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CYP1A gene expression in adipose fin of rainbow trout (*Oncorhynchus mykiss* Walbaum) exposed to benzo[*a*]pyrene

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ABSTRACT

Proximate to the environment, adipose fin of fish may be considered as a lipid storing tissue, and thus can be a target for either waterborne or dietary polycyclic aromatic compounds (PACs). We determined the effects of benzo[*a*]pyrene (B[*a*]P), a model PAC member, on CYP1A gene expression in adipose fin and compared that with the effects in gill of juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum) using the quantitative reverse transcription polymerase chain reaction (Q-RT--PCR). The results of the study demonstrated that constitutive CYP1A mRNA was present in adipose fin of rainbow trout, but the transcripts were far less abundant than those in gill tissue. We confirmed high CYP1A gene induction potential of the gills in rainbow trout injected with benzo[*a*]pyrene, but also showed moderately and transiently induced CYP1A mRNA in adipose fin. The modest and transitory gene expression may preclude rainbow trout adipose fin CYP1A mRNA levels from using it as an indicator of sustained exposure of fish to the polycyclic aromatic compounds.

INTRODUCTION

The small, non-rayed adipose fin is present in eight extant orders of fishes, including Salmoniformes (Nelson 1994), but neither functional significance of the trait is known, nor physiology of the tissue forming the fin is understood. Recent evidence suggests a hydrodynamic function of the adipose fin in juvenile salmonids (Reimchen and Temple 2004), and observations of sexually dimorphic adipose fin expression and female preference for the trait indicate a role in reproduction by mature individuals (Helfman et al. 1997; Næsje et al. 1988).

On the other hand, in an increasingly chemical environment, the adipose fin (Figure 1) might serve as a site of molecular biomarkers for certain classes of organic compounds. The proximate to the environment adipose fin may be considered as a lipid storing tissue, and therefore can easily be a target for either waterborne or dietary polycyclic aromatic compounds (PACs). It is known that in other tissues which directly contact the contaminated environment, such as the gill and intestine, highly lipophilic PACs traverse rapidly lipoid barriers (Van Veld et al. 1988). Furthermore, a number of enzyme systems exist that allow the tissues to eliminate these chemicals, thus preventing them from accumulating with detrimental consequences to the organism (Ioannindes 2007). In the present study, we were interested whether the biotransformation systems present in other tissues occur also in adipose fin, and if so, might the molecular responses be indicative of the exposure and effect. Among a number of enzyme systems that may be contributing to the biotransformation of PAC chemicals, the most important is that of cytochrome P450 (Ioannides 2007).

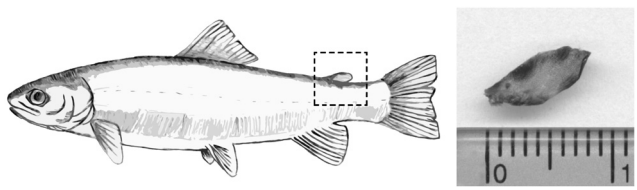

Figure 1. Adipose fin shown on rainbow trout illustration (left) and a photograph of a representative fin sampled in this study (right).

The PAC-dependent P4501A (CYP1A) gene expression (mRNA) primarily studied in fish liver as a biomarker to identify exposure to a number of PACs (Stegeman et al. 1992; Van der Oost et al. 2003), has also been measured in a number of other tissues including brain (Huang et al. 2000), pituitary (Andersson et al. 1993), intestine (Van Veld et al. 1988), gills (Levine and Oris 1999), heart (Andreasen et al. 2002), head kidney (Brzuzan et al. 2006), or eye (Wang et al. 2006).

Studies in some species including salmonids indicate also that CYP1A enzyme activities are induced in olfactory organs of fish exposed to PACs (Chung-Davidson et al. 2004), thus serving the first protection line in the brain for the xenobiotics. Once determined and confirmed, high level of CYP1A gene induction in adipose fin of fish exposed to polycyclic aromatic compounds could indicate its role in biomonitoring studies of dietary and environmental contamination with PACs.

Therefore, in the present study we determined the effects of benzo[*a*]pyrene (B[*a*]P), a model PAC member, on CYP1A gene expression in adipose fin and compared that with the effects in gill of juvenile rainbow trout (*Oncorhynchus mykiss* Walbaum) using the quantitative reverse transcription polymerase chain reaction (Q-RT-PCR).

MATERIAL AND METHODS

A group of hatchery raised rainbow trout, with an average body mass of 49.4 g and total length of 15.5 cm were given an intraperitoneal injection (10 mg•kg^{-1}) of B[*a*]P in corn oil (2 mg B[*a*]P•ml^{-1} corn oil) or corn oil alone (Control). After 8 and 24 h (T = 7°C), trout gills and adipose fins (n = 4) were immediately stored in RNA stabilizing buffer (RNA Later™, Qiagen, Hilden, Germany) at -20°C. The fish were treated in accordance with the rules approved by the Local Ethical Commission No. 38/N issued on 29.07.2004 (conforming to principles of Laboratory Animal Care, NIH publication No. 86-23, revised in 1985).

Total RNA isolation, quantification and storage were described recently (Brzuzan et al. 2007), except that RNALater™ preserved whole adipose fins (13.0 ± 1.0 mg) or small pieces of gills (15.0 ± 2.0 mg) were used. The total RNA was used to synthesize cDNA with a commercially available RevertAid™ First Strand cDNA Synthesis Kit (Fermentas; Burlington, Canada). Mixture of each sample contained 1 μg of total RNA and 0.5 μg of oligo(dT)$_{18}$ primers, and the reaction was performed according to the manufacturer's recommendations. cDNA samples were stored at -20°C until used.

We examined CYP1A mRNA levels in either adipose fin or gill tissue of control and exposed rainbow trout using relative quantification method implemented in Relative Expression Software Tool (REST©, version 2; Pfaffl et al. 2002), based on the data obtained from the Real-Time PCR assay. Real-Time PCR primers for CYP1A (forward WML158 5'-CCA ACT TAC CTC TGC TGG AAG C-3' and reverse WML159 5'-GGT GAA CGG CAG GAA GGA-3') were taken from the paper of Rees and Li (2004). To avoid misinterpretation of the derived expression profiles of the target gene two combined endogenous controls were used to normalize the results in either tissue. The primers used for amplification of β-actin were ENDO1F (5'-GTG GCG CTG GAC TTT GAG CA-3') and ENDO1R (5'- ACC GAG GAA GGA GGG CTG GA-3'), previously designed for whitefish cDNA (Brzuzan et al. 2005). The other reference gene, *rpl19*, encoding rainbow trout ribosomal protein L19 (RPL19-F: 5'-GTC ACG GTG CAC TCT CGC GC-3'; RPL19-R: 5'-CGG GCA TTG GCT GTA CCC TT-3') was chosen because it was found to be expressed at a range closer to the *Ct* (threshold cycle) of the target gene than *β-actin*. In similar *Ct* range, reference and target undergo similar cycle condition with respect to the reaction product inhibition (Kainz 2000).

Real-Time PCR assays were performed in MicroAmp™ optical tubes (Applied Biosystems; Foster City, CA) using the ABI 7500 Real-Time PCR system (Applied Biosystems). All samples were analyzed in duplicate in the singleplex mode (1 pair of primers per well). On the plate, negative water controls and genomic DNA control were included to rule out the possibility of PCR amplification resulting from upstream contamination. Each sample reaction mixture contained 10 μl of 2x FastStart SYBR® Green Master ROX mix (Roche Diagnostics; Mannheim, Germany), 5 pmol of forward and reverse primer, 2 μl of cDNA as a template, and PCR-grade water adjusted to a final volume of 20 μl. The reaction was performed in standard thermal conditions: 50°C for 2 min, 95°C for 10 min, then 40 cycles of 95°C for 15 s and 60°C for 1 min. To test for the quality of the PCR products, a melting curve analysis was performed after each run. Additionally, specificity of all PCR products was documented by electrophoresis in standard 2% gels each containing 40 ng of ethidium bromide per 100 ml of an agarose gel, and visualization under UV light (Gel Logic 200 Imaging System, Kodak).

The relative expression ratio of *CYP1A* was computed, based on single individual real time PCR efficiency (E), and the threshold cycle *Ct* difference (Δ) of an unknown sample versus a control ($\Delta Ct_{\text{control – sample}}$) according to the mathematical model given by Pfaffl (2001):

$$Ratio = \frac{(E_{target})^{\Delta Ct_{target}\ [\text{MEAN}_{\text{control}} - \text{MEAN}_{\text{sample}}]}}{(E_{ref})^{\Delta Ct_{ref}\ [\text{MEAN}_{\text{control}} - \text{MEAN}_{\text{sample}}]}}$$

Before using the above method for quantification, we performed a validation experiment to verify that efficiencies of target (CYP1A) and either reference gene, *β-actin* or *rpl19* (endogenous controls) are approximately equal. We performed dilution series of different input amounts (1, 0.1, 0.01, and 0.001 per tube; n = 3) of cDNA (reverse transcribed total RNA) extracts of either adipose fin or gill, and spiked into separate tubes. The PCR reactions on serially diluted cDNA templates were monitored in real time by fluorescence with the incorporation of the dye, SYBR® Green. Ct versus cDNA were plotted to calculate respective slope values (data not shown). The corresponding real-time efficiencies were calculated according to the equation: $E = 10^{[-1/\text{slope}]}$ (Pfaffl 2001). The resulting E-values were high (from 1.95 to 1.99) and were the basis for the calculation and further randomization tests with REST© (Pfaffl et al. 2002).

Differences in CYP1A expression between control and B[*a*]P treated samples were assessed in group means for statistical

significance by randomization tests (REST©). Randomization tests are a useful alternative to more standard parametric tests for analyzing experimental data. A statistical test is based on the probability of an effect as large as that observed occurring under the null hypothesis of no treatment effect. It is assumed that if the hypothesis is true, the values in one treatment group were just likely to have occurred in the other group. The randomization test repeatedly and randomly reallocates the observed values to the two groups and notes the effect each time. The proportion of the effects that are as great as that observed in the experiment gives the *P*-value of the test (Pfaffl et al. 2002). In the study for each particular data set 2000 randomizations were performed.

RESULTS AND DISCUSSION

At similar efficiencies of PCR amplification of every two genes compared, without normalizing by another reference gene, the lower value of the mean *Ct* represents higher mRNA expression. As shown in Table 1, variations of sample mean *Ct*s for investigated transcripts remained stable, with a CV ranging from 0.85 to 6.06%. For either tissue analyzed, single individual constitutive mRNA expression of β-actin or rpl19, measured in control samples, were not significantly different between the studied time points of the experiment (REST©, $P > 0.05$). Furthermore, the reference genes showed no changes in their mRNA levels as a result of treatment with B[*a*]P. The constitutive levels of CYP1A mRNA, as measured in control fish, were significantly lower in adipose fin compared to those in gill at either 8 or 24 h of the experiment (REST©; $P = 0.003$, $P = 0.046$, respectively).

Real time PCR results for CYP1A gene for two examined tissues, time points and treatments are given in Figure 2. The data were presented as the mean CYP1A expression ratio (ER) for each sample, normalized by expression of either, β-actin or rpl19, or both. B[*a*]P exposure for 8 h caused 2-4 fold increase of expression ratio (ER) of CYP1A mRNA levels in either tissue (adipose fin, ER = 2.84 ± 1.85; $P = 0.067$; gill, ER = 2.79 ± 2.09; $P = 0.250$). After 24 h of the treatment, only CYP1A gene in gill was significantly induced by B[*a*]P (ER = 14.12 ± 12.25; $P = 0.001$), whereas the CYP1A mRNA in adipose fin decreased to control levels (ER = 1.46 ± 0.79; $P = 0.596$).

The results of this study demonstrate that constitutive CYP1A mRNA was present in adipose fin of rainbow trout, but the transcripts were far less abundant than those in gill tissue. Furthermore, the CYP1A gene in adipose fin was moderately induced by B[*a*]P after 8 h of the treatment, at a rate comparable to that in gill (~2-4 fold increase, depending on the reference gene expression used for normalization; Figure 2). We expected this because B[*a*]P is known to be distributed rapidly within the body (Moir et al. 1998) and its metabolites can readily penetrate cell membranes (Brown and Chee 1992). However, whereas gill CYP1A increased significantly (~14 fold) after 24 h of the experiment, CYP1A mRNA levels in adipose fin did not. Therefore, it is very likely that accumulation of adipose fin (and gill) CYP1A mRNAs during the first 8 hours of exposure appeared primarily as a result of *de novo* synthesis, through an intracellular receptor complex (the aryl hydrocarbon receptor, AhR; Hahn et al. 2005), rather than from CYP1A mRNA stabilization or from decreased CYP1A mRNA degradation rates. The cause for the decrease of rainbow trout CYP1A mRNA in adipose fin

Table 1. Variation in the number of threshold cycles (*Ct*s) for β-actin, rpl19 and CYP1A determined in adipose fin and gill of rainbow trout by real time PCR. Given are the mean, standard error (S.E.), coefficient of variation (CV), each one based on $n = 4$ for controls and B[*a*]P-treated fish for 8 and 24 h.

Treatment/hour		Adipose fin			Gill		
		β-actin	rpl19	CYP1A	β-actin	rpl19	CYP1A
Control/8	**mean**	15.67	16.82	24.31	14.15	17.53	20.84
	S.E.	0.31	0.43	0.61	0.52	0.42	0.50
	CV (%)	1.97	2.57	2.51	3.64	2.37	2.42
B[*a*]P/8	**mean**	15.15	17.54	22.84	13.10	16.93	18.53
	S.E.	0.37	0.24	0.59	0.35	0.42	0.75
	CV (%)	2.42	1.38	2.58	2.67	2.49	4.02
Control/24	**mean**	14.98	17.08	23.22	13.17	16.68	21.34
	S.E.	0.33	0.28	0.22	0.21	0.17	0.30
	CV (%)	2.19	1.64	0.94	1.57	1.03	1.41
B[*a*]P/24	**mean**	14.41	16.61	22.40	13.43	16.69	17.65
	S.E.	0.13	0.14	0.68	0.55	0.55	1.07
	CV (%)	0.89	0.85	3.03	4.12	3.29	6.06

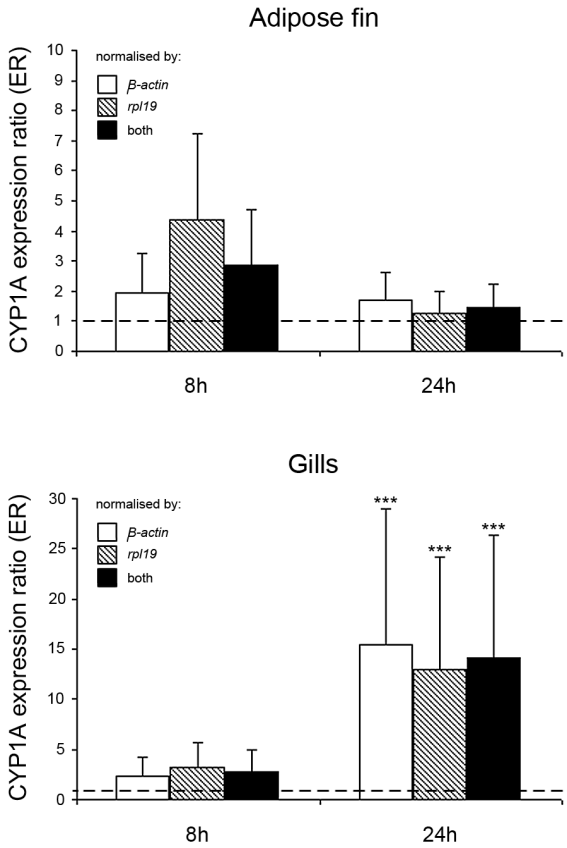

Figure 2. Expression pattern of CYP1A mRNA levels in adipose fin and gill of B[*a*]P-treated rainbow trout at 8 and 24 h of the experiment. Bars represent mean values of expression ratios (ER) with their respective standard errors of the mean (S.E.; $n = 4$), normalized by different endogenous references: β-actin, rpl19 or combined (both) and relative to a control sample (ER = 1.00; dashed line) of a respective tissue and time of the experiment. The ER values were calculated by REST© from Table 1. Tripple asterisk indicates significant difference ($P = 0.001$) between gill CYP1A expression of control and that of B[*a*]P-treated rainbow trout after 24 h of the study.

to a basal level between the 8 and 24 h of exposure remains unknown. We hypothesize that the decrease in CYP1A mRNA level in adipose fin may be in part due to an increased rate of CYP1A-catalyzed first-pass metabolism of parent B[*a*]P by the liver, and possibly other biotransformation systems, that lowered concentration of parent B[*a*]P reaching the adipose tissue. Further studies may determine if the observed decrease can occur due to either increased breakdown of CYP1A mRNA molecules, rapid saturation of Ah receptors present in the tissue, or increased rate of B[*a*]P metabolism.

With regard to the CYP1A expression in gill tissue of rainbow trout, the results of the present experiment almost replicated previously reported data by Levine and Oris (1999). The authors have shown that rainbow trout exposed to waterborne B[*a*]P concentrations of 0.78 ± 0.11 μg/l had gill CYP1A mRNA levels significantly induced after 6 h of exposure and the tissue remained at this high level of induction through 120 h. Interestingly, the apparently different routes of B[*a*]P exposure, i.e. intraperitoneal, (this study) or waterborne (Levine and Oris 1999), produced similar effects on CYP1A mRNA levels in rainbow trout gill within the comparable exposure period.

In summary, our results not only confirmed high CYP1A gene induction potential of the gills in rainbow trout injected with benzo[*a*]pyrene, but also showed moderately induced CYP1A mRNA in adipose fin. The observed CYP1A mRNA levels in adipose fin of rainbow trout may be used as a surrogate for CYP1A enzyme abundance in the tissue, and thus may add to results of previous investigations that have shown that tissues other than the liver, contain active CYP1A enzyme systems that can modify the absorption, toxicity and fate of CYP1A substrates (Andersson and Pratt 1989; Kennedy and Walsh 1994; Miller et al. 1989; Ueng et al. 1992; Van Veld et al. 1997; Wang et al. 2006). Unfortunately, the transitory CYP1A gene induction, decreasing through the 24 h of exposure, may preclude from using rainbow trout adipose fin CYP1A mRNA levels as an indicator of sustained exposure to polycyclic aromatic compounds.

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