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Effects of tributyltin, triphenyltin and atrazine on plasma vitellogenin concentration in Japanese medaka fish *Oryzias latipes*

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The Japanese medaka fish, *Oryzias latipes*, was used to determine the influence of exposure to chemicals on plasma vitellogenin (VTG) concentration using anti-medaka VTG antibody. When females were exposed to tributyltin (TBT) and triphenyltin (TPT) for 7 days, dose-related decrease of plasma VTG was observed. Significant decrease was observed with 1 ppt TBT and TPT, and TPT was more effective than TBT. Addition of 10 ppt 17β-estradiol to 1 ppt TPT gave no increase, but rather decrease in female plasma VTG. When males were treated with 1 ppt TBT and TPT or 1 ppt TPT + 10 ppt 17β-estradiol for 7 days, no VTG was detected in plasma. When females were exposed to 10 and 100 ppt atrazine, plasma VTG tended to be decreased. When males were treated with 10 and 100 ppt atrazine, no VTG was detected in plasma. The ecological significance of these findings and implications for the development of the plasma VTG tests using Japanese medaka fish are important.

Key words: medaka fish, plasma vitellogenin, triburyltin, triphenyltin

Introduction

A number of substances have been detected in the environment that can affect the endocrine system [1]. These endocrine-disrupting chemicals, such as organotin, atrazine, and 17β-estradiol discharged from industries and municipal sewage treatment plants ultimately reach coastal areas [2]. These chemicals have been widely distributed in sediments, fish, and mammals, despite regulation or prohibition of the use. Recent studies have noted that endocrine-disrupting chemicals lead to abnormal physiological response and cause adverse effects on the reproductive system of fish [3-5]. Estrogenic chemicals, such as 17β-estradiol and atrazine, induce intersex conditions, such as the development of oocytes within testicular tissue (testis-ova)[6-8]. Induction of testis-ova is one indicator of exposure to estrogenic chemicals in male medaka.

Organotin compounds, such as tributyltin (TBT) and triphenyltin (TPT) have been widely used as biocides, agriculture fungicides, wood preservatives, disinfecting agents in circulating industrial cooling waters, and as antifouling paints for marine vessels [9,10]. Organotin compounds are a typical endocrine-disrupting chemical that can induce imposex in mollusks [11,12]. However, few studies addressing the effect of organotin compounds in fish have been reported. Nakayama *et al*. administered TBT to medaka for 3 weeks and indicated that TBT affected sexual behavior and reproduction in male medaka [13].

Atrazine (Atz), 2-chloro-4-ethyl-amino-6-isopropylamino-S-triazine, is probably the most widely used herbicide in the world and one of the most common contaminants in ground and surface water. Recent studies indicate that Atz may be a potent endocrine-disrupting compound in frogs exposed to part-per-billion (μg/L) concentrations [14].

Among populations of fish with gonadal intersex, significant levels of vitellogenin (VTG) were reported in male fish. The egg precursor protein VTG has been frequently used as a biomarker of exposure of male fish to estrogenic substances. VTG is normally only present in the plasma of mature female fish, but exposure to estrogenic substances can induce VTG synthesis in male fish and in juvenile females [15].

Previously we found that exposure of male medaka fish to 17β-estradiol induced a 200-kDa protein in blood plasma and liver [16]. The 200-kDa protein was supposed to be VTG, but was not identified. We here measured the plasma VTG level using anti-medaka VTG antibody, and found that exposure of female medaka fish to TBT and TPT decreased the plasma VTG. The decrease of the plasma VTG was also observed with female medaka fish exposed to Atz.

Materials and methods

Materials

Medaka fish was purchased from Shimizu Suisan Co., Kyoto and usually kept in the glass-made 57 liter aquarium (vessel) filled with natural water (tap water, after leaving for 2 days) under a controlled environment as follows: light 14 h, dark 10 h, water temperature 26°C, fed with a commercial fishfood twice a day [16]. TBT chloride, TPT chloride, and atrazine were obtained from Wako Pure Chemical Industries, Ltd., and 17β-estradiol was from Nacalai Tesque. An enhanced chemiluminescence (ECL) kit was purchased from Amersham Biosciences.

Blood sampling

Adult fish was kept in a 200 ml glass-made beaker filled with the natural water containing TBT chloride, TPT chloride, 17β-estradiol and atrazine at concentrations indicated under the controlled environment.

Tail was cut off from fish body and blood exuded was put into microtubes filled with a buffer consisting 25 mM Tris-HCl (pH 7.2), 150 mM NaCl, and 5 mM EDTA. Supernatant was obtained by centrifugation of blood at 12,000×g and 4°C for 15 min. The protein concentration was determined by the Micro BCA Protein Assay Reagent (Pierce).

Measurement of plasma vitellogenin

The plasma vitellogenin was detected around 200 kDa by SDS-PAGE [17]. The separation gel was prepared with 8% polyacrylamide, and plasma sample (0.45 μg of protein) was subjected to electrophoresis. After equilibration in transfer buffer, proteins were electrophoretically transferred onto PVDF membranes. Membranes were blocked in 3% gelatin in PBS for 1 h, and incubated with 1:500 diluted anti-medaka VTG antibody (Enbio Tec Laboratories), then washed with PBS containing 0.1% Tween 20, incubated further with horseradish peroxidase-linked anti-mouse IgG (Amersham Biosciences). The protein bands were visualized with ECL.

Statistical analysis

All results are expressed as the means ± SD. Student's *t*-test was used to determine the statistical significance.

Results

Effect of TBT and TPT on plasma VTG in female medaka fish

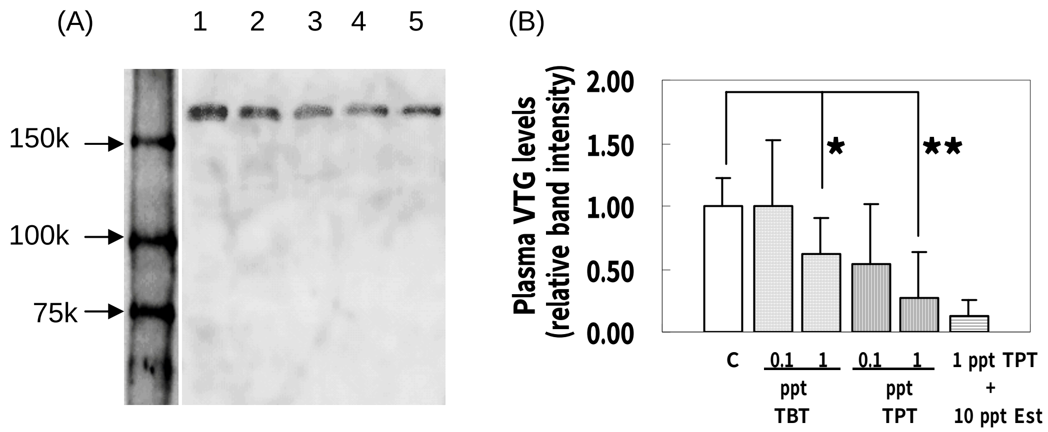

Fig. 1. Relative plasma vitellogenin (VTG) levels in the tributyltin (TBT) chloride- and triphenyltin (TPT) chloride-treated female medaka. *Panel A*, females were kept with 0.0001% DMSO (lanes 1 and 2) or 1 ppt TBT chloride (lanes 3-5) for 7 days. Plasma samples were separated on a SDS-polyacrylamide gel, and Western blotting was performed using anti-medaka VTG antibody. Bands were visualized using the ECL system according to the manufacture's instructions. *Panel B*, females (N=5-9) were kept with 0.0001% DMSO (C), 0.1 and 1 ppt TBT chloride, 0.1 and 1 ppt TPT chloride, or 1 ppt TPT chloride and 10 ppt 17β-estradiol (Est) for 7 days. Relative amounts of vitellogenin in plasma were determined by Western blot. * and ** indicate a statistically significant difference at $p < 0.03$ and $p < 0.003$, respectively, compared with the levels in the control.

When females were exposed to TBT and TPT for 7 days, dose-related decrease of plasma VTG was observed (Fig.1). Significant decrease was observed with 1 ppt TBT and TPT, and TPT was more effective than TBT. Addition of 10 ppt 17β-estradiol, which can induce plasma VTG in male medaka fish, to 1 ppt TPT gave no increase, but rather decrease in female plasma VTG.

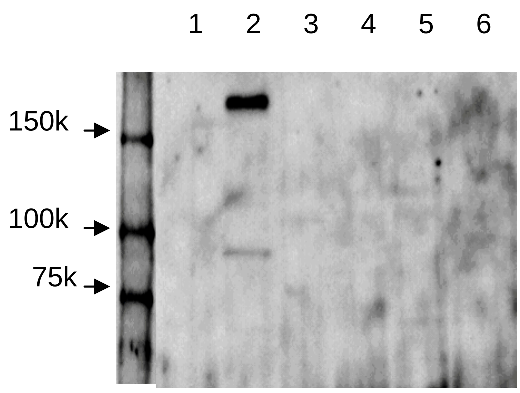

Fig. 2. Relative VTG levels in the TPT chloride-treated male medaka. Males were kept with 1 ppt TPT for 7days, and plasma VTG bands were visualized as in Fig. 1. Lane 1: control male, lane 2: control female, lanes 3-6: males treated with 1 ppt TPT.

When males were treated with 1 ppt TBT and TPT or 1 ppt TPT + 10 ppt 17β-estradiol for 7 days, no VTG was detected in plasma (Fig. 2 and data not shown).

Effect of Atz on plasma VTG in female medaka fish

When females were exposed to 10 and 100 ppt Atz, plasma VTG tended to be decreased (Fig. 3). When males were treated with 10 and 100 ppt Atz, no VTG was detected in plasma (data not shown).

Discussion

The present results indicated that organotin compounds, such as TBT and TPT reduced the plasma VTG in female medaka fish. It is reported that fertilization success

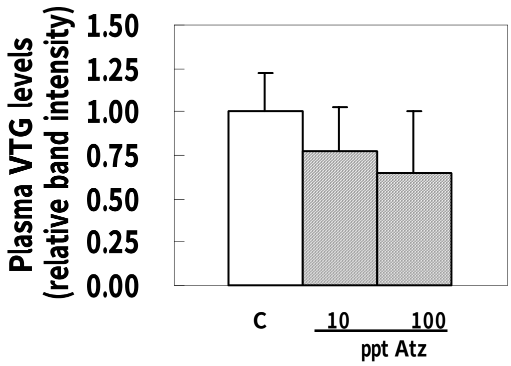

Fig. 3. Relative plasma vitellogenin (VTG) levels in the atrazine (Atz)-treated female medaka. Females (N=4-5) were kept with 0.0001% DMSO (C), 10 and 100 ppt atrazine for 7 days. Relative amounts of VTG in plasma were determined as in Fig 1.

TPT was effective than TBT, that may be caused by that TBT is more easily metabolized than TPT, and that net bioaccumulation of TPT is greater than that of TBT [18]. Exposure to Atz also tended to produce the decreased plasma VTG in female medaka fish.

Organotin compounds are under intense debate because they were recognized in the 1990s for a variety of disastrous effects in the marine ecosystem. TBT, which is used in huge amounts in antifouling paints for large ships, has been called “the most toxic chemical ever deliberately released into the seas” (World Wildlife Fund). Organotin compounds have been recognized as endocrine-disrupting chemicals because numerous marine organisms have been shown to exhibit sexual abnormalities following exposure to TBT and TPT. In gastropod mollusks, TBT has been demonstrated to induce the superimposition of male sex organs, a phenomenon known as imposex. Several reports have suggested that TBT inhibits the enzyme aromatase which catalyzes androgen to estrogen [19,20]. Therefore, it has been theorized that TBT increases androgen levels through inhibition of aromatase activity and/or a suppression of androgen excretion. However, no clear evidence has been provided to verify this hypothesis.

and the numbers of males that performed the sexual behavior such as following and dancing were significantly decreased in male medaka exposed to TBT [13], and TBT induced abnormal development of the eyes, reduced hatchability and increased swim-up failure in medaka [21]. Previously we suggested that TBT induced the expression of a 200 kDa protein in blood plasma of male medaka, but the 200 kDa protein was not identified [16]. We here did not detect VTG in plasma of male medaka exposed to TBT and TPT, using anti-medaka VTG antibody. Nozaka *et al.* also observed no significant VTG response in male medaka exposed to TBT, using an enzyme-linked immunosorbent assay [22]. Toxicity of organotin to male medaka should be further studied. Although the production of VTG in males and immature females has been successfully exploited as a biomarker of exposure specific for estrogenic compounds in a variety of fish species recommended by Organization for Economic Cooperation and Development (OECD) for the validation [23], effects on the VTG response and sexual behavior in fish may be different depending on the conditions (e.g. exposure time). It may be important to clarify whether it is needed to consider normal physiological rhythms such as daily and seasonal differences.

Previous studies have been suggested that Atz is an endocrine disruptor. Atz has been linked to decreased reproductive output in bluegill sunfish, *L. macrochirus* [24]. Developmental arrest in Japanese medaka embryos was observed when embryos were exposed to sublethal concentrations (25-100 ppm) of Atz [25]. Atz may affect mammals and amphibians by altering aromatase activity, but these effects have been observed in a single strain of rat or were produced only at high doses.

We here observed the VTG response in female medaka exposed to TBT, TPT and Atz. The mechanisms of the reduced plasma VTG in females remain unclear, but the ecological significance of these findings and implications for the development of the plasma VTG tests using Japanese medaka fish are important.

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