In Vivo Testing System for Determining the Estrogenic Activity of Endocrine-Disrupting Chemicals (EDCs) in Goldfish (*Carassius auratus*)

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Vitellogenin (VTG) is believed to be an effective and sensitive biomarker to detect the effects of endocrine-disrupting chemicals (EDCs) on fish. An enzyme-linked immunosorbent assay (ELISA) was developed in this study for the quantification of VTG in goldfish (*Carassius auratus*) blood plasma using a monoclonal antibody against carp lipovitellin which is known to cross-react with goldfish VTG, and a working range of 7.8 to 500 ng VTG/ml was established. A laboratory study involving the dosing of male goldfish for 28 days with a range of bisphenol-A (BPA) concentrations was conducted, and the established ELISA was used. There was no significant induction of VTG at the concentrations of 1 and 10 μg/l. Exposure to 100 μg/l and 1000 μg/l BPA for 28 days significantly elevated plasma VTG concentrations to 201 $\pm$ 90 μg/ml and 104552 $\pm$ 24920 μg/ml, respectively. To confirm the effect of temperature on VTG induction, male goldfish were exposed to the nominal concentration of 17β-estradiol (E2) 100 μg/l at 10° and 30°C for 10 days. Plasma VTG concentration in male goldfish increased more quickly in the fish exposed at 30°C than in those at 10°C. In addition, the plasma VTG concentration in the 30°C group (10463 $\pm$ 3268 μg/ml) was higher than that of the 10°C group (1.42 $\pm$ 1.6 μg/ml) after 1 day of exposure. Thus goldfish are useful for the investigation of EDCs under various conditions since they are relatively small in size, and the ELISA established in the present study will contribute to various types of research.

Key words —— vitellogenin, endocrine-disrupting chemical, ELISA, bisphenol-A, biomarker, goldfish

INTRODUCTION

It has been suggested that endocrine-disrupting chemicals (EDCs) pose a potential risk and can alter the hormone balance in humans and wildlife as hormone agonists, antagonists, *etc*. More than 70 chemicals have been cited as potential EDCs, including industrial contaminants, insecticides, and herbicides. Reported phenomena possibly caused by EDCs in fish, reptiles, birds, and other wildlife include abnormal reproductive function, abnormal reproductive behavior, demasculinization, and decreased hatching success.[1,2] The number of these reports has increased sharply since the early 1990s. Although the cause of these abnormalities has yet to be fully elucidated, it is strongly suspected that exposure to DDT and nonylphenols used in surface-active agents are contributing factors.[3,4] The extent of their effects is largely unknown at present.

Recently, the monitoring of sex steroid hormones, such as 17β-estradiol (E2) and 11-ketotestosterone in fish has been used to assess biological effects and exposure to environmental contamination. In addition to sex hormone levels, vitellogenin (VTG), an estrogen-inducible phosphoprotein, can also be used as a biomarker of contaminant exposure in fish and in other oviparous vertebrates. VTG is normally synthesized in the liver of female oviparous vertebrates during oogenesis as a precursor of egg yolk, and is transported to the ovary through the bloodstream to be incorporated into the oocytes. Although VTG is regarded as a female-specific protein, a male fish can also produce VTG in the liver if exposed to a certain amount of substance(s) with estrogenic activity. Therefore the measurement of male plasma VTG concentration has been used as a sensitive biomarker in studies of artificial estrogenic compounds in aquatic environments.[5]

Goldfish (*Carassius auratus*) are comparatively

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small even when mature and are suitable for the evaluation of environmental chemicals in both field and laboratory studies. Although goldfish have been used to test the estrogenic activity of some chemicals,[6] no enzyme-linked immunosorbent assay (ELISA) system had been established for sensitive determination of goldfish VTG. Therefore in this study, an ELISA system was developed for the determination of VTG concentration in goldfish plasma using a monoclonal antibody against carp lipovitellin, which is known to cross-react with goldfish VTG, to promote the investigation of estrogenic activity in aquatic environments.

Bisphenol-A (BPA) is a synthetic chemical used in the production of polycarbonate for manufacturing a wide variety of plastics and other products, and has been detected in food and water consumed by animals and people. BPA is also known to bind competitively to sex steroid-binding protein in humans and rainbow trout[7] and was found to have estrogenic activity in a yeast two-hybrid assay.[8] High doses of BPA cause reproductive toxicity and affect cellular development in rats,[9] and an apparent dose-response induction of VTG and eggshell zona radiata proteins levels were observed in BPA-treated juvenile salmon.[10]

In addition to the establishment of ELISA in this report, the effects of BPA concentration and temperature on VTG production in male goldfish were examined using the newly developed ELISA system.

MATERIALS AND METHODS

Fish and Sample Collection —— Two-year-old (mature) male goldfish were purchased from a local dealer and used. Blood was taken from the caudal blood vessel with a cold, heparinized syringe and was immediately transferred into a centrifuge tube to mix with a 0.1 volume of saline containing 10000 KIU/ml aprotinin, 0.1% phenylmethylsulfonyl fluoride, and 1 U/ml heparin. The blood was centrifuged at 3000 rpm for 20 min to separate plasma, and the resultant plasma was immediately used for analysis or stored at –30°C until analysis. All preparative procedures were carried out at 4°C. After removal of the gonad and hepatopancreas the body weight was measured, and the organs were rapidly frozen in liquid nitrogen and stored at –80°C until analysis. The gonadosomatic index (GSI) and hepatosomatic index (HSI) were calculated as (gonad or liver weight/body weight) × 100.

Preparation of Purified Goldfish VTG —— Goldfish VTG was isolated according to methods described for the purification of VTG in goldfish.[11] Initially, proteins in plasma of E2-exposed male goldfish were separated on an anion-exchange column (POROS-HQ column, PerSeptive Biosystems) connected to a high-performance liquid chromatography system (HPLC). The main peak, with a molecular weight of about 120 kDa, was used as a purified VTG. Its protein concentration was determined by the Bradford assay[12] with bovine serum albumin as the standards.

Analysis of Specificity of Antibody Against Goldfish VTG by SDS-PAGE and Western Blotting —— SDS-PAGE was performed according to the method of Laemmli[13] using 7% polyacrylamide gel under reducing conditions. Coomassie Brilliant Blue G-250 staining was used to detect protein bands in the SDS-PAGE gel. For Western blotting analysis, proteins separated by SDS-PAGE were transferred to a nitrocellulose filter at 50 mA for 1 hr at room temperature with a semidry-type electrotransfer unit. Transfer buffer was 25 mM Tris containing 20% (v/v) methanol. After transfer, the nitrocellulose filter was incubated with 100% nonfat Block Ace (Dainippon Pharmaceutical Co., Ltd., Japan) solution for 1 hr at room temperature. The filter was incubated a further for 2 hr at room temperature with shaking in Tween-PBS [phosphate-buffered saline containing 20% (v/v) Tween 20] containing 0.5 μg/ml anti-carp lipovitellin mouse monoclonal antibody and 25% nonfat Block Ace. The filter was then washed three times with Tween-PBS for 20 min with shaking, and was subjected to reaction with anti-mouse IgG goat antibody (2500-fold diluted), to which horseradish peroxidase (HRP) was conjugated, for 1 hr at room temperature. After the reaction, the filter was washed (20 min, with shaking) three times with Tween-PBS, and goldfish VTG on the filter was detected by an ECL Western blotting detection system (Amersham Pharmacia Biotech, Japan).

Determination of Goldfish VTG by ELISA ——

Preparation of ELISA Plate: Anti-carp lipovitellin antibody (Transgenic Inc., Japan) was diluted to 5.0 μl/ml with phosphate-buffered saline (PBS), and the diluted antibody solution was dispensed into 96-well microtiter plates (100 μl/well) followed by incubation at 4°C overnight. The plates were then washed with Tween-PBS, blocked with 200 μl per well of bovine serum albumin 5 mg/ml in

Tween-PBS for 1 hr at room temperature, and thoroughly washed again three times with Tween-PBS.

Sample Dilution: Plasma samples were diluted from 1 : 5 to 1 : 1280 in bovine serum albumin 1 mg/ml in Tween-PBS. Purified goldfish VTG was diluted serially to 7.8 to 500 ng/ml with Tween-PBS and used as the standard sample.

ELISA: One hundred microliters of diluted samples was dispensed in duplicate into wells of the microtiter plates for ELISA, and the plates were incubated for 2 hr at room temperature, followed by washing three times with Tween-PBS. HRP-conjugated anti-carp VTG rabbit polyclonal antibody (Transgenic Inc., Japan) was diluted to 1 μg/ml with Tween-PBS containing bovine serum albumin 1 mg/ml, and 100 μl of the diluted antibody solution was added to each well. The plates were incubated for 1 hr at room temperature and washed thoroughly with Tween-PBS. *O*-phenylenediamine 100 μl (Wako Pure Chemical Industries, Ltd., Japan) in substrate solution (pH 5.0) was added to each well, and the plate was incubated for exactly 15 min at room temperature, followed by the addition of 2 N sulfuric acid 50 μl/well to stop the reaction of color development, which is dependent on the amount of VTG in each well. The intensity of orange color that developed was measured at 490 nm with a microplate reader. VTG concentrations in diluted plasma samples were calculated by the standard curve after subtracting the small value of nonspecific color development with a Tween-PBS blank.

Exposure of Goldfish to Chemicals ——

Bisphenol-A: Two-year-old male goldfish were exposed to the nominal concentrations of 1, 10, 100, and 1000 μg/l of BPA (Wako Pure Chemical Industries, Ltd., Japan) dissolved in dechlorinated tap water at 21–23°C for 28 days. The control fish were exposed to the solvent carrier only (ethanol 0.1 ml/l). Seven goldfish in each group were maintained in a fish tank (25 l). The water in the tanks was changed every 72 hr. The photoperiod was 12 : 12 light : dark, and the fish were not fed during the experiment. The blood from 7 fish was collected at 0 (initial control), 7, and 28 days after BPA treatment. At the end of exposure, the blood and liver were sampled, and the weight and length of each fish were measured.

17β-Estradiol: Two-year-old male goldfish were exposed to the nominal concentration of E2 100 μg/l (Sigma Chemical Industries, Ltd., Japan) dissolved in dechlorinated tap water at 10° and 30°C for 10 days. The VTG concentration in plasma was assayed at 0, 1, 5, and 10 days. The control fish were exposed to the solvent carrier only (ethanol 0.1 ml/l). Other conditions were same as those in the BPA experiment, except that the number of fish in each group was 3 and the water in tanks was changed every day.

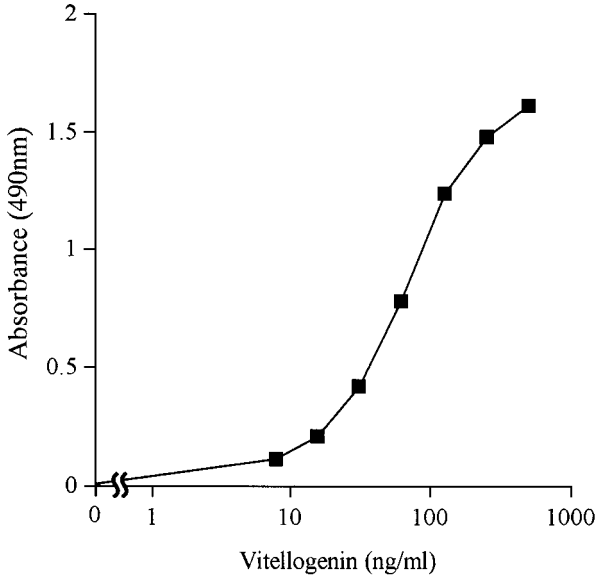

Fig. 1. Standard Curves for the Determination of VTG in Goldfish Plasma by ELISA

Purified VTG from E2-exposed male goldfish was used as standard. The absorbance of blank sample at 490 nm was 0.001 abs.

RESULTS

Evaluation of Purified Goldfish VTG and Its Cross-Reactivity with Anti-Carp VTG Antibody

VTG was purified from the plasma of E2-exposed male goldfish by HPLC. The main peak of retention time at about 9 min was analyzed. The fraction contained high levels of VTG, as indicated by its greenish color and the presence of a large amount of a 120-kDa protein on SDS-PAGE. The fraction was also evaluated by Western blot analysis using monoclonal antibody against carp lipovitellin. The antibody strongly cross-reacted with the VTG band of goldfish, but did not react with other plasma proteins (data not shown). The VTG band was not detected in the plasma of control fish.

Figure 1 shows a standard curve for the ELISA. Each data point is the average of two separate experiments. The lowest measurable concentration of VTG was approximately 1 ng/ml with purified VTG diluted in Tween-PBS. Due to interference by com-

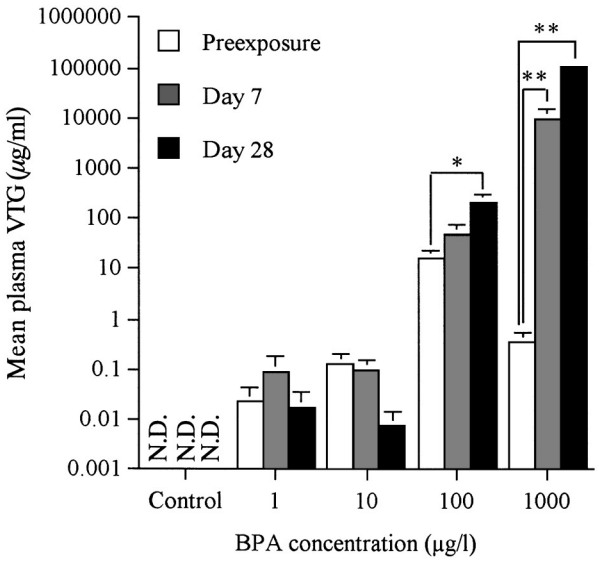

Fig. 2. Induction of Vitellogenin by BPA in Male Goldfish

Male goldfish were exposed to the nominal concentrations of BPA 1, 10, 100, and 100 μg/l for 28 days. The control goldfish were exposed to the solvent carrier only (ethanol 0.1 ml/l). N.D. = not detected. *Significant difference from preexposure at $p < 0.05$ level. **Significant difference from preexposure at $p < 0.01$ level. Error bar represents SEM.

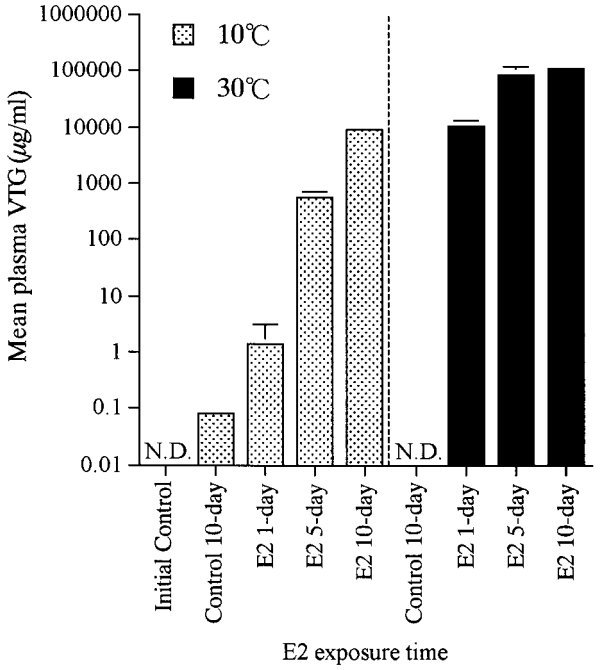

Fig. 3. Effect of Temperature on Plasma VTG Induction in Male Goldfish

Male goldfish were exposed to the nominal concentration of E2 100 μg/l at 10° and 30°C for 10 days. The control goldfish were exposed to the solvent carrier only (ethanol 0.1 ml /l). N.D. = not detected. Error bar represents standard SEM.

ponents in plasma, however, samples had to be diluted at least 5-fold, and the sensitivity was about 5 ng VTG/ml of plasma. This level of sensitivity is sufficient to detect abnormal levels of VTG in male plasma. Analysis of the plasma VTG in 20 samples using the present protocol required about 1 day. ELISA included a positive control plasma, for which the VTG concentration is known, to test inter- and intraassay variation. The coefficient of variation (CV) was calculated for each duplicate sample, and VTG determination was carried out again for samples in which the CV exceeded 10%. Standard curves fit to linear regression were used to calculate the VTG concentration, of which the R^2 values were usually between 0.95 and 0.99. The established ELISA has a working range of 7.8–500 ng/ml, and its sensitivity is 5 ng/ml when 200 μl of sample is assayed.

VTG Induction in Bisphenol-Exposed Goldfish

Male goldfish were exposed to BPA for 28 days. Plasma concentrations of VTG in the goldfish are shown in Fig. 2. VTG was not detected in the plasma of control fish. Exposure to BPA 1 or 10 μg/l did not increase plasma VTG levels. BPA 100 and 1000 μg/l increased the plasma VTG concentration to 201 ± 90 μg/ml and 104552 ± 24920 μg/ml, respectively, after 28 days of exposure. There was no effect of BPA on GSI and HSI (data not shown).

Effect of Water Temperature on VTG Induction by E2 Exposure

To confirm the effect of temperature on VTG induction by E2, male goldfish were exposed to the nominal concentration of E2 100 μg/l at 10° and 30°C for 10 days. Plasma concentrations of VTG in the goldfish are shown in Fig. 3. Control fish were maintained in dechlorinated tap water for 10 days. The mean plasma VTG concentrations of control fish maintained at 10° or 30°C were 0.08 μg/ml and not detectable, respectively. Exposure to E2 100 μg/l increased the plasma VTG in a time-dependent manner at both 10° and 30°C. However, the increase was more rapid at 30°C than that at 10°C, and the plasma VTG concentration after 1 day of E2 exposure was much higher in fish exposed at 30°C (10463 ± 3268 μg/ml) than in fish exposed at 10°C (1.42 ± 1.6 μg/ml).

DISCUSSION

An ELISA system was developed for the determination of VTG in the plasma of goldfish as a biomarker for environmental estrogens, including estrogen mimickers in the aqueous environment.

Lomax *et al*.[14] reported that ELISA assay conditions detected VTG in the concentration range of 10–450 ng/ml (85–20% of binding) of diluted sample. Sherry *et al*.[15] also reported that ELISA performance was optimized and characterized and the assay working range was 25–500 ng/ml with sensitivity of 10.5 ng/ml. In this study, the assay working range of 7.8–500 ng/ml was finally achieved for goldfish VTG. The lower detection limit of plasma VTG in this ELISA system was comparable to that in the previous reports, although anti-carp lipovitellin monoclonal antibody was used for goldfish VTG. Tyler *et al*.[16] reported that ELISA based on carp VTG antibody was developed to quantify VTG in the fathead minnow (*Pimephales promelas*). We have developed in the present study as ELISA system using anti-carp lipovitellin monoclonal antibody to determine goldfish VTG. This antibody could be used for VTG detection in various cyprinid species.

To check the sensitivity of male goldfish to an estrogenic compound, male goldfish were exposed to BPA for 28 days. Plasma VTG concentrations were increased by the exposure to BPA 100 and 1000 μg/l. We also confirmed in another experiment that exposure to BPA 40 μg/l increased plasma VTG concentration (unpublished data). Tabata *et al*.[17] reported that the female-specific protein in the blood solution was induced, when male medaka (*Oryzias latipes*) was exposed to BPA 100 μg/l for 14 days. In comparison with this result, goldfish seemed to have similar sensitivity to BPA as medaka. However, BPA concentrations that increased plasma VTG under the present experimental conditions were much higher than those detected in river water in Japan. To confirm the usefulness of goldfish for field research on EDCs, further experiments are required under various conditions, *i.e*., exposure to other EDCs such as nonylphenol and octylphenol, which induce plasma VTG in male rainbow trout,[18] interactions among EDCs and E2, *etc*.

Temperature is considered to be an important proximate factor regulating teleost reproductive processes. Korsgaard *et al*.[19] analyzed the effects of low (3°C) and high (10°C) temperatures on the vitellogenic response to E2 treatment in Atlantic salmon (*Salmo salar*) postsmolts. The level of VTG in the serum or hepatic RNA did not increase compared with the levels in untreated controls in previously warm-acclimated smolts if the fish were exposed to 3°C water during treatment with E2. Conversely, cold-acclimated smolts had increased levels of hepatic RNA and circulating VTG only when transferred to 10°C water during the experiment. Pawlowski *et al*.[20] also reported that RT-PCR analysis revealed increased amounts of VTG mRNA and estrogen receptor mRNA after 12 and 24 hr of E2 exposure at 18°C as compared with those at 14°C. In the present study, the higher temperature increased the response of goldfish to E2 exposure as evaluated by plasma VTG. Therefore it is necessary to control the water temperature in EDC exposure experiments in fish.

In this study, an *in vivo* testing system was developed for the evaluation of estrogenic activity of EDCs under experimental conditions and for investigations of EDCs in the aquatic environment using goldfish and plasma VTG as a biomarker. Although further studies are required to confirm the usefulness of goldfish, the present results suggest that goldfish could be a good model for EDC research.

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