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Effects of atrazine on hepatic metabolism and endocrine homeostasis in rainbow trout (*Oncorhynchus mykiss*)

Iurgi Salaberria [a,b,*], Bjørn Henrik Hansen [c], Vega Asensio [a], Pål A. Olsvik [d], Rolf A. Andersen [b], Bjørn Munro Jenssen [b]

[a] Department of Zoology and Animal Cell Biology, University of the Basque Country, Apdo. 644, E-48080 Bilbao, Spain
[b] Department of Biology, Norwegian University of Science and Technology, N-7491 Trondheim, Norway
[c] SINTEF Materials and Chemistry, Marine Environmental Technology, N-7465 Trondheim, Norway
[d] National Institute of Nutrition and Seafood Research (NIFES), Nordnes, N-5817 Bergen, Norway

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ABSTRACT

The herbicide atrazine (ATZ) is one of the most widely used pesticides in the world and is now under scrutiny for its alleged capacity to disrupt the endocrine system. Exhibiting negligible interaction with the estrogen receptor (ER), ATZ's mode of action remains to be elucidated. ATZ may act as an inducer of the enzyme aromatase, which converts androgens to estrogens, although other mechanisms should also be taken into consideration such as impairment of hepatic metabolism. Therefore we administered juvenile rainbow trout (*Oncorhynchus mykiss*) a dose of either 2 or 200 μg ATZ/kg, or of carrier control phosphate buffered saline (PBS) and we measured plasma concentrations of testosterone (T), 17beta-estradiol (E2) and vitellogenin (Vtg) 6 days after exposure. Simultaneously we analyzed hepatic gene expression of cytochrome *P450* (*CYP*) *1A* and pi-class glutathione S-transferase (*GST-P*), and catalase (CAT) activity. Although sex steroid levels showed no significant alterations, we found a dose-dependent increase in Vtg and a concomitant decrease in CYP1A. There was no effect of ATZ on GST-P mRNA levels but GST-P was positively correlated with CYP1A. Also, CYP1A was negatively correlated with liver CAT and E2, and varied with T concentrations in a hormetic manner. The results showed that ATZ can alter hepatic metabolism, induce estrogenic effects and oxidative stress *in vivo*, and that these effects are linked.

Introduction

Since its introduction in the 1950s, the herbicide atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine) (ATZ) has become one of the most commonly used pesticides worldwide. It was recently banned in the EU (2004/248/EC) and replaced by the nearly identical terbuthylazine. However in the rest of the world the use of ATZ is continued with few, if any, restrictions. ATZ has been reported to affect fish kidney morphology (Fischer-Scherl et al., 1991) and swimming behavior (Saglio and Trijasse, 1998), and to alter hormonal pathways in various taxa, including fish (Moore and Waring, 1998; Spanò et al., 2004; Thibaut and Porte, 2004).

Various mechanisms of action have been proposed by which contaminants can disrupt the endocrine system. During the last decade there has been much focus on interaction of xenobiotics with hormone-binding proteins, which can lead to induction of, e.g., estrogen receptor (ER)-responsive processes such as vitellogenesis (Arukwe and Goksøyr, 2003). The vitellogenin (Vtg) gene is assumed to be naturally quiescent in juvenile and male fish until exposed to an estrogenic agent. Consequently Vtg has been widely used as a biomarker for (xeno)estrogenic exposure (Heppell et al., 1995). However, the affinity of some endocrine disrupters, including ATZ, for hormone receptors and transport proteins is very low or non-existent (e.g., Gale et al., 2004). Therefore other modes of action should also be considered to explain the endocrine disruptive effects of ATZ. Recently an increasing number of studies report endocrine disruption as a result of altered hormone synthesis and/or metabolism (e.g., Thibaut and Porte, 2004).

ATZ has been suggested to induce activity of aromatase (cytochrome *P450* (*CYP*)*19*) (Sanderson et al., 2001; Hayes et al., 2002; Spanò et al., 2004). Aromatase is the rate-limiting enzyme in the synthesis of C_{18} estrogens, such as 17beta-estradiol (E2), from C_{19} androgens such as testosterone (T). However, studies have also reported no effects of ATZ on aromatase activity (Coady et al., 2005; Hinfray et al., 2006) or even reported aromatase inhibition by ATZ (Benachour et al., 2007).

ATZ may also affect hepatic metabolism. Phase I and phase II enzymes regulate homeostasis of sex steroids. Also, these enzymes metabolize a large number of exogenous compounds. Hence,

* Corresponding author. Department of Zoology and Animal Cell Biology, University of the Basque Country, Apdo. 644, E-48080 Bilbao, Spain.
E-mail addresses: iurgi.salaberria@hotmail.com, iurgi.salaverria@bio.ntnu.no (I. Salaberria).

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Table 1
PCR primers and TaqMan MGB probes used to amplify the studied genes

Gene	Accession no.	Forward primer	Reverse primer	TaqMan MGB probe	Amplicon size (bp)
CYP1A	AF364076	TGGAGATCTTCCGGCACTCT	CAGGTGTCCTTGGGAATGGA	CCGTTCACCATCCCACAC	101
GST-P	BQ036247	ATTTTGGGACGGGCTGACA	CCTGGTGCTCTGCTCCAGTT	TTCTCGACAAAGCTC	81
18S rRNA		CACGCGAGATGGAGCAATAA	CGCAGAGTAGACACACGCTGAT	TGCCCTTAGATGTCC	98

xenobiotic exposure can lead to ligand competition for metabolizing enzymes between endogenous and exogenous compounds, thereby altering steroid homeostasis (Förlin and Haux, 1985).

Phase I metabolism of ATZ includes N-dealkylation and hydroxylation, mediated by CYP1, 2 and 3 in mammals (Lang et al., 1997; Hanioka et al., 1998; Islam et al., 2002). In humans CYP1A2 has been identified as the major phase I enzyme involved in the metabolism of ATZ (Lang et al., 1997). In fish, however, little is known about CYP1A involvement in ATZ biotransformation. ATZ did not alter hepatic CYP1A1 activity in rainbow trout (*Oncorhynchus mykiss*) (Egaas et al., 1993), and induced CYP1A1 mRNA levels in carp (*Cyprinus carpio*) only after prolonged exposure (Chang et al., 2005). In mammals CYP1, 2 and 3 also catalyze steroid metabolism. More specifically, in human liver, E2 is predominantly hydroxylated by CYP1A2 (Tsuchiya et al., 2005). While in fish interactions between CYP1A and E2 have been shown (Förlin and Haux, 1985; Gray et al., 1991; Anderson et al., 1996a, 1996b; Navas and Segner, 2000, 2001), the role of CYP1A in steroid metabolism remains unclear.

Phase II metabolism of ATZ involves glutathione (GSH) conjugation by glutathione S-transferase of the pi class (GST-P) in mammals (Islam et al., 2002). GST-P metabolizes E2 (Hachey et al., 2003) and reduces oxidative stress (Yin et al., 2000) by the same mechanism. In salmonids the major hepatic GST is homologous to the mammalian GST-P (e.g., Dominey et al., 1991). Nevertheless, in fish GST activity appears only to be induced by relatively high levels of ATZ (Egaas et al., 1993; Wiegand et al., 2001).

It also remains to be established whether ATZ metabolism affects the endocrine control of maturation of fish. Indeed, developmental stages that involve important hormonal changes such as early development and puberty are most prone to endocrine disruption. However, predominantly immature 1st year fish are used in experiments studying mechanisms of endocrine disruption. Endocrine parameters in these immature fish are relatively constant and are therefore easy to assess for xenobiotic-induced alterations. An important drawback of using immature fish as a model to study effects of endocrine disrupters is that this model does not allow for assessment of reproductive impairment. Thus in the present study we investigated endocrine disruptive effects of ATZ and effects on hepatic metabolism at the onset of puberty in rainbow trout.

We measured plasma concentrations of sex steroids E2 and T, and of Vtg as well as hepatic *CYP1A*- and *GST-P* gene expression in early maturing rainbow trout of mixed gender during endogenous vitellogenesis (Van Bohemen and Lambert, 1981) 6 days after a single dose of ATZ, or of carrier control phosphate buffered saline (PBS). Fish were injected either with a low ATZ dose, 2 μg/kg, which is the equivalent of the Canadian water quality guideline (i.e., 2 μg/l or 2 ppb), or a high ATZ dose, 200 μg/kg, for comparison. Since GST activity is closely linked with oxidative stress (Yin et al., 2000) we also measured hepatic catalase (CAT) activity, a biomarker of oxidative stress (Matés, 2000).

Materials and methods

Fish and husbandry. Ca. 16 month old rainbow trout (AquaGen strain) of mixed gender (640±267 g; 37.2±4.6 cm) were obtained from a local fish farm (Vikan Settefisk AS, Hitra, Norway). After a 40-day acclimatization period in a 3000 l recirculating tank the fish were transferred to 500 l flow-through tanks for intraperitoneal (ip) injection. From the 3rd day post-injection onwards fish were transferred back into the recirculating tank until sampling. Controls were kept in separate flow-through tanks at identical flow rate and temperature conditions. All trout were exposed and sampled during May 2004. All tanks were supplied with City of Trondheim tap water and the light:dark regime was set at 13.5:10.5 h. Fish were fed a commercial diet (Atlantic 4 mm, Skretting AS) at a rate of 1% body weight per day by automated feeders.

Xenobiotic injection. Fish were administered a single ip injection of either 2 or 200 μg atrazine/kg (Riedel-de Haën, Seelze, Germany) dissolved in phosphate buffered saline (PBS) (0.01 M phosphate buffer, 0.0027 M KCl, 0.137 M NaCl, pH=7.4 at 25 °C; Sigma-Aldrich, Steinheim, Germany). Each treatment group consisted of five individuals. The control group consisted of fish injected with PBS only (n=5). Standard 2-ml injection syringes were used (21GA1 1/2 needles). The total injected volume corresponded to 1% of the body mass. Fish were monitored for fluid loss at injection site.

Sampling. To insure that the trout were sampled at the onset of puberty, 10 control fish were sampled at 3, 6, 9, 12 and 19 days after injection. All fish were anaesthetized by an overdose MS-222 and killed with a blow to the head. Fish were sampled during the light period of the day to reduce variation in steroid hormone concentrations due to circadian cycles. Blood samples were collected by caudal venipuncture using heparinized syringes, and transferred to micro-centrifuge tubes. Plasma was separated by centrifugation at 4 °C for 5 min at 13000 rpm (Eppendorf MiniSpin Plus) and stored at −80 °C until analysis. Whole liver and gonad were excised, weighed, wrapped in aluminum foil, immediately frozen in liquid nitrogen and stored at −80 °C. Fish were treated humanely, in compliance with Norwegian regulations regarding experimental animals.

Results showed that in control females the gonadosomatic index (GSI) and plasma concentrations of E2 and Vtg started to increase from day 9 after initiation of the experiment. Control female fish sampled 19 days after initiation of the experiment showed relatively high Vtg concentrations (330.5±185.6 μg/ml). Thus, to insure that the fish in the present study were sampled at the start of puberty onset, further endpoint analyses were only performed on fish sampled 6 days after ATZ injection. In addition, since more than 3 days are required to induce plasma Vtg protein concentrations in fish exposed to a relatively weak xenoestrogen (e.g., Arukwe et al., 2001), sampling 6 days after ATZ administration allows for the study of ATZ-mediated effects on Vtg protein.

Design of PCR primers and TaqMan MGB probes. PCR primers for amplification of CYP1A and GST, in addition to the reference gene 18S rRNA, were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA). PCR primer sequences used to quantify mRNA levels of CYP1A and GST-P were obtained from GenBank accession numbers AF364076 and BQ036247, respectively, both from Atlantic salmon (Table 1). PCR primers and TaqMan probe for 18S rRNA were obtained by Assay-By-Design service (Applied Biosystems), based on conserved regions of this gene in several fish species. The PCR primers used for amplification were not designed to span exon–exon borders, as they were made from mRNA sequences.