EXPRESSION OF THE ESTROGEN RECEPTOR ALPHA GENE IN THE ANAL FIN OF JAPANESE MEDAKA, ORYZIAS LATIPES, BY ENVIRONMENTAL CONCENTRATIONS OF BISPHENOL A

Hitomi HAYASHI1, Ayano NISHIMOTO1,2, Noriko OSHIMA3 and Shawichi IWAMURO1

1Department of Biology and 3Department of Biomolecular Science, Faculty of Science, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan
2Chiba High School, 1-5-2 Katsuragi, Chuou-Ku, Chiba 260-0853, Japan

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ABSTRACT — The anal fin in Japanese medaka, Oryzias latipes, is a typical sexual secondary character. In the present study, we focused on this organ and examined the effects of low doses of a natural estrogen, 17β-estradiol (E2), and an environmental xenoestrogen, bisphenol A (BPA), in vivo by monitoring estrogen receptor (ER) α gene expression. Groups of adult male and female medaka were immersed in 10⁻⁹ M E₂ or 10⁻¹⁰ to 10⁻⁸ M BPA and the levels of ERα gene transcripts in the anal fins were measured by semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). One day of treatment with each concentration of BPA examined and 10⁻⁹ M E₂ increased the levels of ERα mRNA in female anal fins by 3-fold as compared with controls. In the male specimens, neither 10⁻⁹ M E₂ nor 10⁻¹⁰ M BPA showed remarkable effects on the anal fins as compared with the results in females, but 10⁻⁹ and 10⁻⁸ M BPA increased the levels of ERα mRNA by 2.3- and 3.3-fold with 1 day of exposure, respectively. The present results showed that medaka anal fins may be a sensitive bio-indicator for screening of environmental estrogenic chemicals.

KEY WORDS: Anal fin, Bisphenol A, Estrogen receptor α, Medaka

INTRODUCTION

Bisphenol A (BPA) is a known environmental estrogen that is used as a monomer in the manufacture of polycarbonate plastic. This chemical has been studied mainly with regard to its effects on animal development and reproductive systems (Yokota et al., 2000; Kang et al., 2002). BPA has been described in previous reports as a very “weak” xenoestrogen because these assay systems, especially in vivo, required roughly 1,000- to 10,000-fold concentrations of BPA to exhibit estrogenic activities relative to the potency of 17β-estradiol (E₂). Generally, the effects of xenoestrogens on animals are monitored by measuring the levels of estrogen-response gene transcripts or the subsequently produced proteins, such as vitellogenin, luteinizing hormone (LH), and estrogen receptor (ER), which are transcribed/translated via the ER in a ligand-dependent manner. ER is a member of the steroid hormone/thyroid hormone nuclear receptor superfamily proteins, which act as ligand-inducible transcription factors. Thus, estrogenic activities of chemicals can be detected by measuring the levels of ER gene transcripts in appropriate organs and tissues in test animals.

In the present study, we focused on the anal fin of Japanese medaka (Oryzias latipes), a secondary sexual character of this fish species. Due to sexual dimorphism, we expected that these organs could be good bio-indicators to screen for suspected endocrine disrupting chemicals. In fact, exposure of male medaka to E₂ or estrogenic chemicals at early developmental stages could induce the formation of female type anal fin in these animals through a feminization effect (Tabata et al., 2001). Taken together, we investigated the possible role of the medaka anal fin as a bio-indicator for screening of low concentrations of BPA in vivo by monitoring the expression of ERα mRNA in tissue specimens.
MATERIALS AND METHODS

Animals
Adult male and female medaka weighing 110 ± 5 mg were purchased from a pet shop in Chiba, Japan, maintained in dechlorinated water at 25°C and fed with TetraMin purchased from Tetra Japan (Tokyo, Japan) every other day under a 12-hr light-dark cycle. Animals were acclimatized to these conditions for at least two weeks before the experiments. All experiments were approved by the Toho University Bioethics and Animal Ethics Committee and were carried out by authorized investigators.

Treatment with E2 or BPA
17β-Estradiol (E2) and BPA were purchased from Wako (Osaka, Japan). E2 and BPA were dissolved in ethanol at 10⁻⁸ M as stock solutions and diluted in dechlorinated water at appropriate concentrations. In each experiment, care was taken so that both experimental and control solutions contained equal amounts of ethanol. Groups of male or female medaka (n=4 or 5) were immersed in 500 ml of dechlorinated water containing 10⁻⁹ M E2 or 10⁻¹⁰, 10⁻⁹, or 10⁻⁸ M BPA for 1, 3, 7 or 14 days under the above conditions in a 1 L glass beaker with continuous air-supply. The water was changed every day. At the end of each treatment, the animals were anesthetized with MS222 (Sigma, St. Louis, MO, USA) and the anal fins were removed immediately with a scalpel, and then pooled in each group. Total RNA was extracted from the pooled anal fin specimens by the acid phenol/guanidinium isothiocyanate-phenol-chloroform procedure (Chomczynski and Sacchi, 1987).

Semi-quantitative RT-PCR
Analysis was carried out by a modification of a procedure previously described (Iwamuro et al., 2003) using a One-Step RT-PCR kit (Qiagen, Chatsworth, CA, USA). Briefly, aliquots of total RNA (100 ng for ERα, 50 ng for β-actin) and a set of primers for amplification of partial medaka ERα or β-actin cDNA were incubated at 50°C for 30 min for reverse transcription and then at 95°C for 15 min for denaturation of the reverse transcriptase. Subsequently, PCR was performed under the following conditions: 30 s at 94°C, 30 s at 50°C and 1 min at 72°C for 30 (ERα) or 28 (β-actin) cycles. Finally, the reaction mixtures were kept at 72°C for 7 min to complete extension of the DNA. The specific sense (5’-ATGATGAAGGGCGGTGTCCGCAAGG-3’) and antisense (5’-CAACTTCTGACCGAGCA-GAGTATC-3’) primers for the ERα (GenBank Acc. No. D28954) and sense (5’-ATCCAGGCTGTGTCT- GTCCTCT-3’) and antisense (5’-TCCAGGGCGACG- TAGACAG-3’) primers for the β-actin (GenBank Acc. No. S74868) were synthesized by Sigma Genosys Japan (Ishikari, Hokkaido, Japan). The RT-PCR products (10 µl aliquots) were separated on 1.5% agarose gels with a Mupid II electrophoresis apparatus (Advance, Tokyo, Japan) and stained with ethidium bromide. Fluorescent images of the amplified DNAs were recorded and calculated with the Kodak EDAS290 system (Rochester, NY, USA) using Takara DNA ladder markers (Ohtsu, Japan) (100 ng/band) as a reference standard. The electrophoresis apparatus used in the present study allowed separation of at most 8 samples in duplicate on one agarose gel plate. Therefore, the cDNA specimens amplified by RT-PCR from each experimental group were separated on the same gel and the mRNA levels were semi-quantified in duplicate. The relative amounts of ERα were normalized with reference to the amount of β-actin in each sample.

RESULTS AND DISCUSSION
The present study demonstrated the in vivo effects of environmental concentrations of BPA on the medaka anal fin, which is a secondary sexual character in this fish species, by monitoring ERα gene expression. The basal levels of ERα mRNA in the organ were not significantly different between males and females (data not shown). As shown in Fig. 1, BPA even at a concentration of 10⁻¹⁰ M, as well as 10⁻⁹ M E2, increased ERα mRNA levels in the female anal fins with 1 day of treatment by approximately 3-fold as compared with controls. The concentration of 10⁻¹⁰ M was sufficient for BPA to induce ERα mRNA expression at the maximum level in this experimental system, and no further additive enhancement of the gene expression by higher concentrations of BPA (10⁻⁹ and 10⁻⁸ M) were observed. Thereafter, the levels of ERα mRNA in the female anal fins immersed in solutions of 10⁻¹⁰ E2 or 10⁻¹⁰, 10⁻⁹, or 10⁻⁸ M BPA decreased gradually. The levels in the specimens treated for 3 and 7 days were approximately 1.3- to 1.5-fold and 1.0- to 1.2-fold in control specimens for each treatment period, respectively. The levels of ERα mRNA in the anal fin specimens in solutions of E2 and of each concentration of BPA for 14 days again showed slight increases.

Fig. 2 shows the results from the same series of
Expression of ERα gene in the anal fin of medaka by low doses of BPA.

Experiments as shown in Fig. 1 but with male animals. In comparison with the female anal fin, the organ in male medaka was less sensitive to E2 and its agonist. In the specimens treated for 1 day, the levels of ERα mRNA were slightly (1.5-fold) increased by 10^{-9} M E2. Although 10^{-10} M BPA had no effect on the male anal fin, 10^{-9} M and 10^{-8} M BPA were more active than 10^{-9} M E2 and increased the ERα mRNA levels by 2.3- and 3.3-fold as compared with controls, respectively. E2 at a concentration of 10^{-9} M showed a peak in ERα gene expression after 3 days of treatment in the male medaka anal fin. On the other hand, while the anal fin specimens treated with 10^{-9} M BPA for 3 and 7 days retained increased levels of ERα mRNA, those treated with 10^{-8} M BPA declined gradually and became close to the control levels. This tendency was also observed in the E2-treated specimens. No remarkable morphological changes of the male and female medaka anal fins in each treatment were observed through the experiments.

**Fig. 1.** Effects of 10^{-9} M E2 or 10^{-10}, 10^{-9}, or 10^{-8} M BPA on ERα gene expression in female medaka anal fins with treatment for 1, 3, 7, or 14 days. At the end of each period, the anal fin was dissected out at the base and pooled into tubes (4 or 5 anal fins per tube), and then total RNA was extracted and subjected to semi-quantitative RT-PCR. The relative amounts of ERα were normalized with reference to that of β-actin. Each column represents the average value of duplicate determinations. C, control; E2, 10^{-9} M E2; -10B, 10^{-10} M BPA; -9B, 10^{-9} M BPA; -8B, 10^{-8} M BPA.
In general, the liver of the fish is used as the target organ because of its richness of estrogen-responsive genes and their products. Yamaguchi et al. (2005) reported that BPA had acutely (8 hr) potent estrogenic effects on the expression of ERα in adult male medaka liver, but these activities required a high concentration of 8,000 μg/l (3.5 × 10⁻⁵ M), while E₂ was potent at 0.01 μg/l (3.7 × 10⁻¹¹ M). Similarly, Ishibashi et al. (2005) reported that treatment for 21 days with BPA at 1,000 μg/l (4.4 × 10⁻⁶ M) but not 500 μg/l (2.2 × 10⁻⁶ M) induced vitellogenin mRNA expression in the liver of adult male medaka. Expression of ER, cytochrome P450 aromatase, and p53 genes in the female medaka liver were induced by 75 μg/l (5.3 × 10⁻⁷ M) of BPA (Min et al., 2003). These “effective” concentrations of BPA are roughly calculated as ranging from 5.3 × 10⁻⁷ to 3.5 × 10⁻⁵ M. However, environmental concentrations of BPA in water from the Tama River in the Tokyo area, Japan, in 1999, were up to 700 ng/l, which is approximately equivalent to 3.0 × 10⁻¹⁰ M or 0.07 ng/l.

Fig. 2. Effects of 10⁻⁹ M E₂ or 10⁻¹⁰, 10⁻⁹, or 10⁻⁸ M BPA on ERα gene expression in male medaka anal fins with treatment for 1, 3, 7, or 14 days. Total RNA from the male anal fins was prepared in a manner similar to that described in Fig. 2 and subjected to semi-quantitative RT-PCR. The relative amounts of ERα were normalized with reference to that of β-actin. Each column represents the average value of duplicate determinations. C, control; E₂, 10⁻⁹ M E₂; -10B, 10⁻¹⁰ M BPA; -9B, 10⁻⁹ M BPA; -8B, 10⁻⁸ M BPA.
ppb (Masunaga et al., 2000), and is far from the concentrations at which these agents exhibit potent estrogenic activities. In contrast, recent findings based on in vitro experiments demonstrated that “low-dose,” even $10^{-12}$ M or 0.23 ppt, BPA could act as an E$_2$ agonist (reviewed in vom Saal and Hughes, 2005).

Most of the estrogenic actions of endocrine disruptors have been monitored with respect to genomic responses mediated by nuclear ERs. In addition to these classical genomic actions, recent findings established that estrogens could act through nongenomic or membrane-initiated signaling pathways via the plasma membrane form of ER (Nadal et al., 2000, 2001). These nonclassical membrane ER react to very low concentrations of E$_2$, even at $10^{-12}$ M (reviewed in Watson and Gametchu, 2003). It is noteworthy that most studies indicating low-dose effects of xenoestrogens, including BPA, are involved in nonclassical membrane ER-related pathways (Watson et al., 1999; Quesada et al., 2002; Wozniak et al., 2005). The differences in sensitivity to xenoestrogens between in vivo and in vitro assay systems may be due to which ER, the classical or nonclassical pathway, is bound by the test substances. In the present study, it was not clarified whether stimulation of ER$\alpha$ gene expression in medaka anal fin by E$_2$ and BPA described here were mediated via the classical genomic ER or the non-classical membrane ER when considering the range of effective concentrations. However, as the assay system detected estrogen-response gene expression, it is likely that ER$\alpha$ gene expression induced by low doses of BPA may involve the classical pathway. Further studies are required to elucidate the precise mechanism of this pathway.

Although the present in vivo assay system is still not as sensitive as those of in vitro ER-dependent systems, sensitivity of 1,000-fold or more than the previous in vivo systems has been reported. In addition, the system is advantageous from the viewpoint of simplicity and also its experimental conditions are harmless to test animals. Consequently, the present assay system may be useful as a sensitive bio-indicator for screening of environmental estrogenic chemicals, and experiments are currently underway to develop this assay system for this purpose.

REFERENCES


