Alterations in mRNA expression of steroid receptors and heat shock proteins in the liver of rare minnow (*Gobiocypris rarus*) exposed to atrazine and p,p'-DDE

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ABSTRACT

The chaperon role of heat shock proteins (HSPs) throughout the life cycle of steroid receptors have been demonstrated *in vitro*. However, the actions of HSPs in steroid pathways in animals especially in fish were unclear. In this study, sexually mature rare minnows (*Gobiocypris rarus*) were exposed to typical endocrine disruptors (atrazine and p,p'-DDE). Hypertrophy of hepatocytes at the 333 µg/l atrazine treatment and atrophy of hepatocytes in all p,p'-DDE treatments were observed. The expression of liver *hsp70* and *hsp90* in atrazine treatments were significantly up-regulated. Moreover, remarkable increases in the expression of androgen receptor (*ar*) and estrogen receptor (*er*) were observed, while alterations of the glucocorticoid receptor (*gr*) expression was not significant in atrazine exposed fish. The expression of *ar*, *er*, *gr*, *hsp70* and *hsp90* were significantly suppressed following p,p'-DDE exposure. These results demonstrate that the expression of *hsp70* and *hsp90* is altered along with changes of steroid receptors *in vivo*. Therefore, the results are consistent with the possibility that in fish heat shock proteins (HSPs) play chaperon roles for the steroid receptors *in vivo*, which also concurs with previous *in vitro* mammalian studies.

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1. Introduction

In recent years the highly conserved family of heat shock proteins (HSPs) has received extensive attention for their roles in response to stress. A chaperon role of HSPs throughout the life cycle of steroid receptors (the androgen receptor (AR), estrogen receptor (ER) and glucocorticoid receptor (GR), etc.) has been demonstrated *in vitro* by both biochemical experiments (Scherrer et al., 1990; Reik et al., 1991) and RNA interference in mammalian tissue culture cells (Whitesell and Cook, 1996). However, the roles of HSPs in steroid pathways in animals especially in fish were still not clear. Since many endocrine disruptions were thought to result from the interactions between contaminants and the steroid receptors (McLachlan, 1993), it is important to find out the actions of HSPs in steroid pathways when fish were exposed to environmental contaminants.

Atrazine (2-chloro-4-ethylamino-6-isopropyl-amino-s-triazine) is a triazine herbicide widely used for the control of weeds and grasses. Atrazine concentrations up to 108 µg/l have been reported in rivers of North America (USEPA, 2002). In China, atrazine concentrations exceeding the standards for drinking water (3 µg/l) have also been reported in Guanting reservoir

(Ren et al., 2002) and Taihu Lake (Dong et al., 2006). p,p'-DDE (1,1-dichloro-2,2-bis(p-chlorophenyl)-ethylene) is the major metabolite of the insecticide dichlorodiphenyltrichloroethane (DDT) which has been banned in many countries. However, DDT and its degradates (DDE and DDD) are still consistently detected in river water and sediments of many countries (Peris et al., 2005; Xue et al., 2006). For instance, p,p'-DDE concentrations up to 546 ng/l have been recently reported in the Beijing Guanting reservoir, China (Xue et al., 2006). These compounds are prone to accumulate in different tissues (Harshbarger et al., 2000) and thus may pose a significant risk to aquatic organisms. Both atrazine and p,p'-DDE have been demonstrated to cause endocrine disruptions: atrazine was shown to promote the conversion from androgens to estrogens by inducing the activity of aromatase (Crain et al., 1997); while p,p'-DDE turned out to be a potent androgen antagonist (Kelce et al., 1995). However, their effects on HSPs have rarely been reported (Dowling et al., 2006).

Rare minnow (*Gobiocypris rarus*) is considered to be an appropriate species for the assessment of endocrine disrupting chemicals due to its small size, ease of culture, short life cycle and prolific egg production with high fertilization and hatching rates (Wang, 1992; Zha et al., 2007). Effects on the gonadal and thyroid systems by environment pollutants have been demonstrated in rare minnow (Zha et al., 2007; Zhang et al., 2008; Li et al., 2009).

In this study sexually mature rare minnow were exposed to environmentally relevant concentrations of atrazine and p,p'-DDE.

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The objective was to determine the actions of HSPs in steroid signaling pathways in fish. Relative expression of the heat shock proteins (*hsp70* and *hsp90*) and steroid receptors (*ar*, *er* and *gr*) in the liver of rare minnow was determined. In addition, hepatosomatic indices (HSI) and histopathological lesions were examined to evaluate the general status of the liver tissue.

2. Materials and methods

2.1. Test fish and culture conditions

The rare minnow have been maintained in our laboratory for more than 6 years. The brood stock is kept in flow-through system with dechlorinated tap water and a 16:8 h of light:dark photoperiod at $25 \pm 1^\circ\text{C}$ and fed newly hatched brine shrimp (*Artemia nauplii*) twice and granular food (TetraMin, Tetra Werke, Melle, Germany) once a day.

2.2. Chemicals

Atrazine and acetone were purchased from Sigma (Chemical Co., USA), and p,p'-DDE from Chem Service (West Chester, PA, USA).

2.3. Experimental protocol

Fish were kept under flow-through conditions at $25 \pm 1^\circ\text{C}$ with a photoperiod of 16:8 h (light:dark), and generally fed with a commercial granular food (Tetra, Germany) at a rate of 0.1% body weight per day and newly hatched brine shrimp (*A. nauplii*) twice a day. Wastes and residues were removed daily and the test equipment and chambers were cleaned once a week.

Experiment 1: Sexually mature fish ($n = 210$), ~8 months old and the offspring from the same pair of brood stock, were randomly divided into seven groups (sex ratio 1:1). After acclimating for 2 weeks, fish were exposed to various concentrations of atrazine (3, 10, 33, 100, 333 $\mu\text{g/l}$) for 28 days.

Experiment 2: Sexually mature fish ($n = 210$), ~10 months old and the offspring from the same pair of brood stock, were randomly divided into seven groups (sex ratio 1:1). After acclimating for 2 weeks, fish were exposed to various concentrations of p,p'-DDE (0.1, 0.5, 2, 10, 50 $\mu\text{g/l}$) for 28 days.

The solvent control aquaria received a combination of acetone and water (1:100,000, v/v), whereas the water control aquaria received dechlorinated tap water only. The stock solutions were prepared every 2 days in high-performance liquid chromatography-grade acetone and delivered to glass mixing vessels by means of a peristaltic pump at a rate of 1 ml/min. Then, the solutions were diluted with dechlorinated tap water flowing into the mixing vessels at a rate of 4 l/h, which ensured a retention time shorter than 4 h and tank water replacement with at least 6 volumes a day.

Fish were sacrificed at the end of the exposure. Whole-body and liver weights were determined. Liver tissue from four males and four females in each group was fixed in Bouin's solution (71% saturated picric acid, 24% formaldehyde, 5% glacial acetic acid; Sigma-Aldrich, USA), and tissues from the remaining male and female rare minnow were flash-frozen in liquid nitrogen, and stored at -80°C .

2.4. Hepatosomatic indices

Hepatosomatic indices (HSI) of female and male rare minnow were calculated as follows:

$$\text{HSI} = \left(\frac{\text{liver weights (g)}}{\text{body weights (g)}} \right) \times 100$$

Table 1

Sequences of primers used for RT-PCR.

Gene	GenBank no.	Primer sequences (5'-3')	Product size (bp)
<i>er</i>	EF190319	Forward: AACAGACCGCCACATTAG Reverse: GAGCCACCTGAGATTACC	340
<i>ar</i>	GQ241735	Forward: GACTTCAGGTTTCGTAAC Reverse: TGGACCGAGATGATTCTG	178
<i>gr</i>	FJ410931	Forward: GAGAACTCCAGCCAGAACTG Reverse: CCACGCTCAGAGATTATTCAC	126
<i>hsp70</i>	FJ410933	Forward: CTCATTGGGCGACGATT Reverse: CCTTGTATTCAACTGGACCT	110
<i>hsp90</i>	FJ827029	Forward: CACTCTCAGTTCATCGGTACC Reverse: TCCTTGACCTTCTCTCTCTC	297

2.5. Histopathology

After 24 h, liver samples fixed in Bouin's solution were transferred to 70% ethanol and processed according to standard histological methods and embedded in paraffin wax as described by Wolf et al. (2004). The sections were cut at 3–4 μm , stained with hematoxylin and eosin, and observed on a Axioskop 2 mot plus optical microscope (Zeiss, Germany) and digitized with a AxioCam digital camera (Zeiss) using the Application Suite software AxioVision Rel. 4.5 (Zeiss). Diameters of hepatocytes were measured as described by Kokolakis et al. (2008).

2.6. Preparation of cDNA from liver of rare minnow

Total RNA was isolated using SV total RNA isolation system (with DNase I, promega, USA), and the RNA samples were dissolved in ribonuclease-free water and stored at -80°C .

Subsequently, the reverse transcription reaction mixtures containing 10 μl total RNA extract plus 2 μl (0.05 $\mu\text{g}/\mu\text{l}$) of Oligo(dT)₁₅ were heated to 70°C for 5 min and quickly chilled on ice for 30 s. Then a solution containing 50 mM Tris-HCl buffer (pH 8.3), 75 mM KCl, 10 mM dithiothreitol, 3 mM MgCl_2 , 2 mM deoxynucleotide triphosphate (10 mM each), 40 units of RNAasin (RNAase inhibitor; Promega), and 200 U of Moloney Murine Leukemia Virus Reverse Transcriptase (Promega) was added to a total volume of 25 μl and incubated for 1 h at 37°C . Thereafter, the reaction mixture was heated to 70°C for 10 min to inactivate the reverse transcription. The samples were stored at -20°C until analysis.

2.7. Quantitation by real-time PCR

Real-time PCR was performed in Mx3005P real-time quantitative polymerase chain reaction system (Stratagene, USA) in a total volume of 25 μl , comprised of 1 μl cDNA, 12.5 μl Brilliant II SYBR Green QPCR master mix (2 $\times$, Stratagene, USA), 300 nM forward primer and 300 nM reverse primer, and diethyl pyrocarbonate-treated water. The primer pairs used for real-time PCR are shown in Table 1. The cycling conditions used were: an initial denaturation step of 95°C for 10 min, followed by 40 cycles of 95°C for 30 s, 57°C for 40 s and 72°C for 30 s, and the last cycle of 95°C for 30 s, 57°C for 30 s and 72°C for 60 s for dissociation curve. Dissociation curve analysis for each amplification displayed a single peak, indicating that there was no amplification of untargeted genes (data not shown). The control, containing all the reaction components except for the template, was included in all experiments.

All the samples were analyzed twice and each reaction was performed in triplicate. The mean values were used for calculation.

Fig. 1. Microphotographs of the liver from rare minnow (*Grobicypis rarus*). (A) Liver from female control. (B) Liver from female fish exposed to 333 µg/l atrazine. (C) Liver from female fish exposed to 50 µg/l p,p'-DDE. (D) Liver from male control. (E) Liver from male fish exposed to 333 µg/l atrazine. (F) Liver from male fish exposed to 50 µg/l p,p'-DDE. Arrow shows vacuolization; bar = 20 µm.

Fig. 2. Effects of atrazine on the expression of *ar* (A), *er* (B) and *gr* (C) in the liver of rare minnow (*G. rarus*). Horizontal axis represents atrazine concentrations (µg/l); and vertical axis represents relative expression of target genes (fold). Data are expressed as mean ± SEM ($n=6$). Different letters within each series indicate significant difference in relative expression of target genes (Dunnett's test; $p < 0.05$).

response followed a dose-dependent relationship up to the maximum (14-fold increase) at 33 µg/l. In the case of *hsp90*, a 3-fold increase occurred at 3 and 333 µg/l in both males and females ($p < 0.05$) (Fig. 3).

Significant decreases (more than 2-fold) were observed in the expression of liver *ar* (≥ 10 µg/l), *er* (all concentrations), *gr* (≥ 0.5 µg/l) in females ($p < 0.05$), and *ar* (≥ 0.5 µg/l), *er* (≥ 0.5 µg/l), *gr* (all concentrations) in males ($p < 0.05$) after exposure to p,p'-DDE (Fig. 4). Pearson correlation analysis indicated negative relationships between dose and the expression profiles of liver *ar*, *gr* in both males and females and *er* in males ($p < 0.05$); but not in *er* expression in females ($p = 0.97$).

Fig. 3. Effects of atrazine on the expression of *hsp70* (A) and *hsp90* (B) in the liver of rare minnow (*G. rarus*). Horizontal axis represents atrazine concentrations (µg/l) and vertical axis represents relative expression of target genes (fold). Data are expressed as mean ± SEM ($n=6$). Different letters within each series indicate significant difference in relative expression of target genes (Dunnett's test; $p < 0.05$).

Fig. 4. Effects of p,p'-DDE on the expression of *ar* (A), *er* (B) and *gr* (C) in the liver of rare minnow (*G. rarus*). Horizontal axis represents p,p'-DDE concentrations (µg/l) and vertical axis represents relative expression of target genes (fold). Data are expressed as mean ± SEM ($n=6$). Different letters within each series indicate significant difference in relative expression of target genes (Dunnett's test; $p < 0.05$).

More than 5-fold decreases were observed in the expression of liver *hsp70* and *hsp90* in both males and females at all concentrations of p,p'-DDE ($p < 0.01$) (Fig. 5).

4. Discussion

Many endocrine alterations occur by direct interactions between environmental contaminants and steroid receptors (Park et al., 2007) but other mechanisms that influence transcriptional rates are possible. HSPs have been shown *in vitro* to act as steroid receptor chaperons in mammalian systems (Reik et al., 1991). Although their role in fish steroid signaling pathways remains to be fully elucidated, it is possible that endocrine disruptors may act on HSPs and thereby affect genetic transcription. As a first step in this line of investigation, therefore, the relative expression of heat shock proteins (*hsp70* and *hsp90*) was examined in relation to steroid receptors (*ar*, *er* and *gr*) in the liver of rare minnow. Overall, the results are consistent with indirect endocrine modulator effects mediated through HSPs.

HSI and histopathological lesions served in these experiments to evaluate the general status of the liver tissue. HSI have been

Fig. 5. Effects of p,p'-DDE on the expression of *hsp70* (A) and *hsp90* (B) in the liver of rare minnow (*G. rarus*). Horizontal axis represents p,p'-DDE concentrations (µg/l) and vertical axis represents relative expression of target genes (fold). Data are expressed as mean ± SEM ($n=6$). Different letters within each series indicate significant difference in relative expression of target genes (Dunnett's test; $p < 0.05$).

used as physiological biomarkers to reflect responses following toxicant exposure and provide information on energy reserves and the general health of the organism (Van der Oost et al., 2003). In the present study, HSI showed a dose-dependent increase in atrazine treatments (Table 2). Zhong et al. (2004) and Zha et al. (2007) have reported increased HSI in rare minnow exposed to diethylstilbestrol for 21 days and ethynylestradiol for 28 days, respectively. The increases of HSI also have been found following exposure to estradiol in male flounder (*Platichthys flesus*) (Korsgaard et al., 1983) and rainbow trout (*Oncorhynchus mykiss*) (Christiansen et al., 1998). The HSI were significantly decreased in all p,p'-DDE treatments in this study (Table 2). Both field and laboratory studies on fish have reported decreases in HSI upon exposure to organic contaminants (Van der Oost et al., 2003). These results suggested that dysfunctions in the liver of rare minnow occurred following exposure to atrazine and p,p'-DDE.

Hypertrophy of hepatocytes indicated by increased cell size was observed at the highest concentration of atrazine (333 µg/l; Fig. 1B and E). According to Zaroogian et al. (2001) and Purdom et al. (1994) the increase of liver cell size was suggested to be an indicator of accelerated hepatic protein synthesis. There have been evidences showing that VTG was the main circulating protein that increased with exogenous estrogen treatment (Schultz et al., 2001; Zha et al., 2007; Zhong et al., 2004). So it was likely that exposure to atrazine induced the synthesis of VTG in the liver of rare minnow, thus resulted in hypertrophy of hepatocytes (presented as increased size), and finally increase of the HSI. Besides atrophy of hepatocytes in all p,p'-DDE treatments (Fig. 1D), vacuolization was also observed in the liver of rare minnow treated by p,p'-DDE (Fig. 1F). According to Hinton and Laurén (1990), these lesions were probably due to the loss of lipids or nuclear and cytoplasmic inclusions caused by oxidative stress. Dowling et al. (2006) and Hoarau et al. (2004) have proved that p,p'-DDE generated reactive oxygen species (i.e. indicated by glutathione S-transferase activity) which

could result in oxidative stress in clam (*Ruditapes decussates*). It was possible that in this study the histopathological lesions and decreased HSI were caused by oxidative stress in the liver of rare minnow generated by p,p'-DDE. All in all, these results suggested that atrazine and p,p'-DDE could cause adverse effects on the liver of rare minnow.

Real-time PCR assays were established to evaluate the relative expression of liver *hsp70*, *hsp90*, *ar*, *er* and *gr* when adult fish were exposed to environmentally relevant concentrations of atrazine and p,p'-DDE. With the advances in molecular approaches, the application of toxicogenomics could be a powerful tool for evaluating the effects and discovering molecular mechanisms underlying toxic response (Nie et al., 2006). Both atrazine and p,p'-DDE have been proved to interfere with the steroid signaling pathways in aquatic organisms (Hayes et al., 2002; Kelce et al., 1995), however their effects on the HSPs have been rarely reported.

According to Crain et al. (1997) atrazine promoted the conversion from androgen to estrogen by inducing the activity of aromatase. The increased HSI and histopathological observations in this study also indicated estrogenic effects on the liver of rare minnow. Real-time PCR results in this study showed dose-dependent increases in liver *ar* and *er* mRNA (Fig. 2), indicating that both estrogen and androgen pathways were promoted at the gene level. Moreover, both changes in the HSI and the expression of *ar* indicated that female rare minnow was more sensitive in response to atrazine exposure. Greater responses to external stimulation in matured females than males have been reported in chinook salmon and rainbow trout (McQuillan et al., 2003). No effect on liver *gr* mRNA was observed in rare minnow treated by atrazine. However, disruptions in synthetic pathway of glucocorticoid have been reported in adrenocortical steroidogenic cells of rainbow trout (Bisson and Hontela, 2002). These results suggested that the interference of atrazine with the adrenal system were tissue specific. Real-time PCR results also indicated increases in the expression of liver *hsp70* mRNA (all treatments except for 333 µg/l in females) and *hsp90* mRNA (3 and 333 µg/l in both males and females) in rare minnow treated by atrazine (Fig. 3). Increased HSPs have been reported in liver, ovary and gill in tilapia (*Oreochromis niloticus*) and rainbow trout (Vijayan et al., 2000). It was supposed that the up-regulation of HSPs might be a mechanism for increasing the stress threshold and enhancing self-protection (Vijayan et al., 2003). According to Welshons et al. (2003), different mechanisms may be in charge of the response to stress by varying doses of environmental contaminants. We suppose that enhanced self-protection was mainly achieved by increasing the expression of *hsp70* which could facilitate the folding of hormone-binding domain and activation of the steroid receptors (Pratt, 1993) at the medium concentrations (10, 33, 100 µg/l) of atrazine, while promotions of *hsp90* which could increase the stability of the heterocomplex by preventing proteasomal degradation of the steroid receptors (Segnitz and Gehring, 1997) were also required at the lower (3 µg/l) and higher (333 µg/l) concentrations. The expression of liver *hsp70* and *hsp90* was altered under the same conditions as changes in *ar* and *er* expression. This suggests either direct or indirect endocrine modulating effects of atrazine and is also consistent with a possible chaperone role of HSPs for estrogen and androgen receptors in the rare minnow.

The metabolite p,p'-DDE has been proven to be a potent androgen antagonist (Kelce et al., 1995). However, Li et al. (2008) found in their studies using a battery of recombinant yeast assay that many pollutants could interfere with more than one receptor. In this study, the expression of *ar*, *er* and *gr* showed dose-dependent decreases in p,p'-DDE treatments. Consistent with our results, Larkin et al. (2002) using gene array technology reported that p,p'-DDE could decrease the expression of *ar*, *er* and *gr* in the liver of female largemouth bass (*Micropterus salmoides*). Park et al. (2007)

reported similar results in their study of wild tilapia, and also found the suppressing effects were tissue specific. As a protective effect of glucocorticoids has been proved against oxidative stress induced by extrinsic contaminant in rats (*Rattus norvegicus*) (Montilla et al., 2004), the decreased HSI and histopathological lesions due to depletion of lipids or nuclear and cytoplasmic inclusions, as previously described, were more likely to be related to the disruption of *gr* signaling pathway in the liver of rare minnow by p,p'-DDE. Meanwhile, *hsp70* and *hsp90* mRNA were both significantly suppressed in the liver of rare minnow exposed to p,p'-DDE, indicating that the protecting mechanism of HSPs had been blocked. Overall, simultaneous down-regulated expression of liver *ar*, *er*, *gr* as well as *hsp70* and *hsp90* suggested that the action on steroid receptors were probably accompanied by p,p'-DDE action on HSPs in the liver of the rare minnow. As in the case of atrazine effects, these results are consistent with direct effects of p,p'-DDE on steroid receptors and possible indirect effects involving the chaperone role of HSPs.

5. Conclusions

The alterations in expression of steroid receptors (*ar*, *er* and *gr*) caused by atrazine and p,p'-DDE exposure were accompanied by changes of *hsp70* and *hsp90* expression in rare minnow. Therefore, the heat shock proteins (HSPs) were likely to play chaperon roles for the steroid receptors *in vivo*, consistent with previous *in vitro* results.

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