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Relative quantification of *CYP1A* gene expression in whitefish (*Coregonus lavaretus*) exposed to benzo[*a*]pyrene

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ABSTRACT

The expression of CYP1A (cytochrome P4501A) can be induced by a number of aromatic compounds in teleost fishes. We developed a real-time PCR assay for measuring relative quantities (RQ) of CYP1A mRNA in whitefish (*Coregonus lavaretus*). To test for the usefulness of the assay we performed a treatment study, using benzo[*a*]pyrene (B[*a*]P) a model CYP1A inducer. Primers for the *CYP1A* gene were adapted from the literature, whereas those for ß-actin (endogenous control) were designed from a region that was found to be conserved among salmonid ß-actin genes. A group of hatchery raised whitefish, with an average body mass of 15 g and total length of 12 cm were given an intraperitoneal injection (10 mg/kg) of B[*a*]P in corn oil (2 mg B[*a*]P/ml corn oil) or corn oil alone (Control). After 48 h, whitefish liver, head kidney and brains were collected for mRNA isolation and analysis. In all three tissues sampled, CYP1A mRNA was affected by treatment with B[*a*]P. Head kidney tissue showed the greatest induction potential (RQ=11.00) from base levels (RQ=1.00), followed by liver (RQ=9.45), and brain (RQ=3.76). These results demonstrated that *CYP1A* was highly inducible by B[*a*]P in whitefish head kidney and liver, and to some extent, in brain tissue. The approach presented here has the advantage of providing rapid and accurate measures of *CYP1A* induction in various tissues of fish responding to PAH contaminant exposure.

INTRODUCTION

Fish are among the organisms most sensitive to the toxicity of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCB's), furans and dioxins, particularly at their early life developmental stages (Incardona et al. 2004; Prasch et al. 2003; Teraoka et al. 2003). Environmental exposures to polycyclic aromatic hydrocarbons (possibly in conjunction with PCBs, and dioxins) were related to epizootic outbreaks of liver neoplasia in feral fish from various regions of the United States (Murchelano and Wolke 1991; Myers et al. 1994).

The PAH compounds stimulate expression of various members of the cytochrome P450 family of genes (CYP), particularly those of the CYP1 family, via the aryl hydrocarbon receptor (AhR) – dependent pathway (Hahn et al. 2005). The induction of hepatic *CYP1A* in fish by certain classes of chemicals has been suggested as an early warning system, a most sensitive biological response for assessing environmental contamination conditions (reviewed by Arinç et al. 2000).

Early work demonstrating induction of CYP1A mRNA in fish in response to PAH species used Northern blot analysis (Stegeman 1995). With advances in molecular biology techniques, determination of CYP1A mRNA levels by quantitative reverse transcription polymerase chain reaction (RT-PCR) has been added to biomonitoring studies (see for example Cao et al. 2000; Rees et al. 2003). A fairly new technology that emerged in the early 1990s is quantitative real-time PCR (Q-PCR). The Q-PCR reaction is monitored in real time by fluorescence either with the incorporation of the SYBR green dye that fluoresces only when it is intercalated into DNA or by a fluorescent probe that is complementary in sequence to the cDNA of interest (Higuchi et al. 1993; Larkin et al. 2003). Recently Rees and Li (2004) have developed and evaluated a real-time quantitative PCR assay for measuring the induction of liver CYP1A mRNA in species of three salmonid genera: *Oncorhynchus*, *Salmo*, and *Salvelinus*.

In this study, our goal was to develop a single real-time quantitative PCR assay and use it to estimate CYP1A levels in another genus of salmonids, Coregonus. As coregonid fish have been found feasible organisms for pollution monitoring in aquatic systems (Mellanen et al. 1999; Soimasuo et al. 1998), the other objective was to use the assay to determine and compare the effect of benzo[*a*]pyrene (B[*a*]P) treatment on CYP1A levels in liver, head kidney and brain tissues of whitefish, *Coregonus lavaretus*.

MATERIAL AND METHODS

The fish were treated in accordance with the rules approved by the Local Ethical Commission No. 38/N dated on 29.07.2004 (conforming to principles of Laboratory Animal Care, NIH publication No. 86-23, revised in 1985). Juvenile, hatchery-reared whitefish (15 g ± 1 g mean weight, 12 cm ± 1 cm mean length) from the Department of the Salmonid Research in Rutki, Inland Fisheries Institute in Olsztyn, Poland, were used in the experiment. Whitefish were fed Aller Safir 2mm-XS-S-M (Aller Aqua, Poland) daily at a level of 1.0% body weight. Two days prior to injection, twenty whitefish were taken off of feed and were acclimated at 7°C. For the gene induction study, 5 randomly chosen individuals were sampled, anesthetized using a dose (0.5 ml/l) of 2% Etomidate to avoid animal suffering, and injected intraperitoneally with B[*a*]P (Fluka, Buchs, Switzerland) in corn oil at a dose of 10 mg/kg body weight (2 mg B[*a*]P/ml corn oil). Another group (Control) consisted of 5 fish that were treated with corn oil alone. Fish were then placed in two 800 l flow-through tanks (well water, 600 l/h) for 48 h at 7°C. Then 2 random fish from each group were taken, sacrificed, and their tissues (liver, head kidney, and brain) were immediately stored in RNA*Later*™ at -20°C (Qiagen, Hilden, Germany).

RNA*Later*™ preserved tissues of one whitefish from either sample were homogenized and extracted for total RNA isolation using Fenzol Reagent. Then the samples were purified using silica columns (A&A Biotechnology, Gdynia, Poland) according to the manufacturer's protocol, and re-suspended in 100 μl of diethylpyrocarbonate treated water (DEPC-H_2O). RNA samples were incubated at 37°C for 30 minutes with RNase-free DNase I (Promega, Madison, WI, USA) to ensure that genomic DNA was not amplified during PCR, and quantified using a Bio*Photometer* (Eppendorf, Hamburg, Germany). All RNA samples were of high quality with A_{260}/A_{280} ratios ranging from 1.8 to 1.9.

Highly purified polyA+ mRNA from liver, head kidney and brain was isolated/extracted by polyA+ selection on oligo(dT) – cellulose suspension (QuickPrep *micro* mRNA Purification Kit, Amersham Biosciences) and quantified. The mRNAs were used to synthesize cDNAs using a commercial kit (RevertAid™ Transcription Kit; Fermentas). 20 μl reaction contained 0.25 μg of mRNA, 0.2 μg of random hexamer primers, 1 mM aqueous solution of each of the four nucleotide triphosphates, 4 mM MgCl2, 50 mM Tris-HCl, 50 mM KCl, 10 mM DTT, 20 units of RiboLockTM Ribonuclease inhibitor and 200 units of M-MuLV reverse transcriptase. The reaction was carried out at 25°C for 10 min, 42°C for 60 min, and then at 70°C for 10 min.

RealTime PCR assay

We examined CYP1A mRNA levels in tissues of treated and control whitefish using relative quantification method based on the data obtained from the RealTime PCR assay. In the assay, the PCR reactions are monitored in real time by fluorescence with the incorporation of the SYBR green dye that fluoresces only when it is intercalated into DNA. Relative quantification describes the change of expression of the target gene in a test sample to a calibrator sample. Relative quantification provides accurate comparison between the initial level of template in each sample. The requirement of the method is that amplification of an endogenous control must be performed to standardize the amount of sample RNA or cDNA added to reaction.

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 recent paper by Rees and Li (2004) and were optimized for quantitative PCR with whitefish cDNA. The Primer Express 1.1 software (Applied Biosystems) was used to select an optimal ß-actin (endogenous control) primer pair (ENDO1F 5'-GTG GCG CTG GAC TTT GAG CA-3' and ENDO1R (5'-ACC GAG GAA GGA GGG CTG GA-3'), based on the homologous mRNA sequences available in GenBank (AF012125, OMY438158, and AF157514). The amplicon sequence (150 bp) of whitefish ß-actin mRNA was deposited in GenBank under the Acc. No. DQ232880.

Each PCR reaction consisted of 10 μl 2x SYBR®GREEN PCR Master Mix (Applied Biosystems; Branchburg, NJ), 2.5 pmole of each primer, 1 μl of cDNA template solution from the first step, and nuclease free water adjusted to a final volume of 20 μl. The target (CYP1A) and endogenous control (ß-actin) amplifications were run in separate tubes. On the plate, negative water controls and genomic DNA control were included to rule out the possibility of PCR amplification resulting from upstream contamination. Tissue extracts of control and B[a]P-treated fish were assayed in duplicate. All real time reactions were run using the universal thermal cycling parameters, that were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Reactions were run and analyzed on an ABI 7500 real-time PCR thermalcycler (Applied Biosystems). After the run a melting curve analysis was performed. To test for the appearance of the PCR products the samples were additionally electrophoresed in a 2% gel containing 40 ng of ethidium bromide per 100 ml of an agarose gel, and visualized under ultraviolet light (Gel Logic 200 Imaging System, Kodak).

Comparative CT method for relative quantification of CYP1A mRNA levels

The comparative CT method (or "delta-delta method"; Livak and Schmittgen 2001) uses an arithmetic formula of $2^{-\Delta\Delta C_T}$, to calculate the amount of target gene normalized to an endogenous reference using data generated during the PCR experiment. In our CYP1A expression study we used PCR data of B[*a*]P-treated whitefish (target), and control whitefish (calibrator). Both data were normalized to the expression of ß-actin gene in a particular sample (endogenous control).

Before using the comparative CT method for quantification, we performed a validation experiment to verify if primer efficiencies between target (CYP1A) and endogenous control (ß-actin) were approximately equal. We performed a dilution series of different input amounts (1, 0.1, 0.01, and 0.001 per tube) of target and endogenous control cDNA obtained from liver mRNA extracts of fish, and spiked into separate tubes. After performing a run the log of the input amount (a dilution series) vs. ΔC_T was plotted, and linear regression was used to approximate a line through the points (data not shown). As a guideline, the absolute value of the slope of log input amount vs. ΔC_T should be less than 0.1 (Livak and Schmittgen 2001). The slope in the figure was -0.084 (<0.1).

Then, we quantified the expression of *CYP1A* genes in a particular tissue relative to that of the liver. The ΔC_T value was determined for all tissues and treatments by subtracting the respective average ß-actin values from the average CYP1A values. The standard deviations of the differences were calculated from the standard deviations of the respective CYP1A and ß-actin values. The calculation of $-\Delta\Delta C_T$ involves subtracting ΔC_T calibrator value (ΔC_T of liver) from the ΔC_T target value. Because this is a subtraction of arbitrary constant, the standard deviation of $\Delta\Delta C_T$ is the same as the standard deviation of the ΔC_T value. Finally, the amounts of CYP1A mRNA and their ranges in different tissues were determined by evaluating the expression: $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T$ + s and $\Delta\Delta C_T$ - s, where s indicates the standard deviation of the $\Delta\Delta C_T$ value.

To compare between different tissues the potential of B[*a*]P to induce CYP1A genes, we quantified, separately for each tissue, the levels of CYP1A expression in B[*a*]P-treated fish relative to that of control sample. In this case the calculation of $-\Delta\Delta C_T$ involved subtracting respective ΔC_T calibrator values, picked from the control samples, from the ΔC_T value of the fish affected by B[*a*]P.

RESULTS AND DISCUSSION

Figure 1 shows a representative amplification plot for whitefish CYP1A and ß-actin cDNA (reversed mRNA), in this case of brain extracts. The PCR reactions were monitored in real time by fluorescence with the incorporation of the dye, SYBR green. During the log-linear phase, the increasing fluorescence signal was directly proportional to the initial amount of target mRNA in the sample. Thus, it was possible to pick the cycle threshold (C_T), a value that was used to calculate the amounts of cDNA in the samples (Table 1).

In the study, liver tissue demonstrated the highest base level of CYP1A mRNA (Table 1). Considerably lower base levels of P450 expression were seen in kidney (6.8% of that in liver) and brain tissue (3.6%) (Table 1). The base levels of CYP1A in whitefish tissues are similar to those reported in Atlantic salmon (Rees et al. 2003) which showed the highest base level in liver followed by head kidney, and brain.

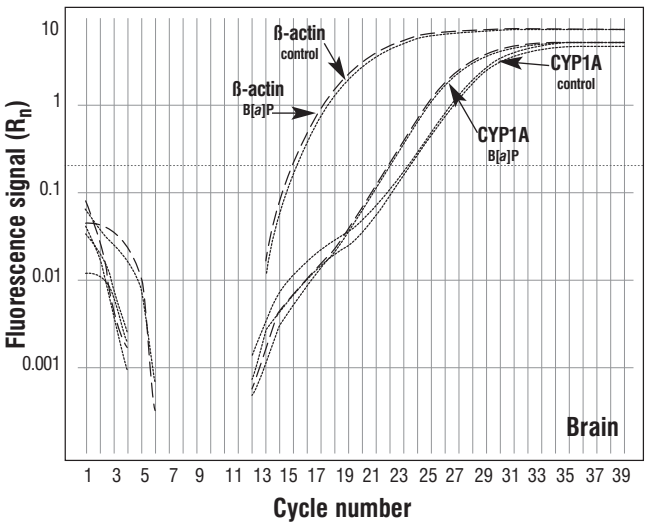

Figure 1. Representative amplification plot for CYP1A and ß-actin cDNA (reversed mRNA) of whitefish brain extracts. The tissue extracts of control and B[*a*]P-treated fish were assayed in duplicate. Each plot represents the increasing fluorescent signal (Rn) of SYBR green dye at each PCR cycle number. A reaction threshold of 0.2 (dashed line) was used for all samples. The cycle number at which this threshold line crosses the amplification curve in its log--linear phase is the cycle threshold (C_T), a value that is used to calculate the amount of cDNA in the sample.

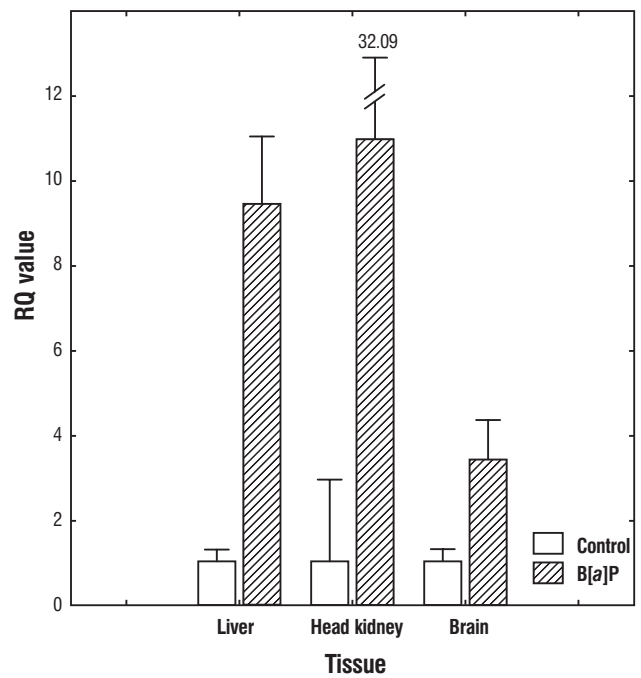

Figure 2. Real-time PCR relative quantification (RQ) of liver, head kidney and brain CYP1A levels in control and B[*a*]P exposed whitefish. Bars represent quantification values of the amount of target CYP1A cDNA, normalized to an endogenous control (ß-actin), and relative to a calibrator (Control sample; RQ=1.00) of a respective tissue. The RQ values were calculated from Table 1.

Table 1. Relative quantification of CYP1A mRNA amounts in tissues of control and B[*a*]P treated whitefish using the comparative C_T method. The table shows the average C_T results for whitefish liver, head kidney and brain samples and how these C_Ts are manipulated to determine ΔC_T, $\Delta\Delta C_T$, and the amount of CYP1A mRNA within the respective tissue relative to that in liver (last column). "s" indicates standard deviation. The ranges given in brackets in the last column were determined by evaluating the expression: $2^{-\Delta\Delta C_T}$ with $\Delta\Delta C_T$ + s and $\Delta\Delta C_T$ - s.

Tissue	Sample	Target CYP1A Average C_T (±s)	Endogenous Control ß-actin Average C_T (±s)	ΔC_T CYP1A ß-actin (±s)	$-\Delta\Delta C_T$ $-(\Delta C_T - \Delta C_{T,\ Liver})$ (±s)	CYP1A Relative to Liver
Liver	Control	20.94±0.14	17.10±0.10	3.84±0.17	0.00±0.17	1.000 (0.888 to 1.125)
	B[*a*]P	17.77±0.25	17.17±0.10	0.60±0.27	0.00±0.27	1.000 (0.829 to 1.206)
Head kidney	Control	28.23±1.02	20.51±1.21	7.72±1.58	-3.88±1.58	0.068 (0.023 to 0.203)
	B[*a*]P	25.29±0.24	21.03±0.15	4.26±0.28	-3.66±0.28	0.079 (0.065 to 0.096)
Brain	Control	24.42±0.17	15.79±0.09	8.63±0.19	-4.79±0.19	0.036 (0.032 to 0.041)
	B[*a*]P	22.89±0.15	16.17±0.09	6.71±0.18	-6.12±0.18	0.014 (0.013 to 0.016)

As is shown in Figure 2, CYP1A mRNA was affected by treatment with B[*a*]P in all three tissues sampled: liver, head kidney, and brain. Head kidney tissue showed the greatest induction potential (RQ=11.00) from base levels (RQ=1.00), it was lower in liver (RQ=9.45), whereas the lowest induction level of *CYP1A* for the tissues studied was in brain (RQ=3.76). These results demonstrate that CYP1A is highly inducible by B[*a*]P in whitefish head kidney and liver, and to some extent, in brain tissue. Interestingly, the apparent differences in potencies of B[*a*]P to induce CYP1A genes in whitefish tissues are correlated with those found in other salmonid fish species exposed to various PAH contaminants (Campbell and Devlin 1996; Rees et al. 2003). For example, Rees et al. (2003) measuring the levels of CYP1A induction in Atlantic salmon (*Salmo salar*) exposed to ß-naphthoflavone, have found these potentials to be: head kidney>liver>brain.

The induction of CYP1A and associated enzyme activities has been confirmed as biomarkers in a number of field studies (e.g. Arinç et al. 2000; Moore et al. 2003; Rees et al. 2003). The real time approach presented here has the advantage of providing rapid and accurate measures of CYP1A induction in various tissues of fish responding to PAH contaminant exposure. Furthermore, it is known that CYP1A activates certain classes of PAH pro-carcinogens and other chemicals by forming oxygenated compounds. Oxygenation of benzo[*a*]pyrene by CYP1A1 in the presence of epoxide hydrolase results, for example, in the formation of the ultimate carcinogen, benzo[*a*]pyrene-7,8-dihydrodiol-9,10-epoxide (BPDE), which forms DNA-adducts (Burczynski and Penning 2000). Thus, greater *CYP1A* induction may result in high levels of activated carcinogens, and consequently to higher degree of persistent DNA-adduct formation, which ultimately may cause a mutation and initiation of cancer (Burczynski and Penning 2000). Parallel real time PCR studies are under way to accurately evaluate the correlation between the induction *CYP1A* with various PAH compounds and their mutagenic activity in rainbow trout. These studies should give us further insight into the mechanism of both toxic and genotoxic potential of the PAH contaminants.

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