 2006; Reynauda and Deschaux 2006). Fish have several mechanisms to protect themselves from exposure to PAHs (Tuvikene 1995).

Retene (7-isopropyl-1-methyl-phenanthrene; RET) is produced from resin acids by anaerobic processes (Tavendale et al. 1997). It can also originate from algal and bacterial precursors (Wen et al. 2000). In sediments of pristine lakes, its concentration ranges from nanograms to micrograms per g dry weight, but can be higher by many orders of magnitude in pulpmill sludges and sediments downstream of wastewater discharges (Leppänen and Oikari 1999, 2001; Leppänen et al. 2000; Meriläinen et al. 2006). Retene is very hydrophobic (log K_{ow} 6.4; Basu et al. 2001), but it can be dissolved in water (Meriläinen et al. 2006). While retene is a good model compound of alkyl-PAHs, pyrene (benzo-phenanthrene; PYR) is usually considered to be a representative of many other unsubstituted PAHs. Pyrene occurs in the environment as a byproduct from the incomplete burning of wood treated with creosote and of a multitude of oil-based products, including gasoline (Burgess et al. 2003; Nahrgang et al. 2009). Pyrene is also relatively hydrophobic (log K_{ow} 4.92; Di Toro et al. 2000), with water solubility about 135 μg/L (Mackay and Shiu 1977). Thus, fish may be exposed to aquaeous retene originated from contaminated sediment (Leppänen and Oikari 1999, Oikari et al. 2002) and aqueous pyrene originated from several sources (Nahrgang et al. 2009).

The aim of this study was to assess the very short-term transcriptomic effects (24-h exposure) of RET and PYR in the liver of juvenile rainbow trout (*Oncorhynchus mykiss*), a cold-water species commonly used as a representative model in various research areas. The liver is known to have a wide variety of physiological functions, and was chosen as the target organ. Juvenile six-month-old fish were used to minimize the effects of early ontogenic development, as

K. Räsänen (✉) · A. Oikari
Department of Biological and Environmental Sciences, Section of Environmental Science and Technology, University of Jyväskylä, Survontie 9, 40014 Jyväskylä, Finland
e-mail: kati.m.rasanen@jyu.fi

T. Arsiola
Department of Biosciences, University of Eastern Finland, Yliopistonranta 1, 70211 Kuopio, Finland

well as those of later sexual maturation, both apparently being life stage with many functional changes. The hypothesis was that RET and PYR affect expressions of genes and functional groups in a dose-response manner in hepatic tissue. Six genes were selected for studying transcriptomic effects by real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) analyses. Cytochrome P450 1A (CYP1A) was selected as an exposure biomarker of PAHs (Whyte et al. 2000). Three genes in the family of heat shock proteins (Hsp), i.e., 30 kDa heat shock protein (Hsp30), 70 kDa heat shock protein 1 (Hsp70), and 78 kDa glucose-regulated protein precursor (Grp78), were chosen to serve as indicators of a stressed condition in liver tissue (Iwama et al. 2004). Two genes, coding for 15 kDa selenoprotein (Sep15) and glutathione peroxidase 1 (GP1), were chosen as parameters of oxidative stress (Boelsterli 2007).

Materials and Methods

Retene (7-isopropyl-1-methylphenanthrene) was obtained from ICN Biomedicals Inc (No. 34943, purity 98 %, USA) and pyrene (benzo-phenanthrene) from Aldrich (No. 18.551-5, purity 98 %, USA).

Juvenile six-month-old rainbow trout were obtained from Hanka-Taimen Ltd. (Venekoski, Finland). The average length of the fish was 9.2 cm (±SD 2.9), and the weight was 9.5 g (±SD 1.0). Animals were transferred into a 500 L all-steel tank and acclimatized in flow-through water (1–2 L/min, oxygen concentration over 8 mg/L, pH 7.6 (±SD 0.1), at temperature 12.0°C (SD 0.1), with a photoperiod of 16 h light:8 h dark for 2 weeks before the initiation of 24-h exposure. Fish were fed every day with pelletized fish food (Royal Plus, 3.5 mm, Raisio, Finland) ad libitum with 0.25 % of fish biomass. Feeding was stopped 4 days before the start of exposures. The experiment was conducted with the licence authorized by Finnish Animal Experiment Board, and it complied with legislation of Finland.

For PAH exposures, randomly separated fish were transferred into 50 L all-glass aquaria (water volume 45 L), three animals in each and as three replicates for one concentration. Tanks were covered with black plastic from each side to protect fish from disturbances. Water oxygen concentration was over 8 mg/L, and temperature 12.3°C (SD ± 0.1). There were three different nominal concentrations of PAHs in water for RET, 1.0 (low), 3.2 (medium) and 10.0 (high) μg/L, being equimolar to PYR, 0.9 (low), 2.9 (medium) and 8.7 (high) μg/L, respectively. The sublethal concentrations were chosen based on the earlier studies where, compared to acute lethality, the degree of sublethality for PYR (25 μg/L) was less than 5 % (Krasnov et al. 2005), whereas for RET no similar comparison can be made as even saturated solution is acutely non-lethal. Stocks of PAHs (1 mg/mL) were dissolved in dimethylsulfoxide (DMSO). Accordingly, a concentration of 0.01 % DMSO was used in control treatments. Water to biomass ratio was approximately 2 L/g fish/d.

In the aquarium room, the treatments received routine visible light (TLD 36 W/950 daylight, Philips, Holland) with the period of 16 h each day (16:8 h L:D cycle) with light intensity quantified with a lux-radiometer (HD 9221, Electronor, Finland). Also, UV-radiation (less than 400 nm) was quantified with a UV-radiometer (RM 21, UV-elektronik GmbH, Dr. Gröbel, Germany). The average light intensity was 699 lux (SD ± 55), and no UV-radiation was detected.

Rainbow trout were exposed to PAHs for 1 day, whereafter fish were netted for sampling, and stunned with a blow to the head. Fish were quickly decapitated, the livers separated and blotted dry from blood, and snap-frozen in liquid nitrogen. The average fish weight and length were 8.6 g (±SD 2.7) and 9.3 cm (±SD 1.1), respectively.

To determine PAH concentrations in water during the experiments, water samples were collected from aquaria of the medium concentrations of RET and PYR, as well as from control aquaria, after 2 and 24 h from the beginning of exposures. Samples of 200 mL were extracted immediately three times with 50 mL hexane (Rathburn Chemicals Ltd, Scotland), using d10-anthracene (Isotec, Ohio, USA) as an internal standard (9.5 μg per sample). Hexane was evaporated with rotavapor (VV 2000, Heidolph, Germany) to 10 mL, and the extracts were stored at −20°C until analysed. Following this, hexane was evaporated with nitrogen gas, and each sample dissolved in 25 μL of hexane. Measurement was with a gas chromatograph-mass spectrophotometer (Hewlett Packard 6890, MS detector Hewlett Packard 5973, Rämänen et al. 2010).

Total RNA was extracted from fish livers with TRI-reagent (Sigma-Aldrich, MO, USA), 1 mL/50–100 mg, according to manufacturer's instructions. Livers were homogenized manually on ice in tubes with a plastic stave. The homogenates were centrifuged for ten minutes at 12,000*g* at 4°C. Chloroform (200 μL/sample) was added to the isolated supernatants, and RNA precipitated from the aqueous phase with 500 μL of isopropanol. RNA pellets were washed and stored in 75 % ethanol at −80°C. Extracted and diluted RNA (total 27 μg) was purified with PureLink™Micro-to-Midi Kit and subjected to DNase I treatment (1 U/μg) (both from Invitrogen, CA, USA) during purification to destroy any traces of genomic DNA. RNA was eluted into RNase-free water and its concentration was measured with a NanoDrop 1000 spectrofotometer (CA, USA). Further, integrity of RNA was checked on agarose gel (1.2 % agarose, 1 % formaldehyde). For quantitative

RT-PCR analyses, equal amounts of hepatic RNA of three fish from the same aquarium were pooled as one sample, yielding three biological replicates per treatment concentration, representing altogether nine individuals.

Six genes were selected for real time quantitative PCR analyses: CYP1A, Hsp30, Hsp70, Grp78, Sep15, and GP1. Primers were designed with OligoPerfect^{TM} Designer (2010) software (Invitrogen, USA) available online (Table 1). Each primer pair showed specific amplification in melting curve analyses. Four candidates for a reference gene were tested, and out of them NADH-ubiquinone oxidoreductase (NUOR) showed superior stability with no effect of PAH treatments on its expression. The other candidates were 18S rRNA (18SrRNA), elongation factor 1 (EF1a), and RNA polymerase 2 (RPL2).

Purified RNA was used for cDNA synthesis with a SuperScript® VILO^{TM} cDNA Synthesis Kit (Invitrogen, CA, USA) in a 20 μL volume, each sample including 2.5 μg RNA. Several dilutions of cDNA were used for calibration. For validation of gene expression, Express SYBR®GreenER^{TM} qPCR SuperMix Universal (Invitrogen, CA, USA) was used in total 15 μL volume in qPCR reactions with 1.4 μL volume of 1:25 diluted cDNA. Reactions were run in duplicates with an ABI Prism 7700 Sequence Detector (Perkin Elmer, MA, USA).

The relative expression levels were calculated using relative standard curve method for each gene. Shortly, standard curves for each target gene as well as for the endogenous reference were prepared by amplifying a serial dilution (1:10, 1:25, 1:100, 1:250, 1:1,000) of the same batch of liver cDNA. The standard curves were prepared as a semi-log regression line plot of C_T value versus log of input cDNA, and the efficiency of each amplification (E) was calculated as

$$E = (10^{-1/\text{slope}} - 1) \times 100$$

All amplification efficiencies were within the acceptable limits of $0.8 < E < 1.1$ (Table 1). Consequently, the quantities of each target and endogenous reference were interpolated from the appropriate standard curves. Further, results were normalized by dividing each target quantity by reference gene quantity in the same sample. Finally, the relative expression values for each target gene were obtained with division of normalized target quantity by quantity of the untreated control, resulting in n-fold differences relative to the control.

SPSS (version 15 for Windows) was used in statistical analyses. For the fold-data from qPCR, the logarithm ($\log_{10}$) was used to make the response scale linear. The normality was tested using the Shapiro–Wilk-test. One-way ANOVA with the Bonferroni test was used to assess the effect of PAH concentration within a group. Non-normally distributed groups of values were tested with the Mann–Whitney non-parametric test. A probability level of $p < 0.05$ was considered to be statistically significant.

Results and Discussion

In aquatic environment, fishes can be exposed to retene or pyrene originated from different sources. In molecular toxicology perspective, the aim here was to collect basic information with laboratory experiments as approximating some realistic environmental conditions in the initial 24-h exposure.

Table 1 Primers designed and used for qPCR

Gene	Sequence (5′ → 3′)	Amplicon size (bp)	Accession number in GenBank	Amplification efficiency
Cytochrome P450 1A1	F: cagtccgccaggctcttatcaagc	94	CA360960	1.06
	R: gccaagctcttgccgtcgttgat			
Heat shock protein 30	F: gtgggcaggaagctgagagtcagt	126	CA355433	1.10
	R: ctcaggattcacgccttcaggcag			
Heat shock 70 kDa protein 1	F: tggcgacaagtctgagaacgtccag	133	EST1-3A_F05	1.06
	R: ggtctgtttggatgggatggtggtg			
78 kDa glucose-regulated protein precursor	F: aggccaagaagaaggagctgg	101	CA368961	1.07
	R: atcctgatctccctctgcctc			
15 kDa selenoprotein	F: gcccagatggagtccaggaagc	58	CA342228	1.05
	R: ttcatccacacacttccaggatgg			
Glutathione peroxidase 1	F: gttcatcatgtggagccctgtc	75	CA369000	0.98
	R: atctccatcaggactgaccagg			
NADH-ubiquinone oxidoreductase	F: caacatagggattggagagctgtacg	119	DW532752	1.07
	R: ttcagagcctcatcttgcctgct			

F forward, *R* reverse

After 2 h, the analyzed concentrations of RET and PYR were 0.9 μg/L (±SD 0.2) and 2.5 μg/L (±SD 0.2), respectively. Nominal values at the start of the exposures were 3.2 μg/L for RET and 2.9 μg/L for PYR. After 24 h, the concentrations from the same treatments were further decreased, revealing for RET 0.5 μg/L (±SD 0.04) and for PYR 1.4 μg/L (± SD 0.2). Thus, after 2- and 24-h the average concentrations of RET appeared to be 28 % and 16 % of the nominal 3.2 μg/L, respectively. Correspondingly, PYR concentrations were 86 % and 48 % of the nominal 2.9 μg/L. PAHs were not detected in control aquaria. None of the concentrations were lethal to fish and there were no apparent behavioral differences between the exposed and control group of rainbow trout.

Despite relatively high water-to-biomass ratios, concentrations of PAHs did not remain stable during the exposures. The distinct decreases in concentrations were most probably due to rapid and strong adsorption of PAHs on the glass walls of the experimental aquaria, explained by the high hydrophobicity of RET (log K_{ow} 6.4, Basu et al. 2001), and the relatively high value of PYR (log K_{ow} 4.92, Di Toro et al. 2000). Decreasing levels of RET in aquaria have also been documented in other studies (Räsänen et al. 2011). For instance, concentrations in water averaged 83 % of the nominal one (100 μg/L) within 2 h following the addition, dropping to 24 % after 24 h in a study where rainbow trout embryos were exposed to retene (Scott et al. 2009). In the present study, though not measured, we assumed that corresponding decreases had taken place in other aquaria as well. However, three different levels of exposure were evident, as indicated by the graded increases in the expression of the exposure biomarker CYP1A with increasing concentration of RET (Fig. 1).

After 24 h of exposure, expression of CYP1A, a major enzyme involved in biotransformation, showed dose-dependent induction at exposure to RET (Bonferroni, $F_{3,8} = 20.666$, $p < 0.05$ when comparing control and medium, $p < 0.01$ when comparing low and high, $p < 0.05$ when comparing low and medium), and was the strongest at the highest concentration added (65-fold, $p < 0.01$). CYP1A did not respond to PYR (Fig. 1).

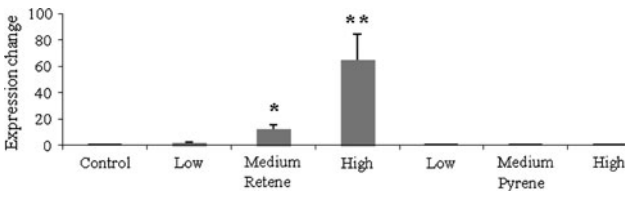

Fig. 1 Expression change of CYP1A in rainbow trout liver by qPCR (mean ± SE). Fish were exposed to RET and PYR in three equimolar nominal concentrations (low, medium, high) at range of 0.9–10 μg/L in 24-h. Controls were treated with 0.01 % DMSO. *Asterisk* denotes significant difference from control (* for $p < 0.05$ and ** for $p < 0.01$)

In this study, RET induced strongly only one gene, CYP1A, in a dose-dependent manner, seen also in CYP1A protein levels (Fragoso et al. 1999; Hawkins et al. 2002; Vehniäinen et al. 2003; Räsänen et al. 2011). The toxicity and metabolism of retene appear to be widely associated with the induction of CYP1A (Fragoso et al. 1999; Hawkins et al. 2002). Recent findings concluded that retene toxicity is associated with the function of aryl hydrocarbon receptor 2 (AhR2) and CYP1A metabolism (Scott et al. 2011). Up-regulation of CYP1A indicated induction of biotransformation with RET at the gene expression level.

In contrast to RET, PYR did not cause CYP1A induction at these concentrations. This is in an agreement with our previous study where PYR, as 10–100 μg/L nominal concentrations, did not induce CYP1A protein in young whitefish, *Coregonus lavaretus* (Räsänen et al. 2011) or it's activity in salmon fry, *Salmo salar* (Honkanen et al. 2008). Also, although PYR is considered to act via the AhR pathway (Incardona et al. 2004; Hendon et al. 2008), it is a weaker AhR agonist than more potent PAHs such as benzo(a)pyrene (Barron et al. 2004). In addition, lack of increase in CYP1A expression in liver of rainbow trout shows a major difference of actions between RET and PYR.

In addition, five selected genes were analyzed by qRT-PCR. There were no significant differences within those gene expressions between different concentrations (Table 2). qPCR revealed degree of variation between replicates, showing lowest variation in control treatments. Hsp30 and Grp78 showed the highest response in low concentrations with both PAHs. Further, inductions of Hsp70 and GP1 were strongest either at low or medium PAH concentrations. Also, the expression of Sep15 was highest at the lowest concentration of retene, but the response to pyrene was reversed.

Genes of 30 kDa heat shock protein (Hsp30), 70 kDa heat shock protein 1 (Hsp70), and 78 kDa glucose-regulated protein precursor (Grp78) were induced differently but not significantly in our results. All three proteins belong to the group of heat shock proteins with chaperone function and are known to respond to cellular stress (Basu et al. 2002). The most widely investigated Hsp70 is affected by diverse stressors, but its specificity is low (Lewis et al. 1999). Basically, RET and PYR can induce Hsp70 proteins in fish (Vehniäinen et al. 2003; Räsänen et al. 2011). Hsp30 is also regulated in a different way after heat shock (Heikkila 2004). The gene for 78 kDa glucose regulated protein, a well-known marker of the endoplasmic reticulum (Lee 2001) is characterized as having high sensitivity in salmonid fish (Skugor et al. 2008).

Both PAHs induced to some extent, but not significantly, the oxidative stress parameter of glutathioine peroxidase and 15 kDa selenoprotein. PYR induced oxidative stress genes, e.g. GP, in rainbow trout fry (Koskinen et al. 2004). It also significantly induced the ROS-metabolizing

Table 2 Expressions of selected genes analyzed by qRT-PCR

	Control	Retene			Pyrene		
		Low	Medium	High	Low	Medium	High
Heat shock protein 30	1.0 (±0.2)	6.6 (±5.3)	2.8 (±2.0)	0.9 (±0.2)	3.0 (±0.7)	1.2 (±0.3)	1.5 (±0.7)
Heat shock protein 70 protein 1	1.0 (±0.1)	0.9 (±0.3)	1.5 (±0.7)	0.9 (±0.2)	0.9 (±0.2)	1.0 (±0.1)	0.9 (±0.2)
78 kDa glucose regulated protein precursor	1.0 (±0.4)	11.0 (±2.7)	4.6 (±3.1)	5.3 (±2.5)	12.7 (±7.8)	7.9 (±7.3)	3.4 (±2.7)
15 kDa selenoprotein	1.0 (±0.2)	2.0 (±0.4)	1.4 (±0.5)	1.4 (±0.1)	0.9 (±0.1)	1.0 (±0.2)	2.8 (±1.9)
Glutathione peroxidase 1	1.0 (±0.3)	1.6 (±0.4)	3.3 (±1.3)	1.5 (±0.5)	3.0 (±2.0)	1.2 (±0.4)	1.3 (±0.5)

Fish were exposed to RET and PYR in three equimolar concentrations (low, medium, high) at range of 0.9–10 μg/L, for 24-h. Data are expression ratio (mean ± SE) of three pooled RNA samples (hepatic RNA of three fish per sample)

enzyme, catalase, in Atlantic salmon fry (Honkanen et al. 2008). Glutathione reductase (GR), another catalyst introduced in GSH-regulation, was induced by PYR in developing post-hatched young pike, *Esox lucius* (Räsänen et al. 2011). In addition, retene did not change whole body lipid peroxide concentrations but had a small effect in the reduction of glutathione level (a parameter of oxidative stress) in rainbow trout (Bauder et al. 2005). Glutathione reductase (GR) was not induced by RET in developing post-hatched young of whitefish but was so to some extent in pike (Räsänen et al. 2011). In addition, retene to some extent induced superoxide dismutase (SOD) in pike (Häkkinen et al. 2004). These increased changes were considered as a sign of oxidative stress which can lead to cellular damage. Induction of oxidative stress by PAHs can vary in different species, and measurement of one or few parameters is not sufficient for definite conclusions.

In this study, we showed that RET and PYR change in two different ways transcriptional activity of genes belonging to various functional groups in hepatic tissue of rainbow trout. For risk assessment, sublethal effects on tissues can be used as early signals of toxicity of these PAHs. However, it is important to realize that transcriptomic changes do not always lead to adverse functional changes in organs or individuals. We found that low, realistic environmental concentrations of RET, but not PYR, rapidly caused a change in CYP1A gene expression, and that such effects can be studied by qPCR.

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