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Bisphenol A Influences the Plasma Calcium Level and Inhibits Calcitonin Secretion in Goldfish

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ABSTRACT—In teleosts, it is well known that plasma calcium levels increase as a result of treatment with estrogen for at least during 2 weeks and that calcitonin secretion is induced by estrogen. The present study examined the influence of bisphenol A on calcium homeostasis in goldfish and compared the above known estrogenic action. In goldfish kept in water containing bisphenol A (10^{-6} M), the plasma calcium concentration increased significantly ($P<0.001$) at 4 days but decreased significantly ($P<0.05$) at 8 days. By the treatment of bisphenol A, calcitonin secretion was not induced until 4 days. At 8 days, however, plasma calcitonin, as well as calcium, decreased significantly ($P<0.05$), although vitellogenin was detected in the plasma. Therefore, bisphenol A influences plasma calcium levels, but its action is different from that of estrogen, which indicates that bisphenol A affects the calcium homeostasis and might bring about abnormal conditions in teleosts.

Key words: bisphenol A, calcium, calcitonin, vitellogenin, goldfish

INTRODUCTION

Bisphenol A, 4,4'-isopropylidenediphenol, is an important industrial compound, a major component of epoxy and polystyrene resins used in food packaging and as a protective coating. This compound has been shown to possess estrogenic properties and is called an endocrine disrupter because it binds to the estrogen receptor (ER) (for a review, see Safe *et al*., 2001). Therefore, the reproductive effect has already been noted (Gould *et al*., 1998; Luconi *et al*., 2001).

Calcitonin, a 32-amino acid peptide hormone, is secreted from the C-cells of the thyroid gland in mammals and from the ultimobranchial gland in non-mammals (Dacke, 1979). In mammals, this hormone has a hypocalcemic action that can mineralize bones by suppressing the activities of osteoclasts (Azria, 1989). In teleosts as well as mammals, we found that this hormone can suppress the activities of osteoclasts in the scale (Suzuki *et al*., 2000a), which is a calcified tissue that contains osteoblasts and osteoclasts, similar to those found in avian and mammalian bone (for a review, see Bereiter-Hahn and Zylberberg, 1993). In eel, furthermore, we demonstrated that plasma calcitonin levels increased with the rise of plasma calcium caused by a dietary uptake of calcium (Suzuki *et al*., 1999). These results indicate that calcitonin is a hypocalcemic hormone in teleosts. On the other hand, this hormone has been closely related to reproductive physiology in female teleosts. Plasma calcitonin levels in some female teleosts increase corresponding to sexual maturation (Watts *et al*., 1975; Yamauchi *et al*., 1978; Norberg *et al*., 1989). An injection of estrogen into rainbow trout induced calcitonin secretion (Björnsson *et al*., 1989). Bisphenol A, a putative endocrine disrupter, might influence plasma calcium and calcitonin levels because it is known that estrogen affects bone metabolism (Komm *et al*., 1988; Eriksen *et al*., 1988; Okazaki *et al*., 2002).

To examine the effect of bisphenol A on calcium homeostasis in teleosts, in the present study, the plasma calcium level was measured in bisphenol A-treated immature goldfish, in which the endogenous effects of sex steroids are negligible. In addition, the plasma calcitonin level was examined and compared with the plasma calcium level. Furthermore, plasma vitellogenin was detected using SDS-polyacrylamide gel electrophoresis (SDS-PAGE) to reconfirm that bisphenol A, as well as estrogen, acts on the liver

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and promotes its synthesis.

This study is the first to demonstrate that bisphenol A affects calcium homeostasis in teleosts.

MATERIALS AND METHODS

Animals

The exact effect of estrogen on the calcium metabolism cannot be examined in matured fish because of the endogenous effects of sex steroids. Using immature fish, therefore, the influence of estrogen on the calcium metabolism has been examined (Mugiya and Watabe, 1977; Björnsson *et al.*, 1989: Persson *et al.*, 1995). In the present study, immature goldfish (*Carassius auratus*) were purchased and used (both sexes, n=56, 6.05±0.51 g).

Blood sampling in goldfish kept in water containing bisphenol A

Goldfish were kept at 25°C during experiment. Blood samples from eight goldfish were taken prior to the start of the experiment (zero hr) after anesthetization with ethyl 3-aminobenzoate, methanesulfonic acid salt (MS-222, Aldrich Chemical Company, Inc., USA) into heparinized hematocrit capillary tubes from the gill. The remaining fish were randomly divided into 2 groups (control and experimental groups, each n=24), and each was kept in an 8 L glass aquarium. Our recent study using immature goldfish of almost the same size indicated that bisphenol A at 10^{-5} M suppressed osteoclastic and osteoblastic activities in cultured scales (Suzuki and Hattori, 2003). In the present study, therefore, we used bisphenol A (Wako Pure Chemical Industries, Ltd., Japan) at 10^{-6} M for 2, 4, and 8 days (each n=8), and the group was compared with a control group kept in tap water. After the exposure, blood samples were taken from the goldfish gill by heparinized hematocrit capillary tubes under anesthesia with MS-222.

Measurement of plasma calcium and calcitonin levels

The plasma calcium concentration was measured using a microplate reader and a modified method of Gitelman (1967).

The plasma calcitonin level was analyzed by the competitive enzyme-linked immunosorbent assay, according to the procedure of Suzuki (2001). The detection limit was 25 pg/ml. The specificity of anti-salmon calcitonin serum (No. 626, Cosmo Bio Co. Ltd., Japan) was checked using peptide hormones (1-34 bovine parathyroid hormone and human calcitonin gene-related peptide). This anti-serum did not cross-react to these peptide hormones.

Detection of plasma vitellogenin using SDS-PAGE in the bisphenol A-treated goldfish

The plasma vitellogenin in goldfish was detected around 140 kDa by SDS-PAGE (De Vlaming *et al.*, 1980). On the basis of the report, the separation gel was prepared with 7.5% polyacrylamide. SDS-PAGE was performed by the method of Laemmli (1970). Five μl of plasma sample at 8 days in either bisphenol A-treated or control goldfish was solbilized in 10 μl of a lysis buffer containing 4% SDS, 4% 2-mercaptoethanol, 8M urea, and 10 mM Tris-HCl (pH 6.8) and subjected to electrophoresis. To calculate the molecular weight of vitellogenin, molecular markers (High Range; Bio-Rad Laboratorys, USA) were used. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 (Research Organics Inc., USA).

Statistical analysis

All results are expressed as the means ± SEM (n=8). Student's *t*-test was used to determine the statistical significance. In all studies, the experimental group was compared to its specific time in the control group. The significance level chosen was $P<0.05$.

RESULTS

Effect of bisphenol A on the plasma calcium level in goldfish

The results are shown in Fig. 1. In the control goldfish, the plasma calcium concentration did not change during 8 days.

Conversely, the plasma calcium concentration increased significantly ($P<0.001$) at 4 days (8.94±0.25 mg/100 ml) by bisphenol A treatment. At 8 days, however, plasma calcium decreased significantly ($P<0.05$)(5.96±0.42 mg/100 ml).

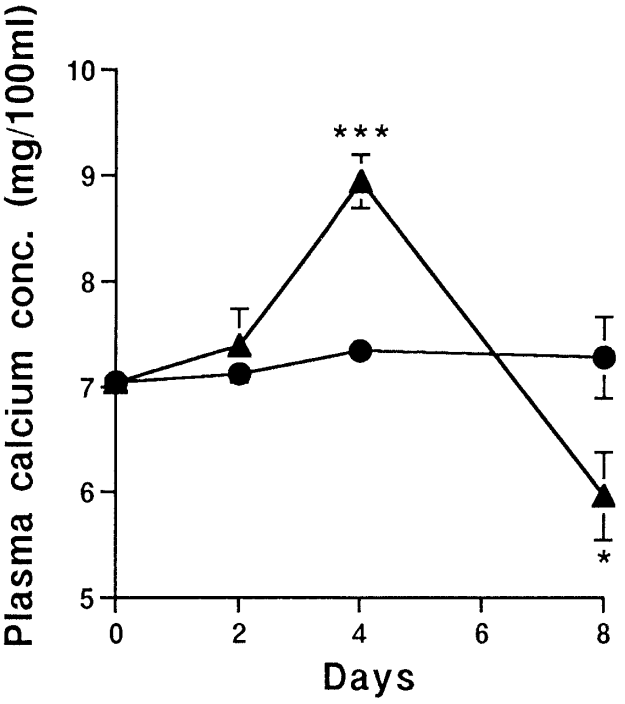


Fig. 1. Plasma calcium levels in the bisphenol A-treated goldfish (▲) and control goldfish (●). Values are means±SEM. *, *** indicate statistically significant differences at $P<0.05$ and $P<0.001$, respectively, compared with the values in the control.

Effect of bisphenol A on the plasma calcitonin level in goldfish

The results are shown in Fig. 2. In the control group, there was not any change for 8 days. The plasma calcitonin levels at 2 and 4 days after bisphenol A treatment were 186.93±11.81 and 168.33±17.50 pg/ml, respectively. At 8 days, however, plasma calcitonin, as well as calcium, decreased significantly ($P<0.05$) in the bisphenol A-treated goldfish (117.50±10.86 pg/ml).

Detection of plasma vitellogenin using SDS-PAGE in the bisphenol A-treated goldfish

The results are shown in Fig. 3. At 8 days, plasma vitellogenin (140 kDa) was detected by SDS-PAGE in the bisphenol A-treated goldfish. However, it was not found in

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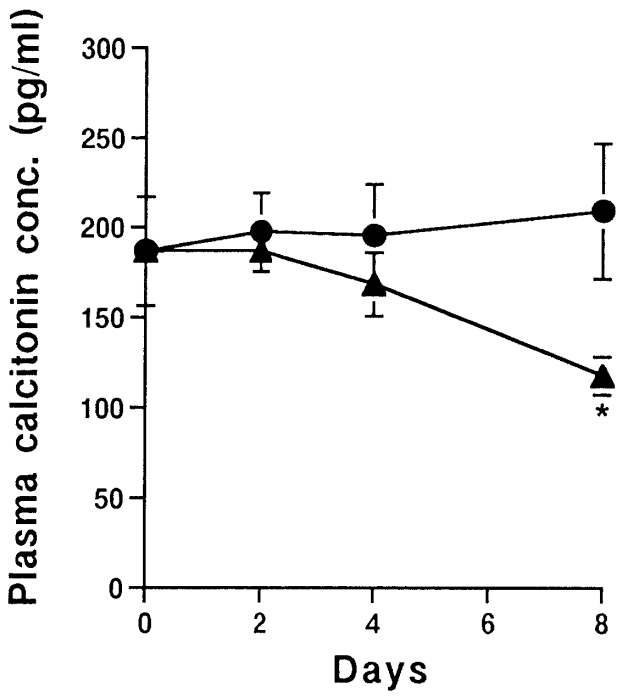


Fig. 2. Plasma calcitonin levels in the bisphenol A-treated goldfish (▲) and control goldfish (●). Values are means±SEM. * indicates a statistically significant difference at $P<0.05$, compared with the values in the control.

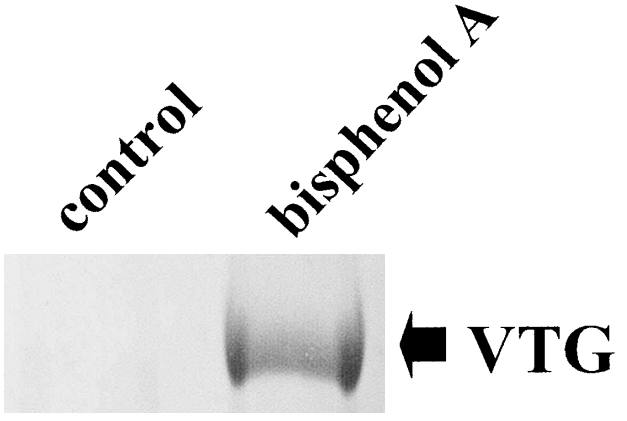


Fig. 3. Detection of plasma vitellogenin using SDS-PAGE in the bisphenol A-treated goldfish and control goldfish. The band of vitellogenin (VTG)(140 kDa) was detected in the plasma of bisphenol A-treated goldfish but not in that of control goldfish.

the plasma of control goldfish.

DISCUSSION

In teleost fish, estrogen is known to be a hypercalcemic hormone that enhances the activities of scale osteoclasts (Persson *et al*., 1995; Suzuki *et al*., 2000a). Several reports have shown that the plasma calcium level increased by treatment with estrogen at least during 2 weeks (Mugiya and Watabe, 1977; Björnsson *et al*., 1989: Persson *et al*., 1995). By the treatment of bisphenol A, the plasma calcium level had increased at 4 days and then dramatically decreased at 8 days in the present study (Fig. 1). Therefore, bisphenol A treatment brought about different actions from estrogen in the goldfish and affected the calcium homeostasis. In addition, we indicated that bisphenol A directly suppressed osteoclastic and osteoblastic activities in the scale in an *in vitro* study (Suzuki and Hattori, 2003). These data show that bisphenol A affects calcium metabolism in teleosts.

During reproductive period, the synthesis of vitellogenin in the liver was induced by estrogen (Kwon *et al*., 1993). In the present study, plasma vitellogenin was detected at 8 days after treatment of bisphenol A (Fig. 3). However, the plasma calcitonin level was inhibited at 8 days after the treatment (Fig. 2), although it has been reported that calcitonin secretion increased by estrogen (Björnsson *et al*., 1989). In plasma calcitonin as well as calcium, therefore, bisphenol A acts differently from estrogen in the goldfish. Furthermore, we demonstrated that calcitonin receptor was expressed in the ovary of teleosts (Suzuki *et al*., 2000b), suggesting that calcitonin has some roles in the maturation and early development of eggs. These results suggest that bisphenol A influences fish reproduction as well as calcium homeostasis. On the other hand, bisphenol A is known to induce larval deformities and growth suppression (for a review, see Vos *et al*., 2000). Our recent *in vitro* study indicated that bisphenol A suppressed bone cell activities (Suzuki and Hattori, 2003). In addition, we have shown in the present study that bisphenol A affects calcium homeostasis. Therefore, bisphenol A might induce the deformity of larvae and growth suppression.

Bisphenol A, similar to estrogen, binds to ER, as is well known in mammals and teleosts (for reviews, see Vos *et al*., 2000; Safe *et al*., 2001). However, a different action caused by bisphenol A and estrogen has been reported, although the detailed mechanism has not been elucidated. For example, bisphenol A had no effect on rat uterine weight (Gould *et al*., 1998). In human spermatozoa, it did not exert any direct effect on progesterone-mediated calcium fluxes, although estrogen had an inhibitory effect on it (Luconi *et al*., 2001). Furthermore, it has been reported that, in the human endometrial carcinoma cell line, estrogen produced a 2-fold increase in the cell number, but bisphenol A did not induce cell proliferation (Bergeron *et al*., 1999). Our recent study shows that bisphenol A significantly suppressed the tartrate-resistant acid phosphatase and alkaline phosphatase activities in the cultured scale, that estrogen stimulated both activities, and that the *insulin-like growth factor-1* mRNA expression decreased as a result of a bisphenol A treatment, which was in contrast to the control and the estrogen treatment (Suzuki and Hattori, 2003).

In the present study, we demonstrated that bisphenol A, an estrogenic endocrine-disrupting chemical, induced an action that was different from that of estrogen, and influenced calcium homeostasis. In reproductive periods, many

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hormones such as the gonad-tropic hormone, are related to the serial phenomena. Therefore, bisphenol A might influence other hormone(s) and affect fish reproduction. Further study will be needed to elucidate the detail effect of bisphenol A on reproduction.

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