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Molecular characterization of cytochrome P450 1A and 3A and the effects of perfluorooctanoic acid on their mRNA levels in rare minnow (*Gobiocypris rarus*) gills

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ABSTRACT

Perfluorooctanoic acid (PFOA), a potentially toxic perfluorinated compound (PFC), has been widely disseminated in the environment. In the present study, rare minnows (*Gobiocypris rarus*) exposed to PFOA exhibited histopathological gill damage, including epithelial hyperplasia of the lamellae, inflammatory cell infiltration, and lamellar fusion. Cytochrome P450s (CYPs) play a central role in the metabolism and biotransformation of a wide range of endogenous substrates and foreign compounds. Thus, we studied the CYPs and the effects of waterborne PFOA on their corresponding mRNA levels in the gills of rare minnows. Two novel CYP cDNAs (CYP1A and CYP3A) were identified in rare minnow and their mRNAs were ubiquitously expressed in all tissues examined. Upregulation of CYP3A mRNA was observed in the gills of male rare minnows exposed to 30 mg/L PFOA, while no significant changes occurred in exposed females. In contrast, down regulation of CYP1A mRNA was detected in the gills of male and female minnows exposed to PFOA. However, the effect of PFOA on gill mRNA levels of their potential regulators, aryl hydrocarbon receptor (AhR) for CYP1A, and pregnane X receptor (PXR) for CYP3A, were not consistent with the observed effects of PFOA on the corresponding CYP mRNA concentrations. This suggests a different or more complex transcriptional regulation of CYP expression following PFOA exposure.

1. Introduction

Perfluorinated compounds (PFCs), such as perfluorooctanoic acid (PFOA), are remarkably stable compounds that do not undergo photolysis, hydrolysis, or biodegradation. Commercial use of PFCs for the past several decades has resulted in a broad distribution of stable precursors/metabolites in wildlife in terrestrial and aquatic environments (Giesy and Kannan, 2001; Prevedouros et al., 2006; Martin et al., 2004; Smithwick et al., 2005; Yeung et al., 2006; Dai et al., 2006). PFOA potentially produces hepatomegaly, induces hepatic peroxisomes, and alters endocrine function in rodents (O'Brien and Wallace, 2004; Seacat et al., 2002; Hanson et al., 2005; Lau et al., 2004). In teleosts, PFOA increases hepatic fatty acyl-CoA oxidase activity, increases oxidative damage, and affects the circulating sex steroid levels (Ankley et al., 2005; Oakes et al., 2004).

Cytochrome P450s (CYPs) are a large superfamily of hemeproteins that play key roles in the biotransformation of xenobiotics and in the synthesis and degradation of physiologically important endogenous substrates (Nelson et al., 1996; Guengerich, 1999). Alteration of the expression of CYPs markedly affects the potential risks and benefits of xenobiotics and, thus, is very important from a toxicological point of view (Williams et al., 1998). On the basis of sequence similarity, CYPs can be classified into various families and subfamilies (Nelson et al., 1993), including CYP1A, CYP3A, and CYP4A. Transcriptional expression of CYP1A and CYP3A are regulated via the aryl hydrocarbon receptor (AhR)-mediated pathway and the pregnane X receptor (PXR) pathway, respectively (Hahn, 2002; Kliewer et al., 2002). Ligand-activated AhR heterodimerizes with aryl hydrocarbon nuclear translocator (ARNT) and activates the transcription of target genes such as CYP1A through xenobiotic response elements (XREs) (Hahn, 2002). Upon activation, PXR, which is a member of the nuclear receptor superfamily, interacts with retinoid X receptor (RXR). This heterodimer binds to specific DNA sequences and regulates transcriptional expression of target genes, including CYP3A (Wang and LeCluyse, 2003; Mikamo et al., 2003).

PFOA is a potent inducer of hepatic CYPs in mice (Permadi et al., 1992). Recent studies using microarray analysis demonstrated that several CYP450 genes (e.g. CYP2B15 and CYP2J4) were induced by PFOA in rat liver (Guruge et al., 2006); however, information on the modulation of CYPs by PFOA in teleosts is relatively limited. Thus, the freshwater teleost rare minnow (*Gobiocypris rarus*) was used

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in the PFOA exposure experiments. Rare minnows are suitable for aquatic toxicity experiments due to their sensitivity to chemicals, small size, wide temperature range, ease of laboratory culture, and short embryonic development period (Qun-Fang et al., 2002; Zhong et al., 2005). We focused on the toxic effect of PFOA on the gills mainly because gills are the tissue involved in gas exchange and one of the primary tissues exposed directly to aquatic xenobiotic compounds. Here, in order to probe the xenobiotic compound response in rare minnow gills, CYP1A, and CYP3A cDNA was isolated, and the modulatory effects of PFOA on the CYP1A and CYP3A signaling pathways were further investigated by quantitative polymerase chain reaction (Q-PCR) method.

2. Materials and methods

2.1. Fish and PFOA exposure experiments

PFOA (98%) was purchased from Acros Organics (Geel, Belgium). All rare minnows were obtained from a laboratory hatchery. Two hundred and forty mature males and females (about 9 months old with body weight of 1.4 ± 0.4 g and total length of 47.7 ± 3.6 mm) were randomly assigned to eight 20 L glass tanks (30 individuals per tank) and acclimated for a week. Fish were supplied with dechlorinated tap water under continuous flow-through conditions at 25 ± 2 °C and subjected to a photoperiod of 16:8 h light:dark. Fish were fed a commercial granular food (Tetra, Melle, Germany) at a daily rate of 0.1% body weight. Waste and uneaten food were removed daily. After a 1-week acclimation period, 15 randomly selected male and 15 randomly selected female rare minnows (gender determined by observing the shape of the abdomen and the distance between the abdomen fin and the stern fin) were assigned to each of the four groups (0, 3, 10, or 30 mg/L PFOA). Each treatment was carried out in duplicate tanks (15 male and 15 female in each tank). These concentrations were selected based on previous reports (Oakes et al., 2004). The flow rate of the test solution was 8 L/h. After a 28-day exposure period, fish were anesthetized on ice. Gills from four male and four female fish from each treatment group were quickly dissected and fixed in 10% formalin for histological analysis. The gills from six male and six female fish in each group were removed and immediately frozen in liquid nitrogen and stored at −80 °C until further real-time PCR analysis. Various tissues, including muscle, liver, brain, gonad, gill, and intestine from two male and two female fish in the control group were used for semi-quantitative PCR analysis. The remaining samples were used for proteomic analysis.

2.2. Histological analysis

Formalin-fixed gills were processed sequentially in ethanol, xylene, and paraffin. Tissues were then embedded in paraffin wax, cut in 4–5-μm slices parallel to the surface, mounted on slides, and stained with hematoxylin and eosin.

2.3. Total RNA isolation and cloning of CYP1A and CYP3A cDNAs from rare minnow gill

Total RNA and first strand complementary DNA (cDNA) synthesis were performed as described in the manufacturer's instructions. Briefly, total RNA was extracted from 20 mg of gill tissue from each individual fish using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and treated with RNase-free DNase I (Qiagen, Hilden, Germany) to remove any remaining genomic DNA. Isolated RNA was quantified based on the A260 value. Approximately 1 μg of total RNA from each sample was reverse transcribed using an oligo $(dT)_{15}$ primer (Promega, Madison, WI, USA) and M-MuLV reverse transcriptase (New England Biolabs, MA, USA) with the following parameters: 42 °C for 60 min followed by 98 °C for 5 min and 4 °C for 15 min.

To obtain full-length rare minnow CYP1A and CYP3A cDNAs sequences, 5′- and 3′-rapid amplification of cDNA ends (RACE) reactions were performed using the SMART™ RACE cDNA Amplification Kit (Clontech Laboratories, USA) according to the manufacturer's protocol. The gene-specific primers (GSPs) were designed and synthesized according to the partial sequences of CYP1A and CYP3A ESTs (GenBank accession numbers EE396294 and EE396426, respectively) which were from our rare minnow adult liver cDNA library. The GSPs for CYP1A and CYP3A are as follows: CYP1A GSP1, 5′-GATTGCTTCCCACCTCCAGCACATTTC-3′; and GSP2, 5′-CAATGGCTCTAACGGTTCTCCCTGTGT-3′. CYP3A GSP1, 5′-GGACACT GCGGATTCTCCTCCAGTCAT-3′; GSP2, 5′-AACATGGGCTCTGCTCGTG CTGTTGGT-3′. For 5′-RACE, the first strand cDNA was synthesized from the total RNA using the 5′-CDS Primer and SMART II A oligo and amplified by PCR using GSP1 and 10× universal primer A mix. For 3′-RACE, the first strand cDNA was synthesized from the total RNA using the 3′-CDS Primer A. The cDNA was amplified by PCR using GSP2 and 10× universal primer A mix. Amplification of cDNA ends was performed for 32 cycles with the following conditions: 94 °C for 30 s, 68 °C for 30 s, and 72 °C for 3 min. The amplified products were cloned into a pGEM-T vector and sequenced.

2.4. Sequence alignment

To examine the sequence conservation of rare minnow CYP1A and CYP3A, their orthologs from other species were retrieved, and their amino acid sequences were aligned using ClustalW multiple alignment software in EBI.

2.5. Semi-quantitative PCR

To determine the tissue distribution of CYP1A and CYP3A mRNA levels, total RNA was isolated from various adult rare minnow tissues including skeletal muscle, liver, brain, gonad, gill, and intestine from two male and two female unexposed fish. The isolated total RNA was treated with DNase I, and reverse transcribed to cDNA. Expression of CYP1A and CYP3A was analyzed individually by semi-quantitative PCR using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal control. The PCR primers for GAPDH were designed according to a GAPDH EST (GenBank Accession Number EE337198) which was from our rare minnow adult liver cDNA library. The primer sequences for CYP1A, CYP3A, and GAPDH are listed in Table 1. To quantitate the expressed mRNA levels, different cycles of semi-quantitative PCRs were performed, and the band densities of the resulting products were measured using Bandleader Software. A cycle number within the exponential phase of

Table 1
Primers used for the analysis of gene expression by PCR

Gene name	5′–3′Primer sequence	Amplicon size (nucleotides)
CYP1A	Forward: CAATCATCGGAAATGTGCTG Reverse: AGAACTCCTCGCCCTGTTTG	171
CYP3A	Forward: CTGTGCTGTGCATCATGGATC Reverse: CGGAAGTTCCTTCTGTTGGTG	88
AhR	Forward: GCTACAGGCTATAAATGGCTT Reverse: ATCCGACTGATGGAACCC	103
PXR	Forward: GACGGGAATTTGGGAGTG Reverse: GCATCAGCACATACTCCTCCT	149
GAPDH	Forward: CGTGCTGCTGTCCAGTCCAA Reverse: GCCGCCTTCTGCCTTAACCT	138

the amplification curve was chosen for quantifying the expression of each gene in subsequent experiments.

2.6. Quantitative real-time PCR

Quantitative PCR was performed to evaluate CYP1A, CYP3A, AhR, and PXR expression in gills of male and female rare minnow exposed to PFOA using a QuantiTect SYBR Green PCR kit (Qiagen, Hilden, Germany) and the Stratagene Mx3000P qPCR system (Stratagene, La Jolla, CA, USA). GAPDH was chosen as an internal control. Reactions were performed in 25 μL total reaction volume that contained 12.5 μL of 2× QuantiTect SYBR Green PCR master mix, 1 μL cDNA template, and 1 μM of each target-specific primer. The primers for rare minnow PXR and AhR were designed from conserved regions of corresponding genes from other teleosts, such as zebrafish (*Danio rerio*) and rainbow trout (*Oncorhynchus mykiss*), and are listed in Table 1. The cDNA from each fish was performed in duplicate in each real-time PCR analysis. Thermal cycling conditions were as follows: 95 °C for 15 mins followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 10 s. After PCR, a melting curve analysis was performed to demonstrate the specificity of the PCR product as displayed by a single peak (data not shown). After verifying that the efficiencies of CYP1A, CYP3A, AhR, PXR, and GAPDH amplifications were approximately equal, differences in mRNA levels were calculated using the $2^{-\Delta\Delta C_t}$ method, where C_t is the threshold cycle, indicating the fractional cycle number at which the amount of amplified target reaches a fixed threshold (Livak and Schmittgen, 2001). The method was based on the equation: the relative mRNA ratio of a target gene $= 2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = (C_{t(\text{target})} - C_{t(\text{GAPDH})})_{\text{treatment}} - (C_{t(\text{target})} - C_{t(\text{GAPDH})})_{\text{control}}$.

2.7. Statistical analyses

All values are expressed as mean ± S.E. from one representative experiment. All experiments were performed two to three times to confirm the results. The Fisher's least significant difference test was used to determine the statistical difference between groups. A p-value of <0.05 was considered significant. All analyses were carried out using SPSS software (Version 13.0).

3. Results and discussion

3.1. Pathological changes in gills following PFOA exposure

Several histopathological studies of PFOA exposure on rodents (Pastoor et al., 1987; Berthiaume and Wallace, 2002) revealed that the liver was the primary target organ for PFOA. Treatment with this compound initiated a characteristic sequence of morphological and biochemical changes in liver due to increased peroxisomes and beta-oxidation of peroxisomal fatty acid. High doses of PFOA resulted in multifocal coagulation and liquefaction necrosis in the liver (Son et al., 2008). Relatively few pathological evaluations of PFOA exposure have been performed in teleosts (Wei et al., 2007). In the present study, we found that fish from PFOA-exposed groups exhibited apparent gill damage, and similar histopathological changes were observed in both males and females (Fig. 1). No detectable changes were observed in fish gills from unexposed groups (Fig. 1A). Branchial impairments in fish increased in severity with increasing doses of PFOA exposure. Gills from fish exposed to 3 mg/L PFOA displayed a slight expansion in the lumen of capillaries and modest blood congestion (Fig. 1B). Widespread

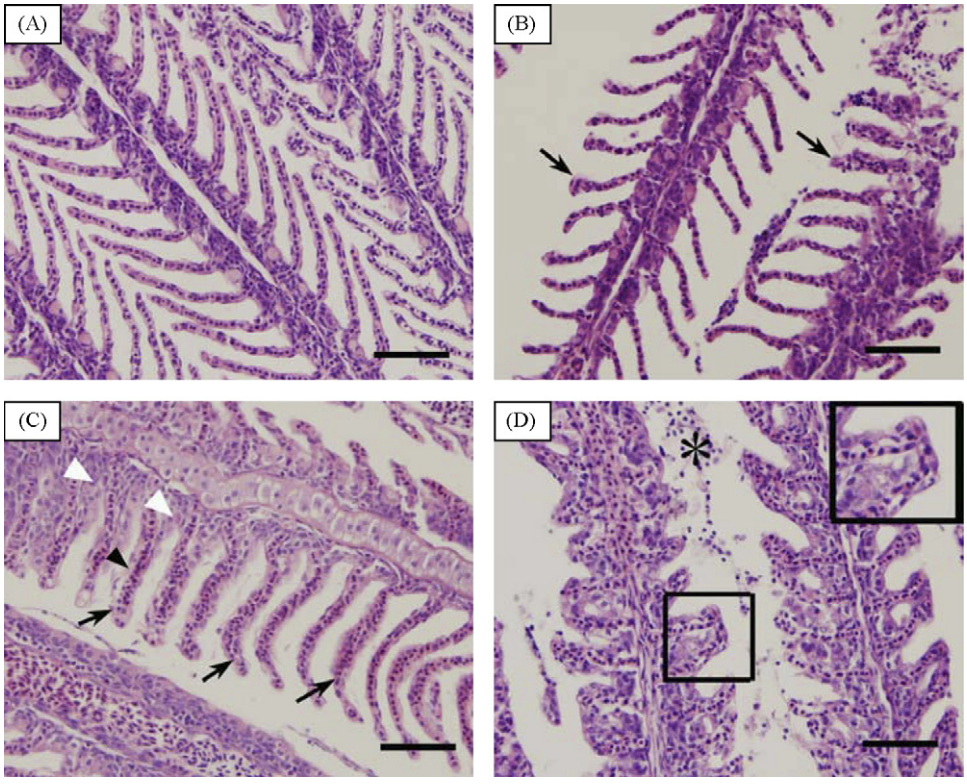

Fig. 1. Gills from rare minnows following PFOA exposure. Photomicrographs of gill sections (4–5 μm) stained with hematoxylin and eosin. (A) Unexposed gills (40×). (B) Gills from fish exposed to 3 mg/L PFOA (40×). Secondary lamellae displayed a slight expansion in the lumen of capillaries and exhibited modest blood congestion (arrow). (C) Gills from fish exposed to 10 mg/L PFOA (40×). Secondary lamellae exhibited widespread dilation of lumens of capillaries with concomitant blood congestion (arrow) as well as hyperplasia of mucous cells (white arrowhead) and epithelia (black arrowhead). (D) Gills from fish exposed to 30 mg/L PFOA (40×). The gills exhibited lamellar fusion (see the rectangle in the middle of panel D and the corresponding magnification at the right top corner) together with inflammatory cell infiltration (asterisk).

capillary lumen dilation with blood congestion and disintegrated pillar cell systems were observed in gills from fish exposed to 10 mg/L PFOA (Fig. 1C). In addition, hyperplastic mucous cells in the primary lamellae as well as epithelial hyperplasia of the secondary lamellae occurred in gills of fish exposed to 10 mg/L PFOA. The most severe damage was observed in gills from fish exposed to 30 mg/L PFOA (Fig. 1D). In these fish, the gills exhibited considerable lamellar fusion together with inflammatory cell infiltration. Moreover, some lamellae appeared distorted and club shaped.

(A)

```
gactcctcagcgcgtgagtgactctgatgcacagaagaggaaccttcatcagcactgtaacagaagaaagca        1
atggctctaacggttctccccgtgttgggtccgatctctgtatccgaaagcctggtggccatcatcaccatatgtgtg  73
M  A  L  T  V  L  P  V  L  G  P  I  S  V  S  E  S  L  V  A  I  I  T  I  C  V
gtgtacctgctcatgcgcctcatgcgtgtgaagatcccagagggcctccacaagctcccggggcccaagcctctccca 151
V  Y  L  L  M  R  L  M  R  V  K  I  P  E  G  L  H  K  L  P  G  P  K  P  L  P
atcatcggaaatgtgctggaggtgggaagcaatccacacctgagcctgaccgccatgagtaagtgctacggccccatc 229
I  I  G  N  V  L  E  V  G  S  N  P  H  L  S  L  T  A  M  S  K  C  Y  G  P  I
ttccagatccagatcggcacgcgtcccgtggtggtgctcagcgggaacgacgtgatccggcaggctctcctcaaacag 307
F  Q  I  Q  I  G  T  R  P  V  V  V  L  S  G  N  D  V  I  R  Q  A  L  L  K  Q
ggcgaggagttctccggccgcccggatctctacagcaccaggtacatcagcgacgggaagagtctggccttcagcacg 385
G  E  E  F  S  G  R  P  D  L  Y  S  T  R  Y  I  S  D  G  K  S  L  A  F  S  T
gatcaggtgggagtgtggcgcgcccgcaggaaactggccctcagcgccctgcgcaccttctccacgctgcagtcccag 463
D  Q  V  G  V  W  R  A  R  R  K  L  A  L  S  A  L  R  T  F  S  T  L  Q  S  Q
agctcccagtattcctgcgctctggaggagcacatcagcaaagagggcctgtacctgatcgagcggctgcactccgtg 541
S  S  Q  Y  S  C  A  L  E  E  H  I  S  K  E  G  L  Y  L  I  E  R  L  H  S  V
atgaagtccgacgggagcttcgacccctccggcacatcgtggtgtccgtggctaacgtgatctgcggaatatgcttc 619
M  K  S  D  G  S  F  D  P  F  R  H  I  V  V  S  V  A  N  V  I  C  G  I  C  F
ggccggcgctacagccacgacgatgatgagctggtgagtctggtcaacccgagcgacgagttcgggaagatcgtggga 697
G  R  R  Y  S  H  D  D  D  E  L  V  S  L  V  N  P  S  D  E  F  G  K  I  V  G
agcgggaaccctgcggatttcatccctatcctgcggatcctgcccagctgctcgatgaagaagttcctggccatcaac 775
S  G  N  P  A  D  F  I  P  I  L  R  I  L  P  S  C  S  M  K  K  F  L  A  I  N
gctcgcttcagcgcgttcatgcagaagatcgtgaaggaccactacgactccttcagcaaggacaacatccgtgacatc 853
A  R  F  S  A  F  M  Q  K  I  V  K  D  H  Y  D  S  F  S  K  D  N  I  R  D  I
accgactcgctgatcgaccactgcgaggaccacaagctggacgagaactccaacgtccaggtgtccgatgagaagatt 931
T  D  S  L  I  D  H  C  E  D  H  K  L  D  E  N  S  N  V  Q  V  S  D  E  K  I
gtgggcatcgtcaatgacctcttcggagctggattcgacaccatcagcacggctctgtcctggtctgtcgtctatctc 1009
V  G  I  V  N  D  L  F  G  A  G  F  D  T  I  S  T  A  L  S  W  S  V  V  Y  L
gtggcgtatcccgagatccaggagcgactgcagagagagctgaatgagaaggtgggaatggagcgcacgccgcgcctg 1087
V  A  Y  P  E  I  Q  E  R  L  Q  R  E  L  N  E  K  V  G  M  E  R  T  P  R  L
tccgacaggaccgagcttccgcttctcgaggccttcatcctggagatcttccggcattcctccttcctcccctcacc 1165
S  D  R  T  E  L  P  L  L  E  A  F  I  L  E  I  F  R  H  S  S  F  L  P  F  T
attcctcactgcacgactaaagacacgtcgctcaacggattcttcattcccaaagacacctgtgtgtttgtgaaccag 1243
I  P  H  C  T  T  K  D  T  S  L  N  G  F  F  I  P  K  D  T  C  V  F  V  N  Q
tggcaggtcaaccacgacccggaactgtggaaagatccgtcctgcttcaacccggaccgcttcctcaccgcagacggg 1321
W  Q  V  N  H  D  P  E  L  W  K  D  P  S  C  F  N  P  D  R  F  L  T  A  D  G
acggacctcaaccggatggagggagagaaggtgctggtgttcggcctggggaagcgccgctgcatcggagagtccatc 1399
T  D  L  N  R  M  E  G  E  K  V  L  V  F  G  L  G  K  R  R  C  I  G  E  S  I
ggacgcgcagaggtcttcctgttcctggccatcctgctgcagaggttagagttcagcgggatgcccggagaactgctg 1477
G  R  A  E  V  F  L  F  L  A  I  L  L  Q  R  L  E  F  S  G  M  P  G  E  L  L
gatatgaccccggagtacgggctgaccatgaagcacaagcgctgtctcctgcgagtggcgccgcgccccggctacgcc 1555
D  M  T  P  E  Y  G  L  T  M  K  H  K  R  C  L  L  R  V  A  P  R  P  G  Y  A
tcgtgatcccacccgggcccagtctccgccggctcgcaatgccaaatatctgaaagatgttcgtctgggttctgatgc 1633
S
actgcagttattcaaccatcataatcagatccgctgtatcacctctatcatctctgctctcagacgcaaacgctgatc 1711
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tcctgtctcattacatcccataatgtgatcaccgtgtgtaaatatacagcctcttctaccatgtgcctgatcttctgt 1867
gtgtacagactgttgccctgctgttttggtatttatcagggacatgatccatgataatgaaggaaatctgaagtactg 1945
aacacatcattcctgtaaatgtgcgctcatttcaactctgcctgcattgtttttgcatagcatattcatgattgccaa 2023
atgattaataaaactttcattcaaaaaaaaaaaaaaaaaaaaaaaaaaaaa  2101
```

Fig. 2. Nucleotide and deduced amino acid sequences of rare minnow CYP1A (A) and CYP3A (B). The nucleotide sequences for rare minnow CYP1A and CYP3A were deposited in GenBank with accession numbers: EU106660 and EU106659, respectively. The numbers at the end of each line refer to the nucleotide positions. The start codon and stop codon are indicated in bold. The characteristic heme-binding domain is indicated with a shadow box. The polyadenylation signal, aataaa, is underlined.

(B)

```
ccagagaccatgagcttcggtctgttcttctccgctgaaacatgggctctgctcgtgctgttggtgacgcttctggtt  1
        M  S  F  G  L  F  F  S  A  E  T  W  A  L  L  V  L  L  V  T  L  L  V
atatatggatcctggactcacagtattttcaagaagttgggaatacagggtcccaaacccatcccgttcttcggaaca  79
I  Y  G  S  W  T  H  S  I  F  K  K  L  G  I  Q  G  P  K  P  I  P  F  F  G  T
atgctgagatatagagaggggtttcataacttcgatctcgagtgtttcaggaagtacggacgagtctggggtatctac  157
M  L  R  Y  R  E  G  F  H  N  F  D  L  E  C  F  R  K  Y  G  R  V  W  G  I  Y
gatgcgaggcagcctgtgctgtgcatcatggatcaatccatcatcaaaaccatcctgattaaagaatgttactctctc  235
D  A  R  Q  P  V  L  C  I  M  D  Q  S  I  I  K  T  I  L  I  K  E  C  Y  S  L
ttcaccaacagaaggaacttccgtctgaacgggcccctgtacgacgctgtgtccatcgttgaagacgatgactggagg  313
F  T  N  R  R  N  F  R  L  N  G  P  L  Y  D  A  V  S  I  V  E  D  D  D  W  R
agaatccgcagtgtcctctcaccctccttcaccagcgggaggttaaaggagatgtttggcatcatgaagacacactct  391
R  I  R  S  V  L  S  P  S  F  T  S  G  R  L  K  E  M  F  G  I  M  K  T  H  S
caaattctagttcagaatctggggaagtcagcaacacgaggagaaaacgtggatattaaagagttcttcggtgcgtac  469
Q  I  L  V  Q  N  L  G  K  S  A  T  R  G  E  N  V  D  I  K  E  F  F  G  A  Y
agtatggatgtggtgaccagcacggcgttcagcgtggacatcgactccctcaacaaccccaaagatccattcgtgacc  547
S  M  D  V  V  T  S  T  A  F  S  V  D  I  D  S  L  N  N  P  K  D  P  F  V  T
aacattaagaaaatgttgaagtttgacttactgaaccctgtgttcctgatcagtgctatgtttcctttcatcaccccc  625
N  I  K  K  M  L  K  F  D  L  L  N  P  V  F  L  I  S  A  M  F  P  F  I  T  P
ctcatggagaaaatggattttgccttttcccgacatctgtgacggatttcttctacgcagccttgcagaagatcaag  703
L  M  E  K  M  D  F  A  F  F  P  T  S  V  T  D  F  F  Y  A  A  L  Q  K  I  K
tctgaaagagtggtcagcgatcacaacaagaagagagttgacttcctgcagctgatggtcgattctcagacggcaggg  781
S  E  R  V  V  S  D  H  N  K  K  R  V  D  F  L  Q  L  M  V  D  S  Q  T  A  G
aaaactctgcctggtgaggaccacacggagaaaggtctgagcgaccacgagatcttatcgcagtccatgatcttcatc  859
K  T  L  P  G  E  D  H  T  E  K  G  L  S  D  H  E  I  L  S  Q  S  M  I  F  I
ttcgccggttacgagaccagcagcagcactctggcgttcttcttctacaatctcgcaaccaacccagaggccatggag  937
F  A  G  Y  E  T  S  S  S  T  L  A  F  F  F  Y  N  L  A  T  N  P  E  A  M  E
aaactgcaggaggagatcgacgagaccttcccgaataaggccccggtggactacgaagctgttatgaacatggactat  1015
K  L  Q  E  E  I  D  E  T  F  P  N  K  A  P  V  D  Y  E  A  V  M  N  M  D  Y
ctggacgctgcgctgaacgagtccctccggctgttccctgttgctgctcgactcgagcgggtctgcaagaaaacagtg  1093
L  D  A  A  L  N  E  S  L  R  L  F  P  V  A  A  R  L  E  R  V  C  K  K  T  V
gacatcaacggcctcctcatccctaaagacatggtcgtcatgatcccgacctacgccctccacagagacccggattac  1171
D  I  N  G  L  L  I  P  K  D  M  V  V  M  I  P  T  Y  A  L  H  R  D  P  D  Y
tggagtgaccccgagagcttcaagccggagaggttcactaaaggaaacaaggagtcgattgacccctacatgtacatg  1249
W  S  D  P  E  S  F  K  P  E  R  F  T  K  G  N  K  E  S  I  D  P  Y  M  Y  M
cccttcggcctcgggcccaggaactgcatcgggatgagatttgctcaggtgaccatgaagctggccatcgtggagatc  1327
P  F  G  L  G  P  R  N  C  I  G  M  R  F  A  Q  V  T  M  K  L  A  I  V  E  I
ctgcagaggttcgacgtctctgtgtgtgcggagacacaggttcctctggagctcggcctcagtggtctactggctccc  1405
L  Q  R  F  D  V  S  V  C  A  E  T  Q  V  P  L  E  L  G  L  S  G  L  L  A  P
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K  D  P  I  K  L  K  L  K  P  R  T  A  P  D  V  C  N  N  T  S
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aattagattaaactgttaagaaattatatggaaatgagcaagttttactttgaaataaagtctgcaaaaaaaaaaaaa  1795
aaaaaaaaaaaaaaa  1873
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Fig. 2. (*Continued*)

3.2. Cloning of CYP1A and CYP3A cDNAs from rare minnow gill

The cDNA sequences obtained based on the corresponding CYP1A and CYP3A ESTs' seq were 2151 bp and 1888 bp and were submitted to the GenBank with Accession Numbers EU106660 and EU106659, respectively. The cloned 2151 bp rare minnow CYP1A cDNA is a full-length includes 72 bp of the 5′-UTR, 1566 bp ORF which encodes for 521 amino acids (aa) and 513 bp of the 3′-UTR (Fig. 2A). Using on-line Compute p*I*/MW program, the predicted molecular weight (MW) for CYP1A protein is 58405.53 and the theoretical p*I* is 6.38. In Fig. 2A, the heme-binding domain, FGLGKRRCIG, is shaded and the polyadenylation signal (aataaa) is underlined. The 1888 bp of the rare minnow CYP3A cDNA includes a reading frame which encodes 512 aa (Fig. 2B). The predicted MW for CYP3A protein is 58404.80 Dalton and the theoretical p*I* is 6.24. In Fig. 2B, the heme-binding domain FGLGPRNCIG is also shaded and the polyadenylation signal (aataaa) is underlined.

3.3. Sequence comparison of rare minnow CYP1A and CYP3A

Extensive phylogenetic analyses of CYP1 and CYP3 gene families in vertebrates have been carried out and these suggest

that there are high similarities among the CYP1A and CYP3A subfamilies, respectively (McArthur et al., 2003; Kubota et al., 2006). The deduced rare minnow CYP1A and CYP3A amino acid sequences were aligned with their orthologs. Based on the putative amino acid sequence, CYP1A is also highly conserved with teleost CYP1A isozymes. Rare minnow CYP1A exhibited 87% identity to zebrafish CYP1A1 (AAL54873), 77% identity to rainbow trout CYP1A3 (AAD45966) and atlantic salmon CYP1A (AAM00254), 76% identity to black porgy CYP1A1 (ABI54450), and 59% identity to baikal seal CYP1A1 (BAF58167). The rare minnow CYP3A exhibited 83% identity to zebrafish CYP3A65 (GenBank accession number AAH72702), 68% identity to rainbow trout CYP3A45 (AAK58569) and 70% identity to killifish CYP3A30 (AAF14117), and 68% identity with largemouth bass CYP3A68 (ABH05129) and Japanese medaka CYP3A40 (AAK37960). Moreover, CYP3A exhibited considerable sequence identity with mammals, including 57% identity to Bolivian squirrel monkey CYP3A4 (ABG79670) and 54% identity to Rhesus monkey CYP3A66 (AAT49270). The apparent high degree of sequence similarity of these proteins in teleosts suggested that CYP1A and CYP3A isozymes were highly conserved during teleost evolution.

3.4. Tissue expression of CYP1A and CYP3A transcripts in rare minnow

The tissue distribution of CYP1A and CYP3A were examined by semi-quantitative PCR in rare minnow. The cDNA samples from rare minnow muscle, liver, brain, gonad, gill, and intestine were amplified for 30 cycles with CYP1A and CYP3A primers. Parallel reactions using GAPDH specific primers were performed for 26 cycles with the same amplification parameters. The CYP1A and CYP3A mRNAs were ubiquitously expressed in the various tissues examined, although CYP3A exhibited relatively weak expression in muscle compared with that in other tissues (Fig. 3). High expression in liver and intestine was also demonstrated for *Rivulus marmoratus* CYP1A (Lee et al., 2005) and zebrafish CYP3A (Tseng et al., 2005).

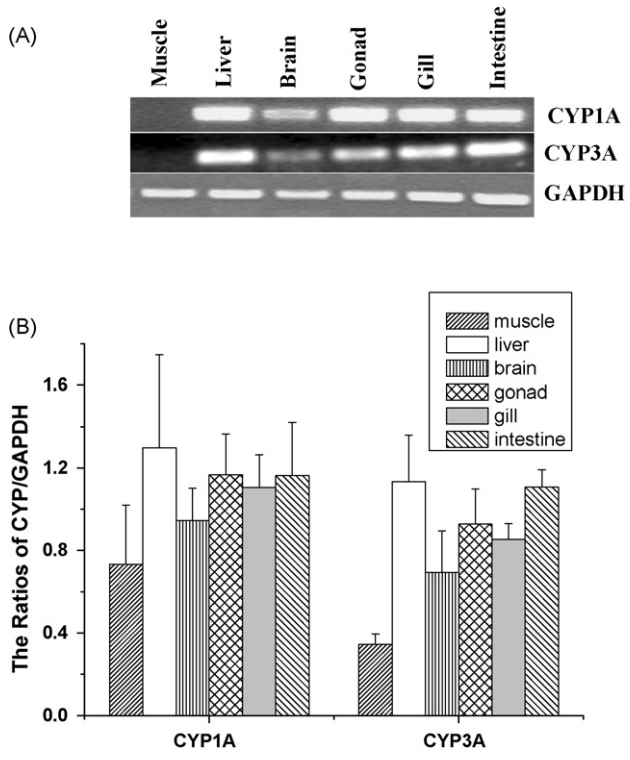

Fig. 3. Examination of rare minnow CYP1A and CYP3A mRNAs in various tissues. (A) 2% gel analysis of the semi-quantitative PCR products for the targeted genes in various tissues as marked. Total RNA extraction and PCR conditions were described in Section 2.5. The intensities of the GAPDH (internal standard) bands were used for normalization. (B) The density of electrophoretically separated PCR products was determined using Bandleader Software and normalized for GAPDH levels. Each value represents the mean ± S.E. from one representative experiment.

3.5. Determining CYP1A and CYP3A mRNA concentrations the gills of rare minnow exposed to PFOA

Perfluoro compounds can inhibit and induce several CYP isozymes and their associated detoxification activities, even though the compounds are not themselves metabolized (Armstrong and Lowe, 1989). In the present study, the levels of CYP1A (Fig. 4A) and CYP3A (Fig. 4B) mRNAs in rare minnow gills exposed to PFOA was examined using real-time qPCR. CYP1A mRNA levels in gills of both sexes of rare minnow were decreased after the PFOA treatments and the decreases were statistically significant in the male after 3 and 10 mg/L exposure and in the female after 30 mg/L treatment. In contrast, exposure to 30 mg/L PFOA significantly elevated the CYP3A transcript levels in male gill samples. The increase and the decrease in the CYP3A mRNA level observed in the 10 and 3 mg/L treatment, respectively, in male gill was not statistically significant.

Cytochrome P450 catalyzes the oxidative and reductive metabolism of many drugs and environmental chemicals as well as of endogenous compounds (Guengerich, 1991; Okey, 1990). The biosynthesis and metabolism of cholesterol, bile acids, and major classes of steroid hormones are essential to maintaining physiological homeostasis. Thus, we speculated that the alteration of CYP expression in rare minnow in response to PFOA exposure may alter the metabolism levels of endogenous substances, such as prostaglandins and steroid hormones, disrupt the physiological homeostasis status, and contribute to the toxic effect of PFOA on rare minnow gills.

3.6. The effect of PFOA on the AhR and PXR mRNAs levels in rare minnow gills

Transcriptional expression of CYP1A and CYP3A may be induced via the AhR- and PXR-mediated pathways, respectively (Hahn, 2002; Kliewer et al., 2002). Therefore, AhR and PXR expression was examined in rare minnow gills exposed to PFOA. Exposure induced both AhR (Fig. 4C) and PXR expression (Fig. 4D). AhR mRNA levels exhibited a 1.8-fold increase in male rare minnows in the 10 and 30 mg/L PFOA treatments compared to the controls. In females, the AhR mRNA levels were increased approximately 2.5-fold in 10 mg/L treatment group. PXR upregulation was statistically significant in the 3 and 30 mg/L treatments in both male and female. A similar trend was also observed in the other treatment groups (10 mg/L male group, $p = 0.084$ and 10 mg/L female group, $p = 0.089$). Interestingly, mRNA expression of the cloned CYPs in rare minnow was not consistent with their corresponding regulators, especially AhR and CYP1A. The expression of AhR in gill samples was upregulated in PFOA treatment groups, while CYP1A transcripts were reduced.

In conclusion, PFOA exposure leads to apparent pathological damage in rare minnow gills. These effects include epithelial hyperplasia and inflammatory cell infiltration. In addition, PFOA exposure alters the transcriptional expression levels of CYP1A and CYP3A, and these changes may further alter their detoxification and metabolism activities to endogenous and exogenous compounds, and contribute partly to the toxic effect of PFOA.

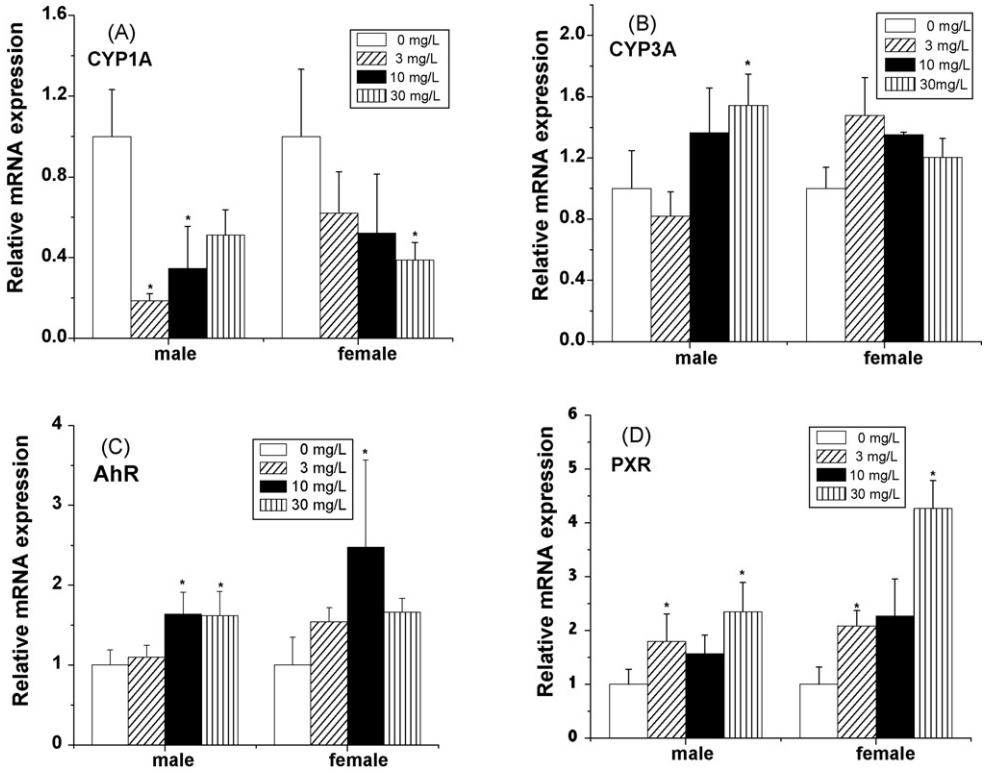

Fig. 4. Quantitative real-time PCR analysis of mRNA levels of CYP1A (A), CYP3A (B), AhR (C), and PXR (D) in rare minnow gills. The expressed mRNA levels in males and females exposed to PFOA for 28 days were quantified. Bars represent the relative mRNA expression in exposed animals versus control animals. GAPDH was used for normalization. Values are presented as the mean ± S.E. for six fish per group. Asterisks denote significant differences ($p < 0.05$) compared with the control.

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