Benzo[*a*]pyrene and cyclopenta[*c*]phenanthrene suppress expression of *p53* in head kidney of rainbow trout

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ABSTRACT

Although p53, a protein of important tumor suppressive function, has been extensively studied in mammals, relatively little is known about the p53 pathways in lower vertebrates. Particularly, limited information exists on possible influences of environmental contaminants on the expression of the *p53* gene in fish. In the current study, we assessed the effects of benzo[*a*]pyrene (B[*a*]P; potent tumor promoter) and cyclopenta[*c*]phenanthrene (CP[*c*]Ph; clastogenic agent) exposure on a 24h profile of *p53* gene expression in head kidney of juvenile rainbow trout (*Oncorhynchus mykiss*). To analyze the *p53* transcription rate, we developed protocol for the examination of both mRNA and heterogeneous nuclear (hn) RNA of the gene, using Real-Time RT-PCR approach. The results show that both compounds are capable of suppressing *p53* transcriptional activity within 12h of the treatment. Our finding supports the idea that structurally different PAHs may influence cell physiologic functions controlled by *p53* in fish, in part, by down-regulating its RNA expression levels.

INTRODUCTION

p53 is one of the most investigated genes in cancer research due to its crucial role in cell death, proliferation, and senescence. The *p53* gene is highly conserved in evolution and has been studied in a wide range of species from human to flies and worms (Jin et al. 2000; Lee et al. 2008; Soussi and May 1996; Sutcliffe and Brehm 2004). The protein p53 protects normal cells from aberrant growth by activating and repressing target genes involved in the control of a variety of cell physiologic functions. Numerous cellular stressors, such as DNA damage, oncogene activation, hypoxia, growth factor stimulation, and oxidative stress can activate mammalian *p53* (Pluquet and Hainaut 2001). Importance of *p53* for cell defense programs has also been shown for fish. For example, knockdown of *p53* in zebrafish (*Danio rerio*) embryos, resulting in *p53* deficiency, reduced apoptosis triggered by either UV treatment or exposure to a DNA damaging agent (Langheinrich et al. 2002).

While it is generally believed that the principal mechanisms governing the activity of *p53* occur at the protein level (Braithwaite et al. 2005), in some models, chemical DNA damage by B[*a*]P for example, seems to modulate *p53* transcription (Lu et al. 2000). Recently, environmental pollutants such as PAHs (B[*a*]P; Brzuzan et al. 2006a), environmental pharmaceuticals (diclofenac; Hong et al. 2007), and environmental toxins (microcystin-LR; Brzuzan et al. 2009) have been reported to modulate p53 mRNA expression in fish and its role as biomarker has also been suggested. From the studies performed by Ray and Swanson (2003, 2004) on human epidermal keratinocytes (HEKs), it is evident also that dioxin, another environmental contaminant and potent tumor promoter, is able to repress both the expression of cyclin dependent kinase inhibitor $p16^{INK4a}$ and *p53*, and the onset of senescence in the cells as well. The authors showed that repression of transcriptional activity of both genes in the HEK cells requires involvement of aryl hydrocarbon receptor (AhR), and the process is accompanied by promoter methylation.

Currently the idea that environmental contaminants, which show potential to induce the AhR, may be involved in promoting DNA methylation of the tumor suppressor

gene (TSG) promoters is plausible. To examine the likely contaminant dependent repressive effect on the *p53* gene expression in fish, rainbow trout was chosen as a model organism for the study, and two of the PAH compounds that were found to be AhR agonists were tested: benzo[*a*]pyrene [B[*a*]P; potent tumor promoter (e.g. Bailey et al. 1996)] and cyclopenta[*c*]phenanthrene [CP[*c*]Ph; clastogenic agent (Brzuzan et al. 2006b)]. We examined the *p53* gene expression in head kidney, a fish multifunctional organ that contains hematopoietic, lymphoid and endocrine tissue with highly proliferating cells, all showing relatively high constitutive level of p53 mRNA compared to other tissues, such as liver or brain (Brzuzan et al. 2006a). Changes of mRNA expression levels may result in a consequence of various events, such as increased transcription, increased stability, decreased breakdown, or altered processing (increased mRNA levels and increased rate of transcription are not always synonymous). Therefore to analyze the rate of transcription of *p53*, we developed a protocol for quantitative examination of mRNA and nascent heterogeneous nuclear (hn) RNA levels (the unspliced transcripts), which may reflect alterations in transcription or the rate of splicing to mature transcripts (Elferink and Reiner 1996).

MATERIAL AND METHODS

Juvenile female trouts (individuals with average body weight of 55g and length of 12cm) were obtained from the Department of Salmonid Research in Rutki (Inland Fisheries Institute in Olsztyn, Poland). All fish were acclimated for a minimum of 2 weeks prior to exposure. For the chemical exposure procedure, individuals were randomly sampled, anesthetized by immersion in etomidate solution (Propiscin®; IRŚ; Poland), and injected intraperitoneally with either benzo[*a*]pyrene (B[*a*]P; Fluka, Germany) or cyclopenta[*c*]phenanthrene (CP[*c*]Ph, synthesized as described in Brzuzan et al. 2006b), each at dose of 10mg·kg^{-1} dissolved in corn oil as a carrier solution. Pure corn oil was used as negative control. For the time point 0h of exposure, tissue samples of 3 random individuals from the control group were taken immediately after the corn oil injection. After 2, 4, 8, 12 and 24h of exposure (T=7°C) 3 individuals were randomly taken from each of the experimental group, then were anesthetized and decapitated by severance of the spinal cord. Head kidney tissues were excised and immediately immersed in the RNA*Later*™ solution (Qiagen; Germany) according to the manufacturers recommendations and stored at -20°C.

RNA*Later*™ preserved head kidney tissues were homogenized and extracted for total RNA isolation using Total RNA Mini isolation kit (A&A Biotechnology; Poland) according to the manufacturer's protocol. To prevent genomic DNA contamination, RNA samples were incubated with RNase-free DNase I (Roche Diagnostics; Germany). Total RNA quality and quantity of all samples were estimated using BioPhotometer (Eppendorf; Germany), and the measured A_{260}/A_{280} ratios were high (>1.8). Total RNA was used to synthesize cDNA with RevertAid™ First Strand cDNA Synthesis Kit (Fermentas; Canada). The cDNA reaction for each sample contained 1μg of total RNA and 0.5μg of oligo(dT)$_{18}$ primers, and the reaction was performed according to the manufacturer's recommendations. At this step, RNA was examined for DNA contamination by performing RT-PCR on all samples in parallel. The first reaction set received reverse transcriptase, while the second set did not. No PCR products were observed in the absence of RT enzyme.

Primer pairs for Real-Time PCR (Table 1; Figure 1) were either chosen from the literature (Brzuzan et al. 2006a) or designed using Primer Express v.2.0 software (Applied Biosystems; USA). The assay was performed on ABI 7500 Real-Time PCR system thermalcycler (Applied Biosystems) in singleplex mode, and all samples were analyzed in duplicates. Each PCR reaction tube contained 10μl of Power SYBR® Green Master mix (Applied Biosystems), 2.5pmol of each (forward and reverse) primer, 2μl of cDNA as a template, and PCR-grade H_2O to final volume of 20μl. The reaction was performed in standard thermal conditions: 95°C for 10min, then 40 cycles of 95°C for 15s and 60°C for 1min.

Table 1. Real-Time PCR primers used in the study.

Primer pair		Sequence (5'→3')	Amplicon length (bp)	Reference
p53	Forward	agtggtacgagccctggcca	148	Brzuzan et al. (this paper)
	Reverse	tgatcgctggttcccctcaac		
p53hn	Forward	acgtggtgagacgctgccct	74	Brzuzan et al. (this paper)
	Reverse	gcctttcccgctgtgttgtg		
β-actin	Forward	gtggcgctggactttgagca	150	Brzuzan et al. 2006a
	Reverse	accgaggaaggagggctgga		

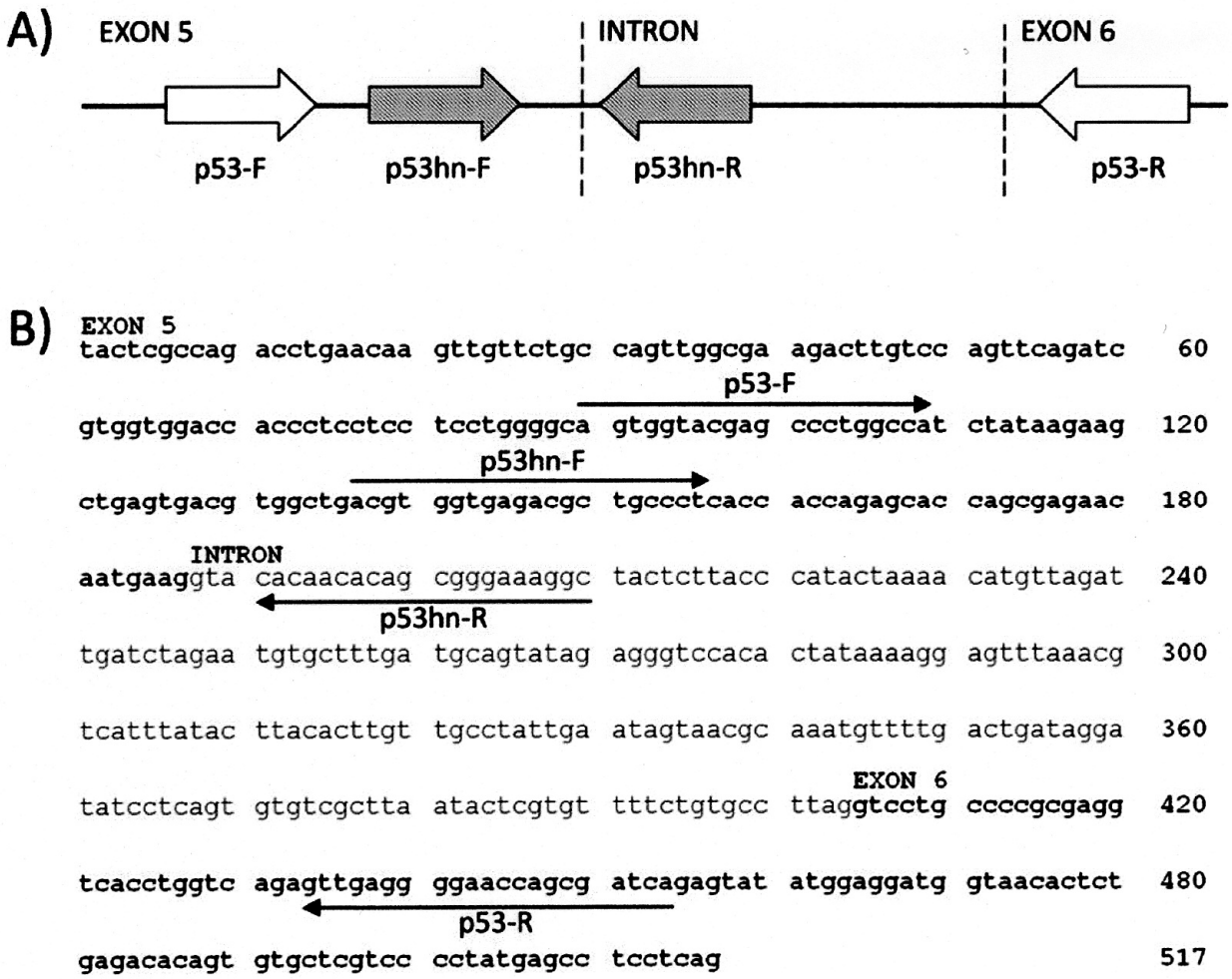

Figure 1. Design of PCR primers for examining of *p53* mRNA and hnRNA expression in head kidney of rainbow trout. (A) The positioning of primers within *p53* gene of rainbow trout is indicated by white and gray arrows. (B) Reference sequence of exons 5, 6 and intron (Kusser et al. 1994), with indicated positions of the primers used for Real-Time PCR assay.

On the plate, negative water controls (NTCs) were included to rule out the possibility of cross-contamination. To verify the quality of PCR products, a melting curve analysis was performed after each run, and the samples were additionally electrophoresed in standard 1.5% agarose gel and visualized with ethidium bromide under UV light. Finally, reverse transcribed DNA of exposed fish were subjected to DNA sequencing and sequence analysis (data not shown).

Data obtained from the assay was used to compute expression ratio of p53 mRNA and hnRNA, relative to β-actin which mRNA level was stable throughout the experiment. Briefly, the calculations were based on gene individual Real-Time PCR efficiency (E), and the threshold cycle difference (ΔCt) of an unknown sample versus a control ($\Delta Ct_{control-sample}$) according to the mathematical model: Expression Ratio (ER) = $[(E_{target})^{\Delta Ct\ target}]\cdot[(E_{reference})^{\Delta Ct\ reference}]^{-1}$ given by Pfaffl (2001). The efficiencies for each gene were estimated by running reactions with a dilution series of cDNA template with primer pairs used in the study, and the threshold cycle (Ct) vs. cDNA concentrations was plotted to calculate respective slope values (data not shown). The corresponding Real-Time PCR efficiencies were calculated according to the equation: $E = 10^{[-1/slope]}$ (Pfaffl 2001). The resulting E-values for each gene examined were the basis for the calculation and further randomization tests with REST© software (Pfaffl 2002), and the differences in target genes expression between control and treated samples were assessed in group means for statistical significance (REST©). In the study for each particular data set 2000 randomizations were performed. Statistical differences among the expression of either mRNA or hnRNA of p53 in the consecutive hours of particular treatments were tested with

analysis of variance (ANOVA) followed by Tukey's *post hoc* multiple comparison test, using Statistica software (version 8; StatSoft Inc., Tulsa, OK, USA). For the statistical analysis, normalized expression ratios were log-transformed and tested for normal distribution (Shapiro-Wilk W test) and for homogeneity of variance (Levene's test).

RESULTS AND DISCUSSION

The analysis of the transcription rate is an important step in understanding how a gene expression is modulated. One of the useful approaches to study gene regulation is RT-PCR method utilization to quantify nascent heterogeneous RNA of target gene (Elfrink and Reiner 1996), as an alternative for *in vitro* nuclear run-on-assays. This report describes the Real-Time RT-PCR assay, modified for the analysis of *p53* hnRNA transcripts. Specificity for *p53* hnRNA was obtained by using an intron-directed primer in the PCR reaction and reverse primer complementary to downstream intronic sequence (Table 1; Figure 1).

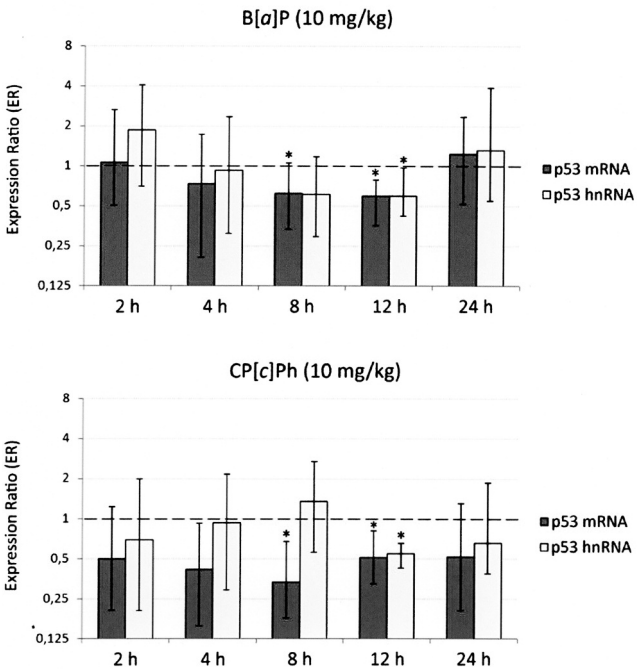

Figure 2. Time course expression patterns of p53 mRNA and hnRNA levels in the head kidney of rainbow trouts exposed to B[*a*]P and CP[*c*]Ph for 24h (*n*=3). The bars represent mean values of expression ratios (±S.E.), normalized by β-actin as an endogenous reference, relative to control sample (*n*=3, ER=1.00; dashed line) of a respective time of the experiment. Data were analyzed using randomization procedure (REST© 2008). Asterisks indicate groups that showed significantly lower levels of either mRNA or hnRNA whith respect to control group (*P*<0.05).

To investigate whether B[*a*]P and CP[*c*]Ph affect the process of *p53* gene transcription, juvenile trouts were exposed to either PAH compound for 24h. The effects of B[*a*]P on the time-profile of *p53* expression in head kidney of the exposed fish were very similar to those of CP[*c*]Ph (Figure 2). The changes of either *p53* mRNA or hnRNA expression levels in the consecutive hours of the treatment with either B[*a*]P or CP[*c*]Ph were modest (ANOVA; $P>0.05$). While the treatment with both compounds did not affect p53 RNA levels at the earliest time points (2, 4h; $P>0.05$), significant inhibition of *p53* gene expression was observed in each fish group for 8 and 12h of the experiment ($P<0.05$). Exposure with either B[*a*]P or CP[*c*]Ph resulted in p53 mRNA level decrease after 8h ($ER_{B[a]P}=0.63$, S.E. $0.33\div1.05$; $ER_{CP[c]Ph}=0.33$, S.E. $0.18\div0.67$), which continued by 12h time point of the treatment ($ER_{B[a]P}=0.59$, S.E. $0.36\div0.79$; $ER_{CP[c]Ph}=0.52$, S.E. $0.33\div0.81$). At this time point significant reductions of nascent hnRNA were observed for either compounds ($ER_{B[a]P}=0.60$, S.E. $0.43\div0.98$; $ER_{CP[c]Ph}=0.55$, S.E. $0.42\div0.67$). After 24h of exposure, the *p53* gene expression returned to the initial levels (Figure 2). A similar pattern of *p53* inhibition was observed in the study of Ray and Swanson (2004), where p53 mRNA level in dioxin treated human keratinocytes was significantly lower at 12h of the exposure as compared to control group, but it returned to an initial level by day 2. Intriguingly, at the later time points of that study, on days 6 and 8, dioxin decreased p53 mRNA levels again (Ray and Swanson 2004).

While the current study demonstrates that B[*a*]P and CP[*c*]Ph may suppress the expression of *p53* in head kidney of rainbow trout and that this occurs at the mRNA and hnRNA levels within 12h, exact mechanism for the gene repression is not known. From studies aiming at inactivation of TSG, it is known that methylation of CpG dinucleotides located in the promoter region is associated with low expression levels of a number of genes (e.g. Herman et al. 1996), including human *p53* (Agirre et al. 2003). Recent research indicates that this modification of the genomic DNA is as important as mutation shutting down TSG. Whereas the circumstances at which the methylated state of the gene promoter is acquired are not entirely resolved, it has become apparent that different environmental contaminants, including dioxin (Ray and Swanson 2003) or benzo[*a*]pyrene diol epoxide (Ye and Xu 2010), may be involved in promoting DNA methylation of an array of genes, including those encoding tumor suppressor proteins. Ray and Swanson (2004) proposed mechanism underlying the impact of dioxin on keratinocyte cells, which involves repression of the expression levels of *p53 via* an increase in *p53* promoter methylation that is mediated through AhR regulatory pathway. Bearing in mind that aryl hydrocarbon receptors are conserved in most of vertebrates (Hahn et al. 2005), it is quite possible that similar gene repression in AhR-dependent manner exists in the cells of lower vertebrates,

including fish. Then, if patterns of hypermethylation of TSG characterized in mammals apply to other vertebrates as well, the finding of this study would introduce mechanism by which the compounds may impact on important cell physiologic functions in the tissues of fish. Whether the transcriptional repression of *p53* in head kidney of rainbow trout by the two polycyclic aromatic hydrocarbons did involve promoter methylation, needs to be further investigated in detail.

In summary, to our knowledge this study is the first to measure changes of gene expression with RT-PCR on p53 heterogeneous nuclear RNA in fish exposed to PAH compounds. Our finding of the B[*a*]P and CP[*c*]Ph ability to repress *p53* in head kidney, a tissue with highly proliferating cells, supports the view that different PAHs may impact *p53* regulatory network in the cell, in part, by reducing the *p53* mRNA expression levels.

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