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Effect of Bisphenol-A on Vitellogenin Synthesis and Estrogen-Estrogen Receptor Binding Activity in the Primary Hepatocyte Cultures of Rainbow Trout, *Oncorhynchus mykiss*

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Effects of bisphenol-A (BPA) on vitellogenin (VTG) synthesis and estrogen-estrogen receptor (E_2-ER) binding activity were examined in primary hepatocyte cultures of rainbow trout, *Oncorhynchus mykiss*. Hepatocytes were precultured for 2 days and then estradiol-17β (E_2, 2×10^{-6} M), BPA (10^{-5}~10^{-8} M) and/or 4-hydroxy-tamoxifen (4-OHT, 10^{-6} M) were simultaneously added to the incubation medium. Hepatocytes were cultured for 5 more days and then spent medium was analyzed by SDS-PAGE for VTG production. The addition of BPA to the incubation medium had no effect on the viability of hepatocytes in the culture. On the other hand, BPA increased VTG production in a concentration-dependent way and a significant increment occurred at BPA concentrations greater than 10^{-6} M. Although VTG was increased by the addition of E_2 (2×10^{-6} M) or BPA (10^{-5} M), its were reduced by a simultaneous 4-OHT (10^{-6} M) addition. BPA inhibited E_2-human ER binding activity by 72% at 10^{-5} M of BPA. These results suggested that BPA induced VTG synthesis by BPA-ER binding activity in the hepatocyte of rainbow trout.

Key words: Bisphenol-A, Vitellogenin, Estrogen, Estrogen receptor

Introduction

Recently, considerable concern has been expressed over the possibility that the estrogenic activity of man-made synthetic chemicals, xenoestrogenic contaminants, may adversely affect reproductive functions in humans and wildlife species (Stone, 1994; Jobling et al., 1996; Smeets et al., 1999). For instance, xenoestrogenic contaminants modified the hepatic metabolism of steroid hormones implicated in the reproduction of aquatic organisms (Jobling et al., 1996; Ashfield et al., 1998), resulting in recruitment failure. In teleosts, as in other oviparous vertebrates, vitellogenesis is an important reproductive function which allows the storage of egg yolk proteins. Vitellogenin (calcium-binding phospholipoglycoproteins, VTG), a precursor molecule of egg yolk, is synthesized in the liver under stimulation of estrogen (estradiol-17β, E_2). The first step of VTG synthesis is initiated by the binding of E_2 to its receptors (ER) in hepatocyte, in which receptors are upregulated by E_2 itself (Lazier and MacKay, 1993). The processes of VTG synthesis is also used the biomarker for determining xenoestrogenic contaminant (Christiansen et al., 1998).

Bisphenol-A (BPA), a monomer of polycarbonate plastics, is one of synthetic chemicals and has structural homology with diethylstilbestrol (DES), a synthetic substance with potent estrogenic activity (Steinmetz et al., 1997). Lindholst et al. (2000) also reported that VTG production is closely related to internal liver concentration of BPA when exposured juvenile rainbow trout to BPA. It is a wondering about that how BPA can induce VTG synthesis in the hepatocyte. In the present study, we examined the effects of BPA on VTG synthesis and E_2-ER binding affinity for determining whether VTG synthesized by the binding activity of BPA to ER in

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the hepatocyte of rainbow trout.

The present study aimed to elucidate any direct effect of BPA on VTG synthesis and E_2-ER binding activity in rainbow trout. Hepatocytes were cultured with BPA, E_2 and/or 4-OHT and then synthesized VTG was electrophoretically analyzed. The effect of BPA on the binding affinity of E_2 to ER were also examined using an enzyme-linked immunosorbent assay (ELISA).

Material and Methods

Rainbow trout, *Oncorhynchus mykiss,* weighing 110~480 g were obtained from a commercial dealer and kept in outdoor ponds with running water at about 14℃. They were fed trout food pellets once a day but were starved on the last day before sampling to reduce the production of bile. Maturing females were not included.

Hepatocyte preparation and incubation

Hepatocytes were prepared following Hayashi and Ooshiro (1975) as described by Kwon et al. (1993). Cell yields and viability were determined by the trypan blue exclusion test.

Cell were planted into 60-mm plastic petri dish with positive charge (Falcon) at a density of $2-5\times10^5$ cell/dish. William's medium E (Life Tech. Inc.) containing 0.2 μM bovine insulin (sigma), streptomycin (100 μg/mL), and penicillin (70 μg/mL) was used for cell culture. All incubations were carried out in 3 mL of the medium at 15 under 5% CO_2.

Effect of BPA on VTG synthesis and cell viability

Cells were precultured for 2 days and then estradiol-17β (E_2, 2×10^{-6} M in 3 μL of 95% ethanol; Sigma) or BPA (3 μL of 95% ethanol; Aldrich Chem. Com. Inc.) were simultaneously added to the dishes. BPA concentrations added were 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M. The effects of BPA on VTG synthesis were examined 5 days after addition, during which time the media were changed daily. Control cultures received the equivalent amount of the solvents only. After culture, the media was analyzed using SDS-PAGE for VTG proteins.

The effects of BPA on cell viability were also examined using crystal violet as described by Mugiya and Tanahashi (1998) and Hwang et al. (2000). Cell viability was taken as the number of living cells on Day 5 expressed as a percentage of the number of living cells just before BPA addition.

Effect of 4-OHT on BPA-induced VTG synthesis

Effect of 4-hydroxy-tamoxifen (4-OHT) on BPA-induced VTG production was examined to elucidate the inducible profile of BPA on VTG synthesis. Cells were precultured for 2 days and then E_2 (2×10^{-6} M), BPA (10^{-5} M) and/or 4-OHT (10^{-6} M in 3 μL of dimethyl sulfoxide, DMSO) were simultaneously added to the dishes. The effects of 4-OHT on BPA-inducted VTG production was examined 5 days, during which time the media were changed daily. After culture, spent media were analyzed by SDS-PAGE for VTG production.

SDS-polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was done as described by Mugiya and Tanahashi (1998). Briefly, proteins were precipitated from the media by cold trichloroacetic acid, dissolved in sample buffer (0.175 M Tris-HCl, 8 M urea, 1% SDS and 0.5% 2-mercaptoethanol, pH 7.4) and subjected to 5~20% gradient SDS-PAGE. The gels were stained with 0.25% Coomassie brilliant blue R-250 (CBB). It was difficult to apply a fixed amount of proteins to each lane, because the present experiment was intended to induce VTG (protein) synthesis. Standard proteins used for molecular weight (MW) determinations were carbonic anhydrase (MW 29,000), ovalbumin (45,000), bovine serum albumin (66,000), phosphorylase b (97,400), β-galactosidase (116,000) and myosin (205,000).

Qualitative and quantitative analyses of VTG

The identification of the VTG band was based on the results of a previous study (Kwon et al., 1993) in which isolated rainbow trout hepatocytes incubated with E_2 synthesized a protein with the same electrophoretic mobility (175 kDa) as in the present study. Kwon et al. (1993) identified this band as VTG (main band) by immunoblot and immunoelectrophoresis.

After SDS-PAGE, the integrated optical density (IOD) of the VTG band was measured by a Bio

Image System (Millipore, Bedford) and was expressed as a percentage of the IOD of the total protein including VTG. Minor subunits of VTG were not considered as VTG, because the subunits constituted only a fairly small part of VTG and overlapped with other proteins (Kwon et al., 1993). An excellent correlation ($r=0.99$) was reported between the amount (100~1600 ng/mL) of VTG applied to the electrophoretic lanes and the IOD (Yeo and Mugiya, 1997).

Effects of BPA on E_2-ER binding affinity

Effects of BPA on the binding activity of E_2 to ER was examined with an ELISA using an ER competitor screening kit (Wako Pure Chem). This kit consists of a 96-well microplate coated with human ER α (hER α) recombinant, reaction solution including fluorescence-labeled E_2 and other necessary reagents such as washing buffer. The assay was done as described by Hwang (2001). Briefly, BPA, E_2 and 4-OHT were dissolved in 6 μL of DMSO, added to the reaction solution in hER α coated wells and incubated for 2 h at room temperature. Final concentrations of these additions were 0, 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M. The control group was incubated with the reaction solution containing DMSO only.

Fluorescence intensity in each well was measured with a microplate photometer (MTP-22, Corona) at 490 nm (excitation) and 530 nm (emission).

Statistical analysis

A one-way ANOVA (Fisher PLSD test) was used for statistical evaluation of mean values. Significance was accepted at $P<0.01$. Percentage data were statistically analyzed after being arcsine transformed.

Results

The collagenase perfusion method yielded about 3×10^8 hepatocytes per fish. Cell viability was estimated to be over 90% by trypan blue staining. After being transferred to a positively charged dish, hepatocytes firmly attached to the dish in the serum-free medium and started to spread within 2 days of preculture. Although hepatocytes maintained good viability for at least 15 days, they spread rather slowly and formed a few aggregations. When expressed as a percentage of the number of living cells on Day 5 after the addition of BPA to the number of living cells just before addition, BPA had no appreciable toxic effect on cell viability (Table 1) showing survival rates of 71~79% regardless of the concentrations used.

Table 1. Effect of BPA on survival rate of hepatocytes on Day 5 after BPA addition in the cultures

BPA Concentration (M)	Survival rate (%)
0	72 ± 8.4[a]
10^{-8}	79 ± 8.1
10^{-7}	76 ± 6.1
10^{-6}	78 ± 9.2
10^{-5}	71 ± 9.8

[a]Percentage of the number of living cells on Day 5 to the number of living cells just before BPA addition (mean ± SE for four individuals).

Effect of BPA on VTG synthesis

After a 2-day preculture, hepatocytes were incubated in the serum-free medium with E_2 or BPA for 5 days and the spent medium was analyzed by

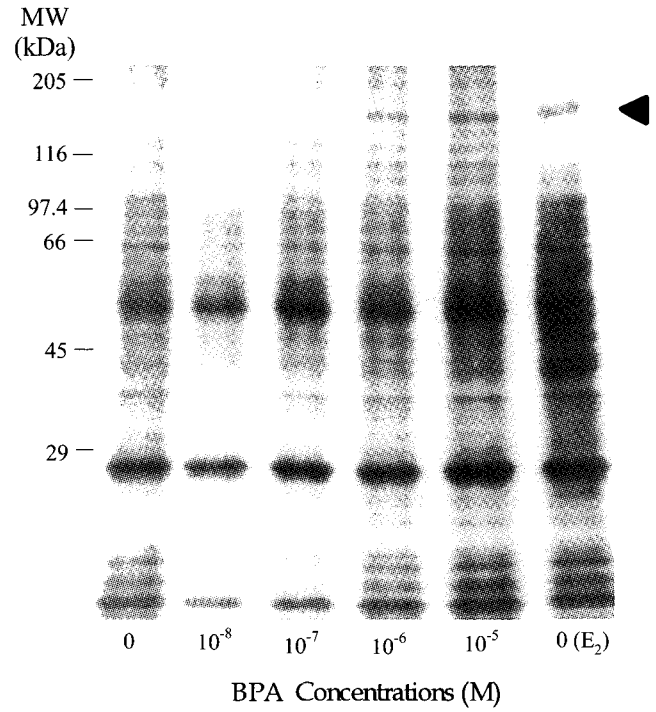

Fig. 1. SDS-PAGE showing the induction of VTG (arrowhead) by BPA in primary hepatocyte cultures in rainbow trout, compared with that by E_2. Spent media were analyzed on Day 5 after BPA or E_2 addition. CBB stain.

SDS-PAGE. A newly synthesized VTG band was detected at a molecular weight position of 175 kDa (Fig. 1). This band was identified as a main VTG band by Kwon et al. (1993) on the basis of immunoblotting and immunoelectrophoresis.

The addition of BPA to the incubation medium increased the intensity of CBB staining for the VTG band in a concentration-dependent fashion (Fig. 1). The band was faintly detected at an BPA concentration of 10^{-6} M and became distinct at 10^{-5} M as the experimemt culture with E_2 (2×10^{-6} M).

The relative amount of VTG was determined by IOD. VTG accounted for 0.34% of the total proteins in the control culture without E_2 and BPA, but this value increased with increasing BPA concentations (Fig. 2). Significant increment was confirmed at BPA concentration of 10^{-6} and 10^{-5} M, in which the percentage was highly increased to 8 times ($P<0.01$) and to 13 times ($P<0.01$) of the control, respectively.

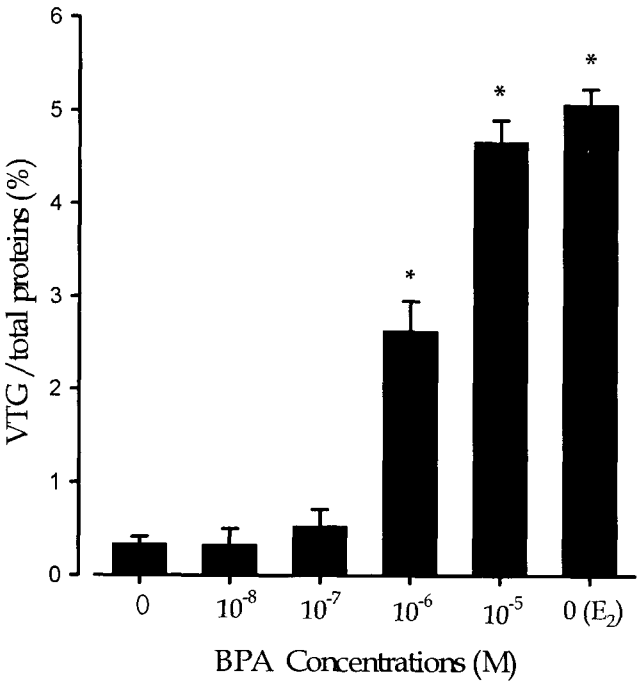

Fig. 2. Concentration-dependent induction of VTG by BPA in primary hepatocyte cultures in rainbow trout. The activity of VTG was estimated as a percentage of VTG to total proteins after SDS-PAGE on Day 5 after BPA or E_2 addition. Vertical bars represent the SE of the mean for three individuals. *$P<0.01$ for control (without BPA and E_2).

Effect of 4-OHT on BPA-induced VTG synthesis

After the addition of E_2, BPA and/or 4-OHT to incubation media for 5 days, spent media was analyzed for VTG synthesis. SDS-PAGE showed that VTG band induced by E_2 and BPA are not shown when 4-OHT was added (Fig. 3).

The VTG bands were quantified by IOD and expressed as a percentage of the IOD of total proteins including VTG (Fig. 4). VTG accounted for almost 0% of the total proteins in the culture with E_2 or BPA, because of the addition 4-OHT to incubation media.

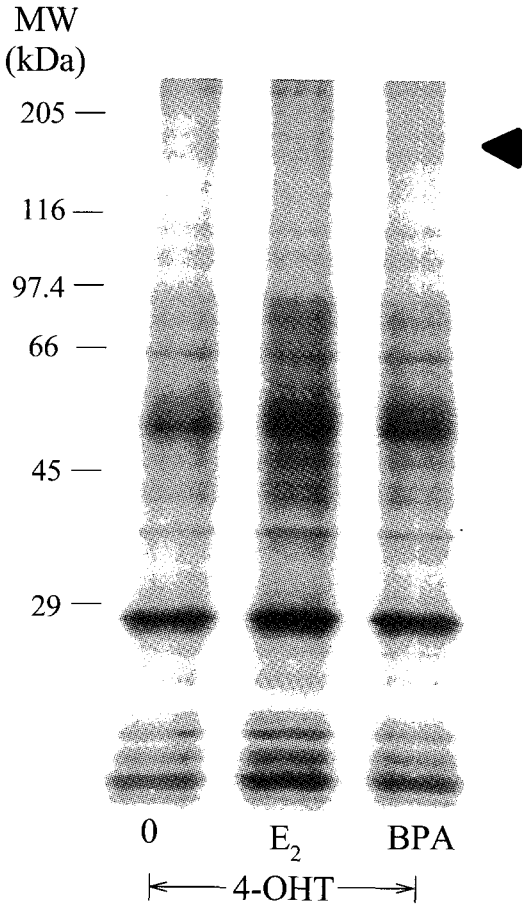

Fig. 3. SDS-PAGE showing the induction of VTG (arrowhead) by BPA (10^{-5} M) or E_2 (2×10^{-6} M) in primary hepatocyte cultures with 4-OHT (10^{-6} M) in rainbow trout. Spent media were analyzed on Day 5 after BPA or E_2 addition. CBB stain.

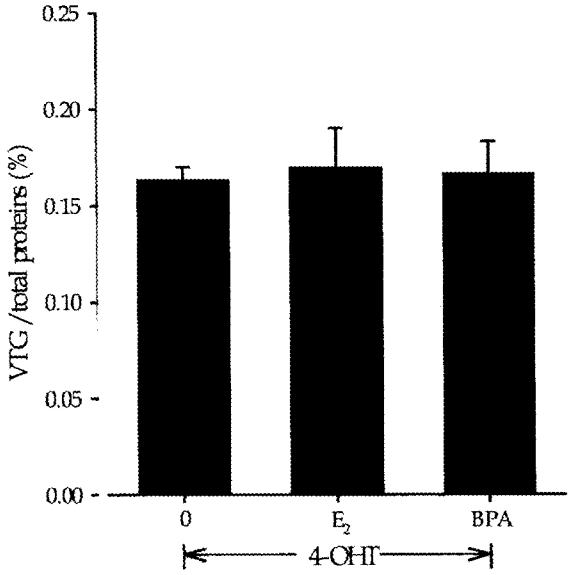

Fig. 4. Induction of VTG by BPA (10^{-5} M) or E_2 (2×10^{-6} M) in primary hepatocyte cultures with 4-OHT (10^{-6} M) in rainbow trout. The activity of VTG was estimated as a percentage of VTG to total proteins after SDS-PAGE on Day 5 after BPA or E_2 addition. Vertical bars represent the SE of the mean for three individuals.

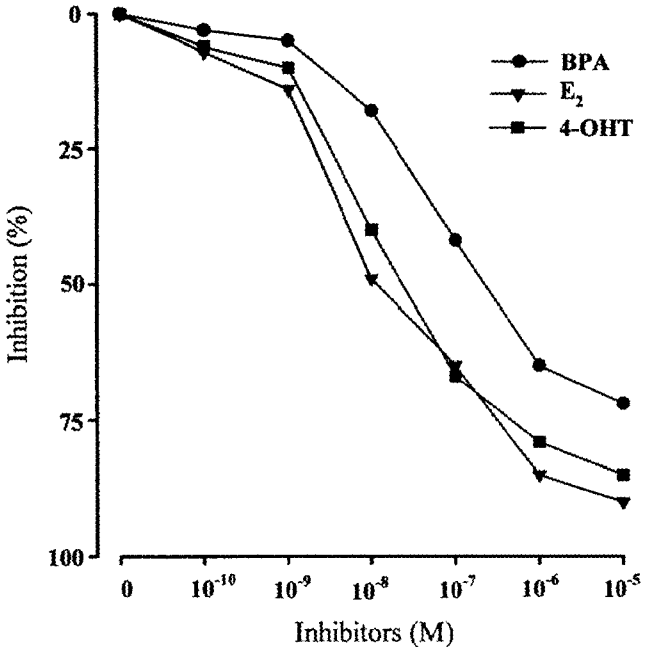

Fig. 5. Inhibitory effects of BPA, E_2 and 4-OHT on the binding activity of fluorescence E_2 to hER coated on a microplates. Each point represents the mean of four determinations. Error bars are omitted.

Effect of BPA on E_2-ER binding affinity

The effects of BPA on the binding affinity of fluorescence-labeled E_2 to hER were examined with an ELISA. The addition of BPA, E_2 or 4-OHT to the wells inhibited the binding of the labeled E_2 to hER in a sigmoid fashion (Fig. 5). It was inhibited by 72, 90 and 85% at 10^{-5} M of BPA, E_2 and 4-OHT, respectively.

Discussion

Many *in vitro* studies on VTG synthesis have been reported in birds (Boehm et al., 1988), reptiles (Ho et al. 1985), and amphibians (Stanchfield and Yager, 1978). It has been widely used for hormonal regulation (Yeo and Mugiya, 1997), gene expression (Flouriot et al., 1996; Hwang et al., 2000) involved in the synthesis of VTG in the fish. Recently, the effects of aquatic pollutants such as metals and synthetic chemicals on VTG and its mRNA induction are also examined using the hepatocyte culture of rainbow trout (Smeets et al., 1999; Hwang et al., 2000). Hepatocyte culture is used for confirm and analyze *in vivo* results directly (Jobling and Sumpter, 1993; Pakdel et al., 1997).

It is reported that the induction of VTG synthesis and inhibition of testicular growth were induced by exposure of male rainbow trout to alkylphenol (Jobling et al., 1996). Lindholst et al. (2000) also reported that the BPA concentration of the internal liver and VTG quantification of serum were increased when rainbow trout were exposed to BPA, and VTG production also increased with correlation with the BPA concentration in the internal liver ($r=0.66$). Similarly, in the present study, VTG was synthesized by the addition of BPA to incubation medium and increased in a concentration-dependent fashion. Although the present results clearly showed that BPA induced VTG synthesis in the hepatocytes of rainbow trout, it remains unknown how BPA can induce VTG synthesis. Therefore, we added 4-hydroxy-tamoxifen (4-OHT, antiestrogen) to incubation medium with BPA for determine whether VTG was synthesized by the estrogenic activity of BPA at the ER level.

4-OHT are molecules which are able to interfere

with E_2 binding to ER, resulting in inhibition of ER expression (Flouriot et al., 1996). One of typical antiestrogens is the 4-OHT which inhibits all cellular processes resulting from E_2-ER interaction in rainbow trout (Flouriot et al., 1996). 4-OHT (Kd= 1.9~3.4 nM) binds to ER avidly, even better than E_2 itself (Kd=2~5 nM) (Lazier and MacKay, 1993). Hwang (2001) reported that increases in 4-OHT concentrations linearly decreased the VTG synthesis and radioactivity of [^{3}H]E_2 to estrogenized hepatocytes in rainbow trout. In the present study, BPA-induced VTG production was reduced by 4-OHT additon as the control culture without BPA and E_2 (only 4-OHT). It seems reasonable to think that VTG synthesis is mediated by the estrogenic activity of BPA at the ER level. Therefore, if BPA bound to ER, the binding of BPA to the ER will reduce the affinity of E_2 for the ER. It is well known that nonylphenol, one of typical chemicals, bind to ER in hepatocyte with a potency approximately 10^{-4} and 10^{-5} times than that of estrogen (Lutz and Kloas, 1999).

We examined the effects of BPA on the binding affinity of E_2 to ER. To examine this binding affinity, we used a hER assay kit because of the structure of rainbow trout (rt) ER genes resemble that of hER genes (Ponglikitmongkol et al., 1988) and the fundamental process of rtER interaction with E_2 response element is similar to that found in birds, frogs and mammals (Lazier and MacKay, 1993). Therefore, the effects of BPA on the binding activity of E_2 to ER may be the same between hER and rtER. In the affinity experiments, BPA characteristically inhibited E_2-ER binding activity as well as in 4-OHT and E_2 (Fig. 5). This result suggest that BPA bind to ER through competition with E_2.

In conclusion, BPA induced VTG synthesis by the binding activity of BPA to ER in the hepatocytes of rainbow trout.

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