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Induction and Inhibition of CYP1A Gene Expression and Steroidogenesis in Olive Flounder *Paralichthys olivaceus* Exposed to Tributyltin and Benzo[*a*]pyrene

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Cytochrome P450 (CYP1A) gene expression in the liver and sex steroid levels in plasma were investigated in olive flounder (*Paralichthys olivaceus)* exposed to tributyltin (TBT) and benzo[*a*]pyrene (B*a*P). We constructed a cDNA library and cloned a 230-base sequence encoding partial CYP1A DNA. The CYP1A gene expression level was estimated using northern blotting. Hepatic CYP1A mRNA levels in fish injected with B*a*P at 10 mg/kg body weight (b.w.) increased for 48 h after injection. However, fish injected with both B*a*P and TBT at 10 mg/kg b.w. showed no significant changes in CYP1A mRNA level after 48 h. Plasma concentrations of testosterone and 17β-estradiol were not significantly different in males and females injected with B*a*P and TBT. We suggest that TBT-induced suppression of B*a*P bioactivity should be interpreted with caution in biomonitoring field studies.

Key words: Benzo[*a*]pyrene, Cytochrome P450, *Paralichthys olivaceus*, Radioimmunoassay, Tributyltin

Introduction

The induction of cytochrome P450 (CYP1A) has been used as an early warning signal, as well as a highly sensitive biological response of fish to exposure to some aryl hydrocarbons such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and dioxins. Through field studies, the CYP1A enzyme has become well established as a biomarker to monitor exposure to such contaminants (Kleinow et al., 1986; Payne et al., 1987). Most studies on the interaction of environmental chemicals with the hepatic microsomal enzyme P450 have focused on the induction of this enzyme system (Stegman and Kloepper-Sams, 1987; Malmström et al., 2004). However, under actual conditions in the marine environment, chemicals may act agonistically or antagonistically. It has been suggested that CYP1A1 induction can be inhibited by some xenobiotics such as tributyltin (TBT) (McClellan-Green and Robbins, 2000; Padrós et al., 2000; Shim et al., 2003). Furthermore, the relationship between chemical mixtures or dosages and gene expression still remains unclear. Benzo[*a*]pyrene (B*a*P), a typical PAH induces CYP1A as phase I of the detoxification process (Van der Weiden et al., 1994). The products of CYP1A metabolism may produce carcinogenic metabolites in the conjugation step (phase II reaction: McElroy et al., 1991; Nicolas, 1999).

TBT is a component of products that are widely used in wood preservation, disinfection of circulating industrial cooling water, and marine antifouling paints. Although, the use of paints containing TBT has been banned in Korea since 2003, high TBT concentrations are found in marine biota, including fish and shellfish (Shim et al., 2005).

TBT has the potential to cause imposex (imposition of male sexual organs on females) and inhibit the activity of P450-dependent aromatase in gastropods (Bettin et al., 1996) and in fish *in vitro* (Fent and Bucheli, 1994). Several lines of evidence suggest that TBT may alter the activity of CYP1A and the bioactivation of carcinogens such as B*a*P.

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However, most studies have involved low concentrations of TBT (0.001-1 mM: Fent and Bucheli, 1994), and measurements of ethoxyresorufin-*O*-deethylase (EROD) activity and P450 content. Only a few *in vivo* studies have reported the effects of xenobiotic mixtures on animals (Rajasekaran, 2000; Vosyliene et al., 2003).

The olive flounder (*Paralichthys olivaceus).* is a demersal marine fish of Korea and is commercially important It feeds on benthic organisms in the sediment, such as small clams, marine worms and small crabs, and thus has maximal exposure to sediment-bound contaminants (Hart, 1973). The species is also an important ecosystem component in some polluted areas off Korea (Shim et al., 2003; Hong et al., 2003). Our aim was to determine if TBT and B*a*P alter the expression of CYP1A and the levels of sex steroid hormones in this demersal marine fish.

Materials and Methods

Experimental animals and exposure

Olive flounder (2 years old), weighing 300 ± 10 g and with total lengths of 35 ± 5.0 cm, were purchased from a local fish farm in Samcheok, Korea. Fish were acclimated for 2 weeks in the laboratory before commencing experimentation to ensure that they were free of disease. There were no significant differences in water conditions during the experimental periods. All experiments involved acute exposures induced by the administration of a single intraperitoneal injection. Fish were injected with TBT (tributyltinchloride: Aldrich. Miwaukee, Misconsin, U.S.A) and B*a*P (Sigma. Dorset, UK) dissolved in dimethylsulfoxide (DMSO: Sigma, Dorset, UK) to yield whole body concentrations of 10 mg/kg. Control fish were sham injected with DMSO or not injected at all. Seven fish were sampled randomly at 72 h following the injection and killed by a blow to the head. Immediately after movement ceased, blood was drawn from the caudal vein using a heparinized syringe and centrifuged at 3,840×*g*, 4°C for 15 min. Plasma was then collected and stored at -80°C until analysis. The liver was flash-frozen in liquid nitrogen and stored at -80°C until analysis.

Primer design

Degenerate oligonucleotide primers for CYP1A and β-actin were designed based on a conserved amino acid sequence region obtained by comparing sequences (NCBI, National Center for Biotechnology Information). Primers were designed using OLIGO software (National Biosciences, Inc., Plymouth, Minnesota, USA). cDNA probes were made using the cloned CYP1A reverse primer (5'-GATGTGCAATG-AGGGATAGTGA-3') and forward primer (5'-GGA-GCTAGAGAACGCGAAT-3').

Construction of cDNA library and sequencing

Olive flounder were sampled at 72 h following the injection of β-naphthoflavone (β-NF). The livers were used for cDNA library construction. The cDNA library was constructed according to the method of Jung et al. (2005). The constructed cDNA library was used to prepare probes.

Sequence analysis

Sequence alignment and comparisons were performed using Genetyx (version 6.1, Genetyx Co.) software. Database searches and multiple local align-

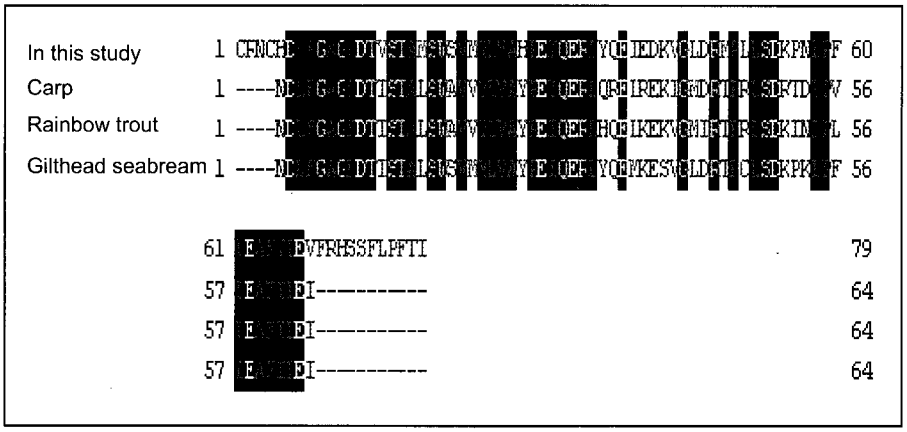

Fig. 1. Alignment of the putative olive flounder *Paralichthys olivaceus* CYP1A sequence with teleost CYP1A. Olive flounder (AT 568556*),* carp *Cyprinus carpio* (AY 775788), rainbow trout *Oncorhynchus mykiss* (U 07055), gilthead seabream *Sparus aurata* (AY 049952).

ments were performed using the BLASTP program (NCBI).

Northern blot analysis

Total RNA was extracted from the tissues of individual liver using ISOGEN kit (Wako, Japan). RNA was pre-hybridized for 3 h in 50% formamide, 5× SSC (sodium chloride-sodium citrate), 5×Denhard's solution, 0.1% SDS (sodium dodecyl sulfate) and 10% blocking regent (Roche, Basel, Switzerland). The cDNA probes were made using the cloned CYP1A reverse primer (5'-GATGTGCAATGAGGG-ATAGTGA-3') and forward primer (5'-GGAGCTA-GAGAACGCGAAT-3'). Templates for each probe were cut from the plasmid vector using EcoRI. These templates were used for the synthesis of α-^{32}P-labeled probes, which were generated using a random primer labeling kit (Takara Co. Japan). Hybridization was performed in a solu-tion containing 50% formamide, 5×SSC, 5×Denhard's solution 0.1% SDS and 10% blocking regent to radio label probes at 42°C for 16 h. Following hy-bridization, blots were washed for 15 min with 2×SSC and 0.5% SDS, and for 15 min with 0.5×SSC and 0.5% SDS at 55°C. The membranes were analyzed using a BAS 2000 Bio-Image Analyzer (Fujix, Japan).

Steroid radioimmunoassay

Plasma levels of 17β-estradiol (E_2) and testosterone (T) were measured using a steroid radioimmunoassay (RIA) according to the method of Aida et al. (1984). The assay system had a working range between 30 and 3,840 pg/mL for both steroids.

Statistical analysis

All data were expressed as the mean ± standard error of mean (SE). Data were compared using analysis of variance (ANOVA), and significant differences were determined using Duncan's multiple range test.

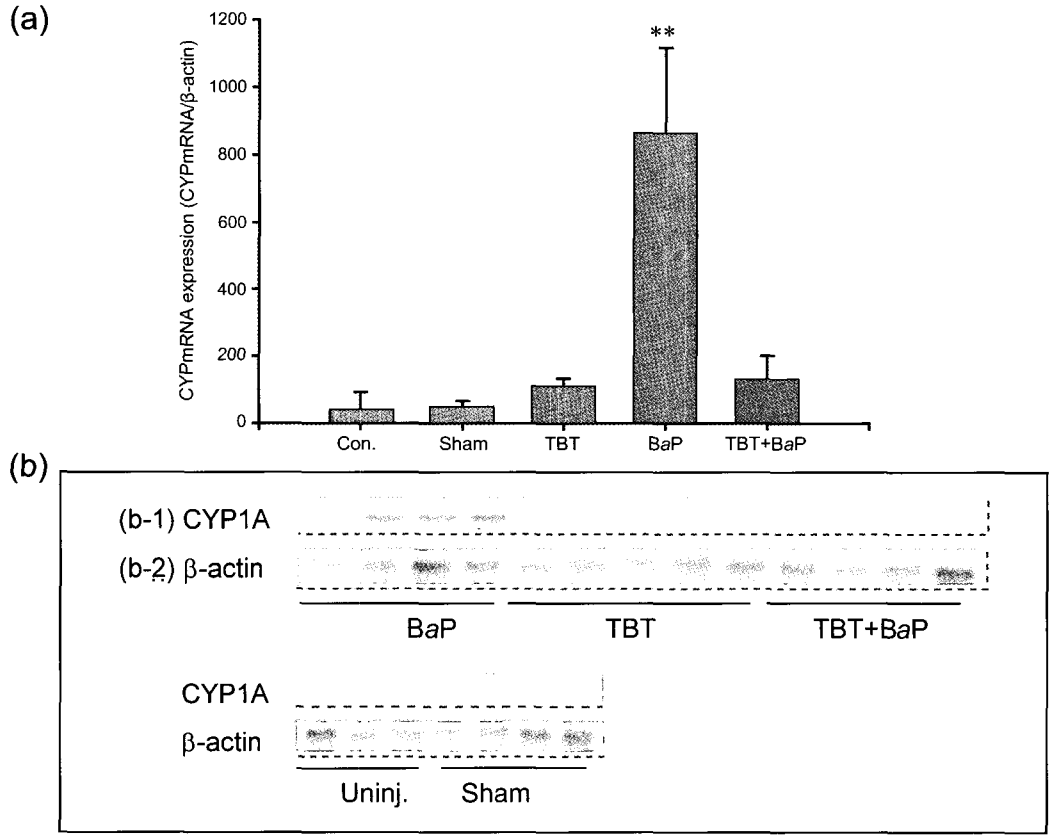

Fig. 2. CYP1A mRNA expression levels in olive flounder *Paralichthys olivaceus* injected with tributyltin (TBT), Benzo[*a*]pyrene (B*a*P) and mixture. (a) Relative mRNA levels of olive flounder CYP1A were normalized to β-actin. Data are the means ± standard error (SE) of six samples. Data were subjected to ANOVA, followed by Duncan's multiple-range test (**P<0.01). (b) Representative northern blot of olive flounder CYP1A mRNA (b-1) and the same northern blot reprobed with β-actin (b-2).

Results and Discussion

A 230-base oligonucleotide encoding a partial CYP1A gene of the olive flounder was amplified and cloned. The deduced amino acid sequence of the 230-base CYP1A mRNA showed high homology to CYP1As of *Cyprinus carpio* (76%) and *Sparus aurata* (73%), respectively (Fig. 1). To determine expression specifically, CYP1A was normalized to β-actin; a primer was designed for a conserved region found in GenBank (Accession no. AY 166590), and 500-base oligonucleotide was cloned. The CYP1A mRNA fragment sequences (210 bases) were submitted to GenBank (Accession No. AY 568556).

The exposure of fish to TBT and B*a*P at 10 mg/kg had no effect on survival for up to 72 h post-exposure. CYP1A mRNA expression in flounder injected with B*a*P increased after 72 h (Fig. 2), supporting previous other studies (Addison et al., 1994; Fent et al., 1998; Basu et al., 2001). In contrast, CYP1A mRNA was not expressed in flounder injected with the TBT-B*a*P mixture. Some PAHs induce CYP1A expressed in fish (Goksøyr and Förlin, 1992). B*a*P can induce neoplasia in fish ; neoplasia it is thought to be caused by strong CYP1A inducers, which are responsible for the high incidence of hepatic neoplasm found at sites with high PAH concentrations (Baumann and Harshbarger, 1995). In contrast to B*a*P, TBT inhibits the CYP1A enzyme (Morcillo and Porte, 1997; McClellan-Green and Robbins, 2000; Padrós et al., 2000; Shim et al., 2003). Northern blots showed that fish exposed to the TBT-B*a*P mixture had no increase in CYP1A mRNA after 72h. TBT appears to specifically inhibit CYP1A expression. Inhibitory effects of TBT on the CYP1A enzyme have been observed previously in *in vivo* and *in vitro* enzyme studies (Reader et al., 1996; Padrós et al., 2000; 2003). However, member of the CYP1A family have different individual functions, and enzyme reaction did not due to single CYP1A for their action. Thus, many previously studies concentrated on isolating and examining the function of each CYP1A isomer using molecular tool (Hasselberg et al., 2004; Yokota et al., 2005).

Rosenberg and Drummond (1983) suggested that the inhibition of CYP1A induction may result from direct damage to the heme moiety of CYP1A or heme binding to amino acids at the active site. Another possibility is that TBT may bind directly to the CYP1A receptor and inhibit transcription *in vitro* (Fent and Stegman, 1991). Recent results support the idea that CYP1A is not significantly involved in the metabolism of TBT *in vitro* (Padrós et al., 2000).

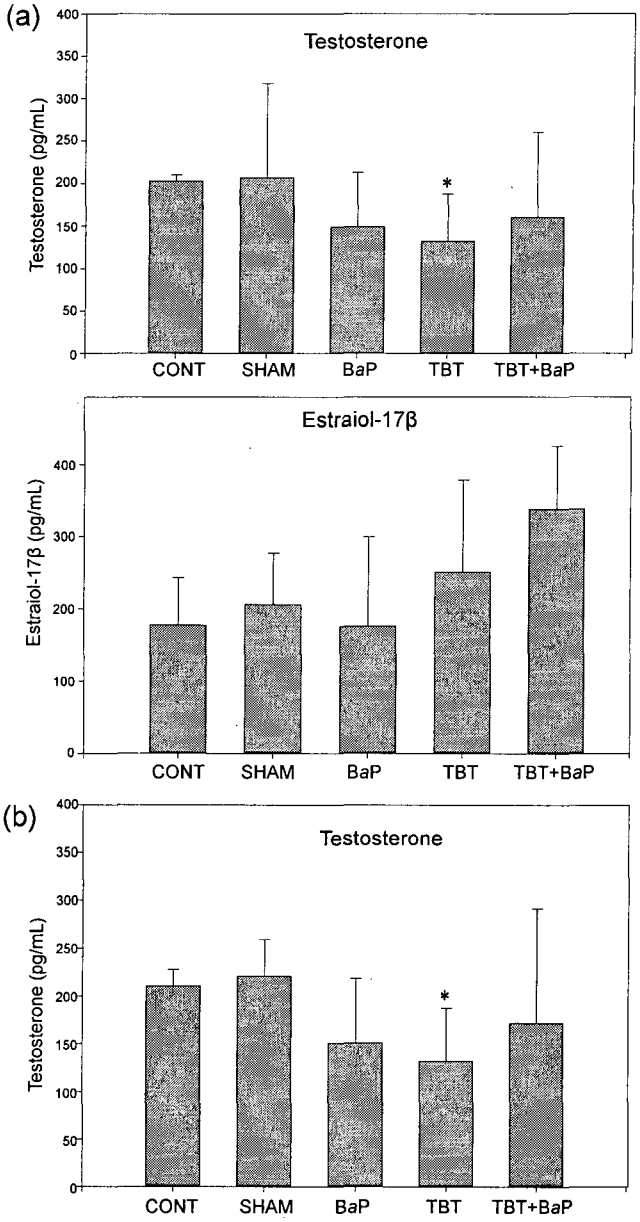

Fig. 3. (a) Plasma testosterone and 17β-estradiol concentrations in female olive flounder (*Paralichthys olivaceus*) following injection with tributyltin (TBT), Benzo[*a*]pyrene (BaP), and a TBT-BaP mixture, mea-sured using radioimmunoassay(RIA). (b) Plasma testosterone concentration, in male olive flounder, following injection with tributyltin (TBT), Benzo[*a*] pyrene (B*a*P), and a TBT-BaP mixture, measured using RIA. Data are the means ± standard error (SE) of seven samples. Data were subjected to ANOVA followed by Duncan's multiple-range test (*P<0.05).

However, *in vitro* studies of inhibitory effects on CYP1A do not truly reflect the *in vivo* situation in fish. Padrós et al., (2000) hypothesize that the inhibitory effect is related to the induction by B*a*P of

CYP1A isoform(s) other than that involved in TBT metabolism. Northern blot analysis, showed that TBT appeared to exert an inhibitory effect on CYP1A only in mixture with B*a*P, demonstrating an interaction between two chemicals and CYP1A. Clearly the inhibition of CYP1A transcription will decrease the detoxifying activity of the liver against other xenobiotics, and could result in the serious disruption of physiological processes.

Exposure to two chemicals (TBT-B*a*P), either separately or together, resulted in no significant change in T in male olive flounder. In females, the plasma concentration of E_2 and T did not differ from levels in the sham groups. CYP1A regulates steroid hormone synthesis and metabolism through, steroid aromatase. Organotins disrupt steroidogenesis, by inhibiting the aromatase enzyme (CYP19), which converts testosterone to estradiol in gastropods and mammals (Fent, 1996). However, this process has yet to be confirmed in fish. We found no significant differences in plasma testosterone and 17β-estradiol levels among treatments. Thus, it appears that fish respond to TBT through steroidogenesis process. That differs from that of gastropods.

Here, we showed the combined effects of two widespread aquatic pollutants and emphasize both the complexity and importance of effects caused by mixtures of pollutants. We suggest that short-term studies using the injection of pollutant mixtures should be emphasized to focus on the combined effects of various pollutant.

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