J. Fish. Sci. Tech. 1(2), 153~158, 1998

Stimulatory Effects of cyclic AMP on Vitellogenin Induction by Estradiol-17β in the Primary Culture of Hepatocytes in the Rainbow Trout *Oncorhynchus mykiss*

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(Received March 1998, Accepted September 1998)

Effects of cyclic (c) AMP and G-protein related reagents (3-isobutyl-1-methyxanthine (IBMX), Forskolin (FSK), cholera toxin (CTX), and pertussis toxin (PTX)) on estradiol-17β induced vitellogenin (VTG) induction were examined in primary hepatocyte cultures in rainbow trout *Oncorhynchus mykiss*. The addition of IBMX, FSK, or CTX to the incubation medium markedly increased VTG production, while PTX was not effective in stimulating the production. It is well known that cAMP regulates phosphorylation and dephosphorylation through mediation of protein kinase A. These results suggest that VTG production is highly dependent on cAMP state in hepatocytes because of its highly phosphorylated nature.

Key words: hepatocyte culture, vitellogenin production, cyclic AMP, 3-isobutyl-1-methyxanthine, Forskolin, cholera toxin, pertussis toxin

Introduction

Vitellogenesis (VTG) is a sex-specific protein appearing in the blood of sexually maturing female fish as well as other oviparous vertebrates. VTG is the egg yolk precursor protein that is synthesized in the liver response to circulating estrogen.

Recently, estradiol-17β (E_2) has been found to stimulate adenylate cyclase and cyclic (c)AMP-mediated gene transcription in human breast cancer cells and rat uterine cells (Aronica *et al.*, 1994). Similarly, Zhou *et al.* (1996) suggested that E_2 can influence the activity of other neuronal transcriptional regulators through a rapid increase in phosphorylation of nucleus-binding protein by cAMP in rats brain. However, the mechanism of cAMP activity by E_2 are not well understood in fish.

Moreover, it was also reported that GH acts on the dissociation of liposis through adenylate cyclase and cAMP systems in the adipose tissue (Swislocki, 1970). Recently, Singh and Thomas (1993) demonstrated that GH treatment stimulates cAMP accumulation in ovarian tissues of trout. Moreover, there is considerable evidence that GH affects gonadal function in mammals (Sheikholislam and Stempfel, 1972) and potentiates the actions of follicle-stimulating hormone on luteinizing hormone receptor induction, cAMP formation, and steroid production in granulosa and Leydig cells (Jia *et al.*, 1986; Hsu and Hammond, 1987; Chatelain *et al.*, 1991).

Although E_2 is the main inducer for VTG production, the previous results showed that the relative amount of VTG to other proteins was higher *in vivo* than *in vitro*. These results suggest that there are some stimulus factors for VTG production other than E_2 *in vivo*. Recently, Kwon and Mugiya (1994) reported that the combination of E_2 with GH dramatically stimulated VTG production in primary hepatocyte culture in the eel. The previous experiments also demonstrated that a higher concentration of GH (100 ng/mℓ) increased VTG production in the hepatocyte culture with E_2. These results suggest that cAMP has a regulatory effect on VTG production as a second messenger, because GH increase cAMP concentrations of intracellular in fish.

The regulation of adenylate cyclase and cAMP activity by hormones is integrated through a G protein complex containing a stimulatory or an inhibitory component (Gilman, 1987; Dachicourt *et al.*, 1996). In the present study, effects of cAMP and G protein-related reagents were examined on E_2-

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induced VTG production.

Materials and Methods

Fish

Immature rainbow trout (*Oncorhynchus mykiss*) weighing 100~250 g were obtained from a trout farm or the Nanae fish culture experimental station, Hokkaido University and maintained at about 14℃ in outdoor ponds. They were fed trout food pellets once a day but starved during the last 3 days before experiments to avoid the induction of bile, which weakens the attachment of hepatocytes to a culture dish.

Hepatocyte Isolation and Culture

Hepatocytes were prepared according to the procedure of Kwon *et al.* (1993). Briefly, after fish were anesthetized with 0.01% 2-phenoxyethanol, the liver 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 the same solution containing collagenase (0.5 mg/mℓ; Wako Pure Chem. Co.) and bovine serum albumin (0.98 mg/mℓ; Sigma) at room temperature (ca. 23℃) for 30 min. The liver was then again perfused with the 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. The culture medium was William's medium (Ca 1.8 mM, Life Technol. Inc.) containing 0.2 μM bovine insulin (Sigma), streptomycin (100 μg/mℓ), penicillin (70 μg/mℓ), and $NaHCO_3$ (23 mM). 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. The medium was changed every day throughout the preculture and experimental periods.

Estradiol- 17β Treatments

Estradiol-17β (E_2, 2×10^{-6} M; Sigma) in 95% ethanol was added to the culture media after 2 days preincubation. The volume of ethanol added was 3 μℓ per dish. The control cultures received ethanol of the same volume.

cAMP and G-Protein Related Reagents

cAMP and G-protein related reagents used were 3-isobutyl-1-methyxanthine (IBMX; Wako Pure Chem. Co.) and forskolin (FSK; Wako Pure Chem. Co.) in 95% ethanol, and cholera toxin (CTX; Wako Pure Chem. Co.) and pertussis toxin (PTX; Sigma) in distilled water. Concentrations of these reagents used were 10^{-5}M~10^{-3}M for IBMX, 10^{-6}M~10^{-4}M for FSK, 0.5μg/mℓ~2.0μg/mℓ for CTX, and 10 ng/mℓ~60 ng/mℓ for PTX.

Effects of these reagents on VTG production were examined on day 7 after E_2 addition.

Qualitative and Quantitative analyses of VTG

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

Proteins were analyzed by 5~20% gradient SDS-PAGE according to the method of Laemmli (1970). After SDS-PAGE, the integrated optical density (IOD) of the main VTG band (175 kDa) 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 cAMP and G-protein related reagents and expressed as the percentage of the experimental to control values. This type of expression has the benefit of excluding effects of variations in the number of cultured cells and in the amount of proteins applied to the lanes of electrophoresis. 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). Our previous experiment showed a highly significant correlation ($r=0.99$, $P<0.01$) between the amount of VTG applied to each lane of SDS-PAGE and the IOD over a wide range of VTG concentrations (100~1600 ng/mℓ, Yeo and Mugiya, 1997).

Statistical analysis

A one-way ANOVA by Fisher's protected least significant difference (PLSD) was used for statistical evaluation of mean values. Significance was accepted at $P<0.05$. Percent data were statistically

analyzed after being arcsine transformed.

Results

Trout hepatocytes were cultured in the serum-free medium with E_2 for 7 days and the spent media were analyzed by SDS-PAGE. A new protein band in the spent media was electrophoretically detected at a molecular weight position of 175 kDa (Fig. 1). This band was identified as VTG by Kwon *et al.* (1993) on the basis of immunoblotting and immunoelectrophoresis. The control culture without E_2 did not induce an equivalent protein.

The addition of IBMX to the incubation medium had no effect on the stainability of VTG band induced at 10^{-5}M (Fig. 1). However, higher concentrations (10^{-4}M and 10^{-3}M) increased the stainability.

IBMX has no effect on the rate of VTG to total protein concentration at 10^{-5}M, but it significantly increased to 11% and 9% of the control at concentrations of 10^{-4}M and 10^{-3}M, respectively (Fig. 2).

The addition of FSK had no effect on VTG production at concentrations of 10^{-6}M and 10^{-5}M (Fig. 3). However a high concentration of FSK (10^{-4} M) only increased VTG production. The rate

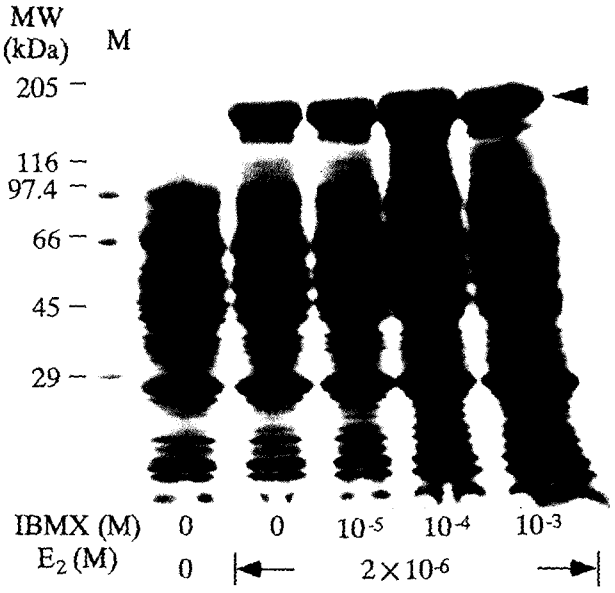

Fig. 1. Gradient SDS-PAGE showing effects of IBMX on the E_2-induced production of VTG (arrowhead) in hepatocyte cultures with E_2. Spent media were analyzed on day 7 in culture. M: molecular weight (MW) marker. CBB stain.

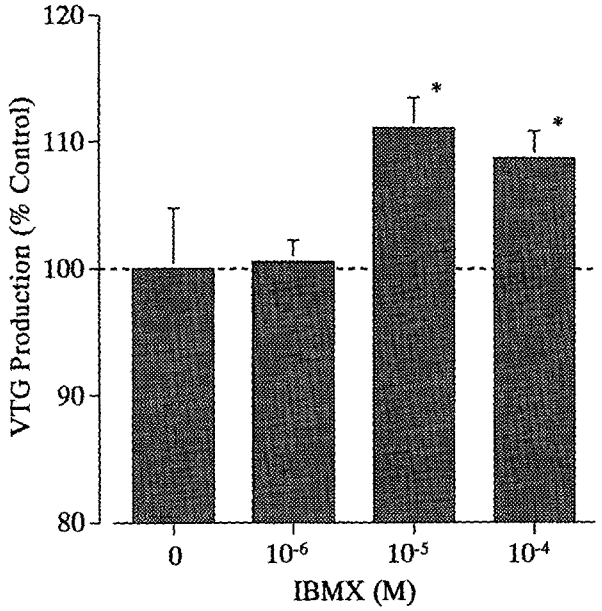

Fig. 2. Effects of IBMX on the E_2-induced production of VTG. Hepatocytes were cultured in the media containing E_2 (2×10^{-6} M) and various concentrations of IBMX for 7 days. Vertical bars represent the SE of mean for six experiments. * $P<0.01$ for E_2 alone.

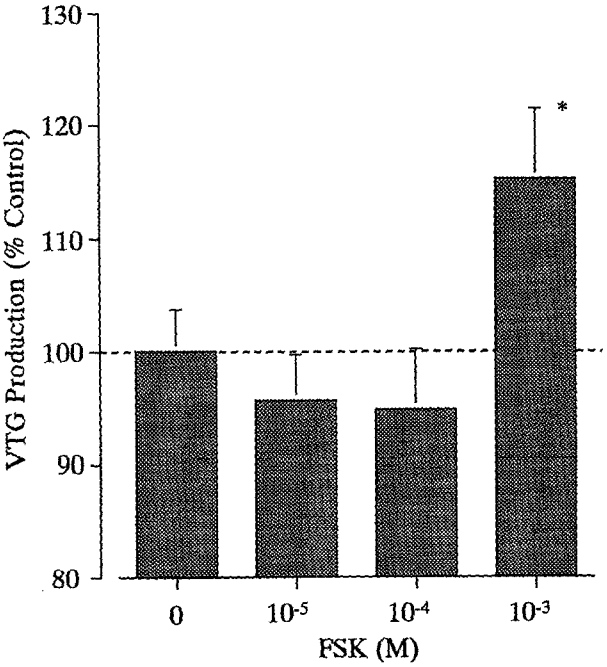

Fig. 3. Effects of FSK on the E_2-induced production of VTG. Hepatocytes were cultured in the media containing E_2 (2×10^{-6}M) and various concentrations of FSK for 7 days. Vertical bars represent the SE of mean for 10 experiments. * $P<0.01$ for E_2 alone.

of increase was about 15% of the control ($P<0.01$).

CTX was not effective in enhancing VTG production at concentrations of 0.5μg/mℓ and 1.0μg/mℓ. However, CTX stimulated VTG production by about 11% of the control ($P<0.01$) at a higher concentration of 2.0μg/mℓ (Fig. 4). The treatments with the lower concentrations had no effect on the production.

The addition of PTX to the incubation medium was not effective in increasing the rate of VTG to total proteins at concentrations of 10~60 ng/mℓ (Fig. 5).

Discussion

It is no doubt that E_2 is the main inducer of VTG production in the liver. It was reported that E_2 has been found to stimulate adenylate cyclase and cAMP- mediated gene transcription in human and rat (Aronica *et al.*, 1994). It is suggested that E_2 can influence the activity of other neuronal transcriptional regulators through a rapid increase in phosphorylation of nucleus-binding protein by cAMP in rat (Zhou *et al.*, 1996). Therefore, we examined the effects of cAMP on the induction of VTG production using cAMP-related reagents in a hepatocyte culture in rainbow trout. cAMP-related reagents used were IBMX, FSK, CTX, and PTX. IBMX is known to inhibit the action of phosphodiesterase, resulting in the accumulation of cAMP in the cytoplasm (Peytremann *et al.*, 1973). FSK is a powerful activator of adenylate cyclase and increases cAMP levels in various cell types (Henquin *et al.*, 1983). CTX also activate adenylate cyclase by the transfer of the ADP-ribosylation of Gs protein and increases cAMP levels in rat hepatocytes (Hülsmann and Dubelaar, 1986), while PTX is known to inhibit the action of adenylate cyclase by the ADP-ribosylation of Gi (Moss *et al.*, 1983). The cAMP-related reagents used are known to change cAMP states in various cells in fish (Alford and Grillner, 1991; Chaang *et al.*, 1992; Molgo *et al.*, 1993; Gleibs *et al.*, 1995).

In the present study, the addition of IBMX, FSK, and CTX to the incubation medium effectively facilitated VTG production, but PTX did not. Among these regents, PTX only has an inhibitory effect for cAMP production (Moss *et al.*, 1983). If E_2 induces VTG production through stimulation of cAMP in hepatocytes, VTG production was expected to

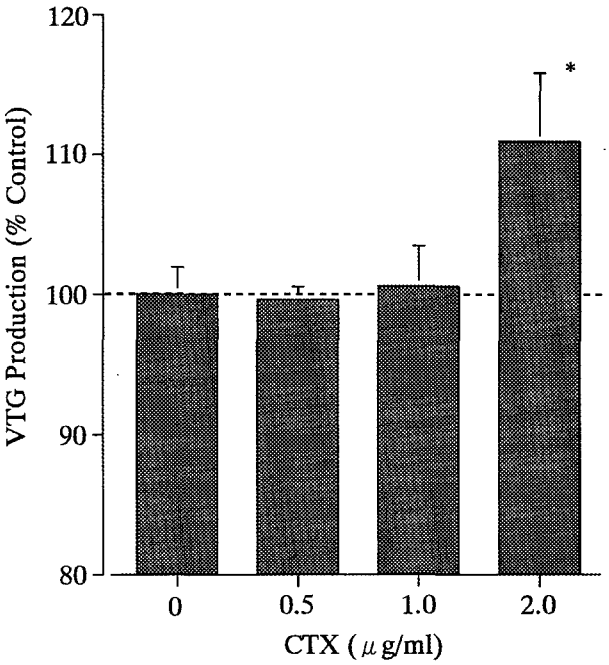

Fig. 4. Effects of CTX on the E_2-induced production of VTG. Hepatocytes were cultured in the media containing E_2 (2×10^{-6}M) and various concentrations of CTX for 7 days. Vertical bars represent the SE of mean for seven experiments. * $P<$ 0.01 for E_2 alone.

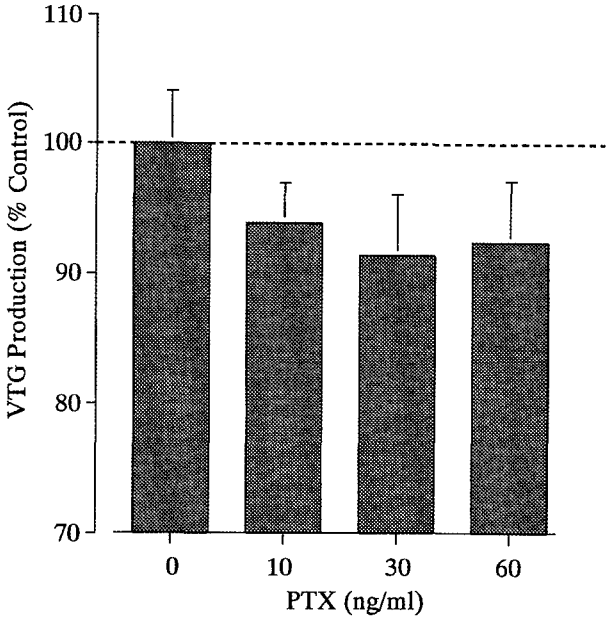

Fig. 5. Effects of PTX on the E_2-induced production of VTG. Hepatocytes were cultured in the media containing E_2 (2×10^{-6}M) and various concentrations of PTX for 7 days. Vertical bars represent the SE of mean for five experiments.

decrease by the inhibitor (PTX) for cAMP production, but this was not the case. Even if the new synthesis of cAMP was inhibited by PTX, the basal level of cAMP in the hepatocytes would be enough for inducing VTG production. Moreover, these results suggest that some factors other than E_2 are required for cAMP synthesis in hepatocytes.

Our previous experiments have shown that a higher concentration of GH increased VTG production in the hepatocyte culture with E_2. Recently, Kwon and Mugiya (1994) reported that reported that the combination of E_2 with GH dramatically stimulated VTG production in primary hepatocyte culture in the eel. 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 increase cAMP concentrations in the ovarian tissue *in vitro*. Therefore, it is possible that a GH-mediated increase in cAMP functions as a second messenger to stimulate VTG production in hepatocytes.

The present study was conducted using cAMP-related reagents. Changes in cAMP induction by hormones remain to be studied for further analyses.

In addition, cAMP regulates phosphorylation and dephosphorylation through mediation of protein kinase A (PKA) (Walsh *et al.*, 1968; Beavo *et al.*, 1974). Traugh and Pendergast (1986) reported that the phosphorylation of ribosomal protein S_6 is stimulated by hormones (e.g. epidermal growth factor, prostaglandin, etc) and other compounds (e.g. cycloheximide, phorbol esters, etc) through increases in intracellular levels of cAMP in the rat liver. Brostrom *et al.* (1987) suggested that cAMP increases the synthesis of protein through phosphorylation of the ribosomal protein S_6. Various cAMP-stimulating reagents enhanced E_2-induced VTG production in the present study. These results suggest that VTG production is highly dependent on cAMP state in hepatocytes.

In summary, VTG production was accelerated by cAMP and G-protein related reagents, which increase cAMP concentrations in cells. This acceleration will be explained by the highly phosphorylated nature of VTG, because cAMP is well known to stimulate phosphorylation. Experiments of the phosphorylation and dephosphorylation of VTG molecules would be useful for determining cAMP acts on VTG synthesis.

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