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Effect of Growth Hormone on Vitellogenin Production by Estradiol-17 β in the Culture of Hepatocytes in the Rainbow Trout *Oncorhynchus mykiss*

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Effects of pituitary and thyroid hormones on estradiol-induced vitellogenin (VTG) induction were electrophoretically examined in primary hepatocyte cultures of rainbow trout. Hepatocytes were precultured for 2 days and then estradiol-17 β (E_2, 2×10^{-6}M), triiodothyronine (T_3, 10^{-8}~10^{-6}M), bovine growth hormone (*b*GH, 10~100 ng/mℓ), ovine prolactin (*o*PRL, 100~500 ng/mℓ), and pituitary extract (PE) of rainbow trout (0.75PE/dish) were added to the incubation medium. The hepatocytes were cultured for 7 more days. The addition of *o*PRL to the incubation medium was not effective in increasing VTG production at any concentrations. The addition of PE to the incubation medium with E_2 was not effective in increasing VTG production. The addition of *b*GH to the incubation medium with E_2 was not effective in increasing the rate of VTG production at concentrations of 10~50 ng/mℓ. However, a higher concentration of *b*GH, 100 ng/mℓ, increased VTG production. The various concentrations of T_3 were ineffective in stimulating VTG production. These results suggest that GH could be one of stimulus factors for VTG production in rainbow trout.

Key words: hepatocyte culture, vitellogenin production, growth hormone, prolactin, thyroid hormones, pituitary extract, rainbow trout

Introduction

Vitellogenin (VTG) is the egg-yolk precursor protein that is synthesized in the liver in response to circulating estradiol-17 β (E_2) and released into the blood. Circulating VTG is transported to the developing ovary and accumulated as yolk proteins in eggs after splitting into lipovitellin, phosvitin, and β-components (Hiramatsu and Hara, 1996).

It is no doubt that E_2 is the main inducer of VTG. However, androgens such as testosterone, methyltestosterone, and methylandrostendiol also stimulate VTG production in goldfish and goby (Hori *et al.*, 1979; Le Menn *et al.*, 1980). Wangh and Schneider (1982) reported that thyroid hormone acts as co-hormones of E_2 for the induction of VTG in the tissue culture in *Xenopus laevis*. The intervention of pituitary hormones such as growth hormone and prolactin in VTG production was demonstrated in reptiles (Ho *et al.*, 1985) and birds (Boehm *et al.*, 1988). Recently, Kwon and Mugiya (1994) reported that VTG production was dramatically stimulated by the combination of E_2 with *b*GH and/or *o*PRL in the primary hepatocyte culture in the eel. However, the direct involvement of pituitary hormones and thyroid hormones in VTG production has not been reported in rainbow trout. In the present study, the involvement of pituitary hormones and thyroid hormones in VTG production by estrogenized hepatocytes was examined in rainbow trout. Hepatocytes were cultured with a combination of E_2 and pituitary or thyroid hormones, and synthesized VTG was electrophoretically analyzed.

Materials and Methods

Immature rainbow trout (*Oncorhynchus mykiss*) weighing 100~150 g were obtained from a trout farm and maintained at about 14℃ in outdoor ponds at our laboratory. They were fed trout food pellets once a day.

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Hepatocyte Preparation and Incubation

Trout hepatocytes were prepared according to the procedure of Kwon *et al.* (1993). To briefly summarize their method, after fish were anesthetized with 0.01% 2-phenoxyethanol, the liver of each fish was exposed and perfused with Ca^{2+}-free saline solution (120 mM NaCl, 1.22 mM $MgSO_4 \cdot 7H_2O$, 4.7 mM KCl, 1.25 mM KH_2PO_4, 23 mM $NaHCO_3$, PH 7.4), followed by perfusion with the same solution containing collagenase (0.5 mg/mℓ; Wako Pure Chem.) and bovine serum albumin (0.98 mg/mℓ; Sigma) at room temperature (ca. 23℃) for 30 min. The liver was then again perfused with a Ca^{2+}- and Mg^{2+}-free solution containing 2 mM EDTA for 10 min. After mincing with scissors and filtering with a nylon gauze, cells were collected by centrifugation and washed three times with the Ca^{2+}-free saline solution. Cell yield and viability were determined by the Trypan Blue exclusion test.

Isolated hepatocytes were plated into a 60 mm plastic petri dish with positive-charge (Falcon) at a density of 3×10^5 cells per dish. William's medium E (Life Technol. Inc.) containing 0.2 μM bovine insulin (Sigma), streptomycin (100 μg/mℓ), penicillin (70 μg/mℓ) was used for cell culture. Hepatocyte culture was carried out in 3 mℓ of the medium at 15 ℃ under 5% CO_2 and air with saturated humidity. Preculture was conducted for 2 days before each experiment.

Hormone Treatment

Isolated cells were precultured in dishes for 2 days. Estradiol-17 β (E_2, 2×10^{-6} M in 3 μℓ of 95% ethanol), triiodothyronine (T_3, $10^{-8}\sim10^{-6}$ M in 3 μℓ of in alkalic ethanol), bovine growth hormone (*b*GH, 10~100 ng/mℓ in 3 μℓ of 0.65% NaCl), and ovine prolactin (*o*PRL, 100~500 ng/mℓ in 3 μℓ of 0.65% NaCl) were then simultaneously added to the dishes. The *b*GH and *o*PRL concentrations used were equivalent to about $2.3\times10^{-6}\sim2.3\times10^{-5}$ M and $4.5\times10^{-6}\sim2.3\times10^{-5}$ M, respectively. The effects of these hormones on VTG synthesis were examined for 7 days after addition, during which time media were changed daily. The control cultures received only the solvents.

In addition, thirty-six pituitary glands of rainbow trout were homogenized in the phosphate buffer (pH 7.4) and centrifuged at 3000 g for 10 min. The supernatant (PE) was added to the incubation medium at a concentration of 0.75 pituitary per dish.

SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Sodium dodecyl sulfate (SDS)-PAGE was performed in the presence of 2-mercaptoethanol on a precast linear gradient slab gel with a 5 to 20% acrylamide gradient (Laemmli, 1970). Spent medium (3 mℓ) was collected and centrifuged to remove any debris in the sample. Total proteins were precipitated by adding cold 50% trichloroacetic acid (TCA) to a final concentration of 10%. The whole precipitate was washed three times with 5 mℓ of 5% TCA, dissolved in 30 μℓ of sample buffer (0.175M Tris-HCl, 8M urea, 1% SDS, 0.5% mercaptoethanol, pH 7.4), and applied to each lane of the gel. It was difficult to apply a fixed amount of protein to each lane, because the present experiment was intended to inhibit VTG production. The gels were stained with 0.25% Coomassie brilliant blue R-250 for 30 min. Standard proteins used for molecular weight (MW) determination 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 VTG bands was based on the results of Kwon *et al.* (1993). Isolated rainbow trout hepatocytes incubated with E_2 produced a protein of electrophoretic mobility of 175 kDa, and Kwon *et al.* (1993) identified this band (the main band) as VTG by immunoblot analysis and immunoelectrophoresis.

After SDS-PAGE, the integrated optical density (IOD) of the main VTG band was measured by a Bio Image (Millipore). The relative optical density of VTG to total proteins was determined on day 7 after the addition of hormones and was expressed as the percentage of the experimental to control values. 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 ($r=0.99$, $P<0.01$) was reported between the amount of VTG applied to each lane of SDS-PAGE and the IOD over a wide range of VTG concentrations (Yeo and Mugiya, 1997)

Statistical analysis

An one-way ANOVA by Fisher's protected least significant difference (PLSD) was used for sta-

tistical evaluation of mean values. Significance was accepted at $P<0.05$. Percent data were statistically analyzed after being arcsine transformed.

Results

The effects of *o*PRL, *b*GH, PE, and T_3 on VTG production were examined in a hepatocyte culture on day 7 after E_2 addition. Figure 1 shows the evaluation of *o*PRL for VTG production as a percentage of VTG predicted compared to that produced by a control (E_2 only). The rate of VTG production was about 5% lower than that of the control at 100~500 ng/mℓ *o*PRL, but its level was not differ significantly from the control level. Therefore, *o*PRL was not effective in increasing VTG production at any concentration.

The addition of *b*GH to the incubation medium also had no effect on the stainability of the VTG band at concentrations of 10 ng/mℓ and 50 ng/mℓ (Fig. 2). However, band stainability became distinct at a concentration of 100 ng/mℓ, compared with the control (GH free). The band of VTG was undetectable when only PE was added to the incubation medium. The addition of PE to the incubation medium without E_2 failed to stimulate VTG production by the hepatocytes. The addition of PE to the incubation medium with E_2 did not increase band stainability (Fig. 2).

The addition of *b*GH to the incubation medium did not increase the rate of VTG production at concentrations of 10 ng/mℓ and 50 ng/mℓ (Fig. 3). The rate of VTG production was about 8% higher than and equal to that of the control at these two respective concentrations. However, a higher concentration of 100 ng/mℓ increased VTG production ($P<0.01$) to about 21 % higher than that of the control. These results suggest that GH stimulates VTG production in rainbow trout.

The addition of PE to the incubation medium with E_2 did not increase VTG production. The addition of PE to the incubation medium without E_2 failed to stimulate VTG production, and the VTG level was not differ from the background level without E_2 (data not shown).

The addition of T_3 had no effect on VTG production at concentrations of 10^{-8} M and 10^{-6} M (Fig. 4). When hepatocytes were cultured with E_2 and 10^{-7} M T_3, the rate of VTG production was about 10% higher than that of the control, but the difference was not significant. Therefore, the present results suggest that T_3 does not increase VTG production in estrogenized hepatocytes.

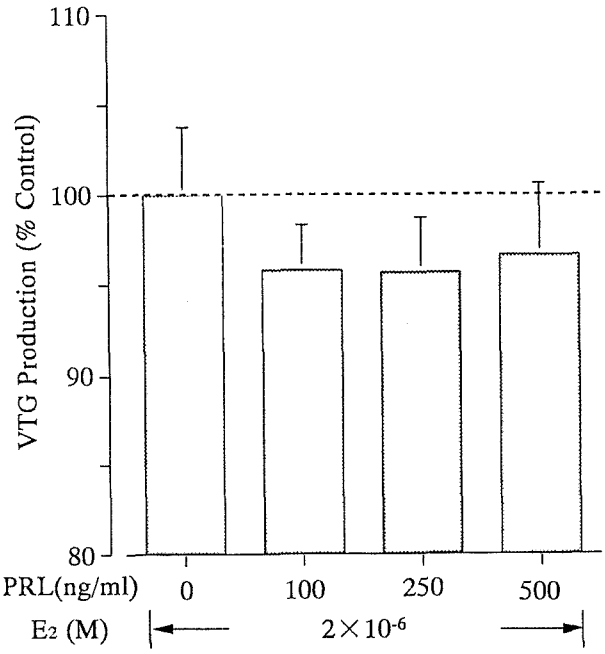

Fig. 1. Effects of various concentrations of *o*PRL on the E_2-induced production of VTG. Hepatocytes were cultured in the media containing E_2 (2×10^{-6}M) and *o*PRL for 7 days. Vertical bars represent the average (mean ± SE) percentage of four experiments.

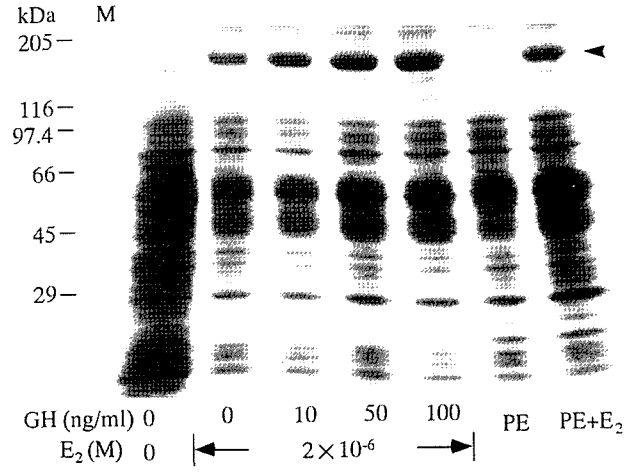

Fig. 2. Effects of *b*GH or PE on the E_2-induced production of VTG (arrowhead) in hepatocyte cultures. Spent media were analyzed on day 7 in culture by gradient SDS-PAGE. M: molecular weight (MW) marker. CBB stain.

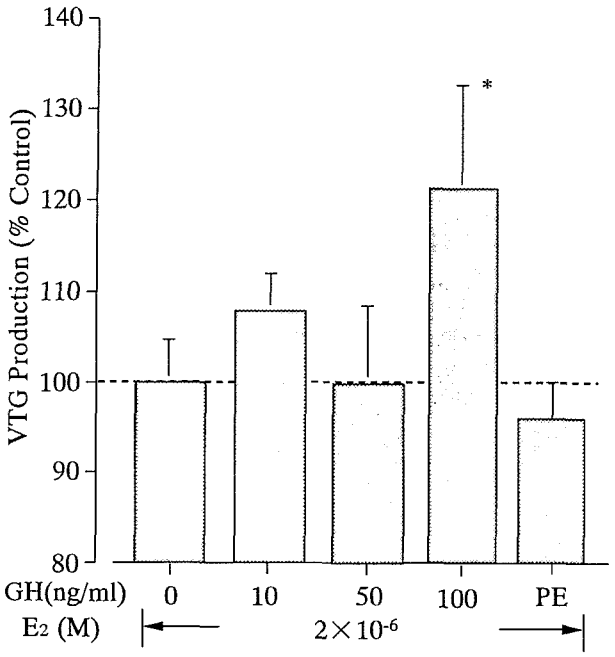

Fig. 3. Effects of various concentrations of *b*GH and PE on the E_2-induced production of VTG. Hepatocytes were cultured in the media containing E_2 (2×10^{-6}M), *b*GH, and PE for 7 days. Vertical bars represent the average (mean ± SE) percentage of six experiments. * $P<0.01$ for E_2 alone.

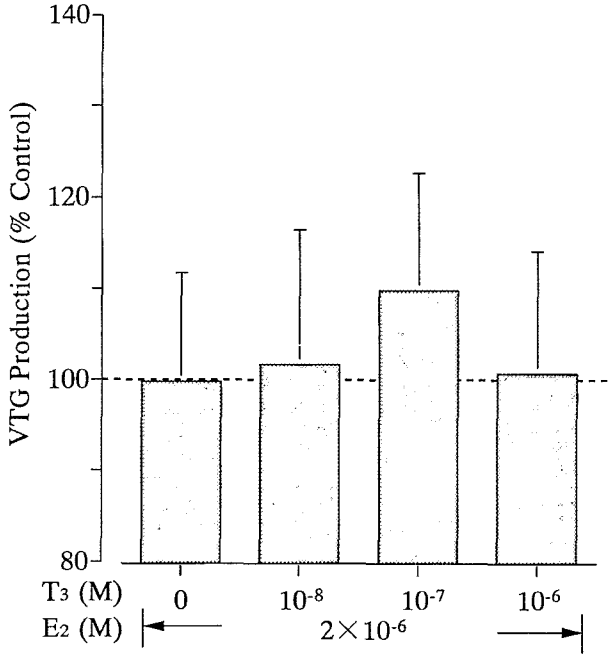

Fig. 4. Effects of various concentrations of T_3 on the E_2-induced production of VTG. Hepatocytes were cultured in the media containing E_2 (2×10^{-6}M) and T_3 for 7 days. Vertical bars represent the average (mean ± SE) percentage of four experiments.

Discussion

Kwon and Mugiya (1994) reported that VTG was minimally synthesized by E_2 alone in hepatocyte cultures in eels and that the combination of E_2 with *b*GH and/or *o*PRL dramatically stimulated the VTG production. Similarly, the stimulation of VTG production by the pituitary hormones was reported in oviparous species other than fish (Ho *et al.*, 1985; Carnevali *et al.*, 1992; Boehm *et al.*, 1988). In the present study, effects of various concentrations of *b*GH and *o*PRL on the induction of VTG production by E_2 in rainbow trout were examined. Additionally, effect of PE on VTG production was also examined. Most hormones tested were ineffective in stimulating VTG production in rainbow trout, but higher concentration of *b*GH (100 ng/mℓ) significantly stimulated VTG production by the hepatocytes, suggesting an involvement of GH for VTG production in rainbow trout.

Although E_2 is the main inducer for VTG production, our previous results showed that the relative amount of VTG to other proteins was higher *in vivo* than *in vitro*. These results suggest that there is a factor other than E_2 that stimulates VTG production *in vivo*. Kwon and Mugiya (1994) reported that VTG production increased dramatically after the combination of E_2 with GH and/or PRL in a primary hepatocyte culture from eels. In the present study, a high concentration of 100 ng/mℓ GH stimulated VTG production. Therefore, GH is a stimulus factor for VTG production in rainbow trout. However, little information is available about why a high concentration of 100 ng/mℓ GH increases VTG production.

The involvement of pituitary hormones in VTG production was confirmed by studies on the hepatic E_2 receptors. The hypophysectomy of female turtles caused a decrease in the number of hepatic E_2 receptors, which was partially restored by GH treatment (Riley and Callard, 1988; Riley *et al.*, 1987). In ovariectomized lizards, the administration of GH with E_2 increased the levels of total receptors in the liver nucleus (Paolucci, 1989). However, no data is available on the multihormonal regulation of E_2 receptors in fishes.

On the other hand, GH is known to activate adenylate cyclase activity and increase cAMP concentrations in the adipose tissue of rats (Swislocki, 1970). Singh and Thomas (1993) also demonstrated that a high concentration of GH rapidly increases cAMP concentrations in ovarian tissue *in vitro*. Therefore, it is possible that a high dose (100 ng/mℓ) of GH secondarily stimulated VTG production by hepatocytes through stimulation of cAMP production in the cells. The effects of cAMP will be studied in further analyses.

Wangh and Schneider (1982) reported that thyroid hormone acts as a co-hormone of E_2 for the induction of VTG in tissue cultures of hepatocytes in *Xenopus laevis*. In contrast, Kwon *et al.* (1993) reported that the addition of T_3 (10^{-7} M) to a culture medium did not enhance E_2-induced VTG production in a hepatocyte culture of rainbow trout. The data presented have also showed that various concentrations of T_3 did not stimulate VTG production. Moreover, we found that the addition of GH, PRL, and T_3 to the incubation medium without E_2 did not stimulate VTG production by the hepatocytes (data not shown). The addition of PE to the incubation medium without E_2 also failed to stimulate VTG production. Therefore, VTG production requires the presence of E_2 in rainbow trout. The variations in the response to pituitary hormones and/or thyroid hormone among different species raise many possibilities for further investigations.

In conclusion, a higher concentration of 100 ng/mℓ *b*GH was effective in stimulating VTG production, but oPRL and T_3 were not. Therefore, GH will be one of stimulus factors for VTG production in rainbow trout.

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