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Effect of Extracellular Calcium on Vitellogenin Production in the Culture of Hepatocytes in the Rainbow Trout, *Oncorhynchus mykiss*

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Effect of extracellular calcium in vitellogenin (VTG) production in response to estradiol-17 β (E_2, 2×10^{-6}M) was examined in primary hepatocyte culture of rainbow trout, *Onchorhynchus mykiss*. Total calcium in estrogenized sera significantly increased, compared with the control, while diffusible calcium was insignificant. However, diffusible calcium in the incubation medium with E_2 was significantly reduced, compared with the control. The uptake of extracellular calcium by cultured hepatocytes significantly increased 90 min after E_2 addition. Moreover, the accumulation of intracellular calcium increased in the cultures with E_2, regardless of the calcium concentrations in the incubation media. In addition, E_2-primed VTG production was significantly decreased by withdrawal of E_2 from the incubation medium. Moreover, VTG production by E_2 -primed hepatocytes was reduced by removing calcium from the incubation medium with or without E_2. These results suggest that the entry of extracellular calcium into the cytoplasm is an important step for VTG production in primary hepatocyte cultures in rainbow trout.

Key words: hepatocyte culture, vitellogenin production, extracellular calcium, calcium uptake, rainbow trout

Introduction

Freshwater teleosts maintain their plasma calcium levels within a narrow range (2~3 mM) in spite of large changes in external calcium concentrations, and obtain calcium mainly from the surrounding (Hwang *et al.*, 1996). However, vitellogenesis increases plasma calcium levels. This is due to the appearance of calcium-binding vitellogenin (VTG) in the plasma to 3~5 folds, suggesting an importance of calcium for VTG production.

VTG, a calcium-binding and highly phosphorylated protein, is synthesized in liver in response to circulating estradiol-17 β (E_2) and released into the blood (Wallace, 1985). VTG contains about 0.7% calcium in the rainbow trout, *Oncorhynchus mykiss* (Fremont and Riazi, 1988) and about 2.0% calcium in bass, *Opsariichthys uncirostris* (Urist and Schjeide, 1961). VTG-binding calcium is generally considered to be incorporated into eggs and to be used as a nutrient through the embryonic to swim-up stage.

Recently, it was reported that VTG production is extracellular calcium-dependent in primary cultures of rainbow trout hepatocytes (Yeo and Mugiya, 1997). Hepatocytes incubated in an EGTA-containing solution reduced the incorporation of amino acids into proteins by a 5-fold, compared with those in the calcium-containing control (Brostrom *et al.*, 1983). Further, Morley *et al.* (1992) found that the addition of 10^{-7} M E_2 to the incubation medium increased intracellular calcium concentrations in chicken granulosa cells. These results suggest that an increase in intracellular calcium concentrations is pre-requested for VTG production.

The present study examined the effect of E_2 on calcium uptake in primary hepatocyte culture of rainbow trout. Effects of calcium on E_2-primed VTG production were also examined.

Materials and Methods

Rainbow trout (*Oncorhynchus mykiss*) weighing 100~250 g were obtained from a trout farm 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

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the induction of bile, which weakens the attachment of hepatocytes to a culture dish.

Hepatocyte Isolation and Culture

Hepatocytes were prepared following Hayashi and Ooshiro (1975) as described by 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. 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.

Determination of DNA

The Schmidt-Thannhauser method, as modified by Munro and Fleck (1966), was used to extract DNA on Days 0 and 7 after E_2 addition. The DNA sample was treated with 0.3 N NaOH for 75 min and then DNA was extracted with 2 N perchloric acid for 60 min at 37℃. DNA was analyzed by ultraviolet absorbance according to Wilder and Stanley (1983).

Calcium Fractions in Serum and Incubation Medium

Male trout were intraperitoneally injected with 1 mg E_2 (Sigma) dissolved in propylene glycol per 100 g body weight. Control fish were given the solvent only. E_2 injections were made three times at three day intervals. Blood was collected on day 10 after E_2 injection from the caudal vessels by cutting the tail of the fish and draining it into 10 mℓ glass test tubes. Serum was separated by centrifugation. Hepatocytes were cultured with E_2 (2×10^{-6}M) for 7 days after 2 days preculture. The whole spend medium (3 mℓ) was centrifuged at 3000 rpm for 20 min to remove any debris.

Four hundred microliters of serum and the incubation medium samples were ultrafiltered (0.45 μm; Centricut; Hokubou) to obtain a protein-free ultrafiltrate. The serum, incubation medium, and ultrafiltrate samples were analyzed by a polarized zeeman atomic absorption spectrophotometer (Hitachi, Z-6100) to determine total and diffusible calcium. One N hydrochloric acid and 10% lanthanum solution were used for dilution to minimize the interference of phosphate.

Calcium Uptake by Estrogenized Hepatocytes

After 2 days preculture, $^{45}CaCl_2$ (0.1 Mbq/mℓ, Dupont/NEN) was added to culture dishes and the cultures were continued until 30, 60, 90, 120, or 180 min, then 1 mℓ of ice-cold solution containing 150 mM KCl, 0.1 mM $LaCl_3$, 20 mM Tris-HCl (pH 7.4) was added to stop calcium influx (Bijvelds *et al.*, 1995). Hepatocytes were then washed three times with 1 mℓ of ice-cold solution, which dissolved with 60% perchloric acid and 30% hydrogen peroxide for over night at 60℃. The samples were added to 12 mℓ Scintisol EX-H (Wako Pure Chem. Co.) to determine their radioactivity using a liquid scintillation spectrophotometer (Beckman, LS6000 1C). Total intracellular calcium concentrations were determined with atomic absorption spectrophotometry after hepatocytes were dissolved as mentioned above.

The uptake of calcium was calculated using the following formula (Tan and Tashjian, 1981);

$$\text{Ca uptake (nmoles/dish)} = \frac{\text{Cell-associated radioactivity (dpm/dish)}}{\text{Medium specific activity (dpm/moles)}}$$

Effects of Calcium on E_2-primed VTG Production

After 2 days preculture, hepatocytes were primed with E_2 for VTG production for 7 days and then incubated in the culture medium containing different calcium concentrations with or without E_2. Calcium free medium was prepared by adding 2 mM EGTA to the original incubation medium.

Qualitative and Quantitative Analyses of VTG

The identification of VTG band was based on

the results of a previous study (Kwon *et al.*, 1993). Isolated rainbow trout hepatocytes that were incubated with E_2 synthesized a protein of molecular weight (175 kDa), and Kwon *et al.* (1993) identified this band as VTG by immunoblot analysis and immunoelectrophoresis.

Total 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) and expressed as a percentage of total proteins including VTG. 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). 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 (100~1600 ng/mℓ, n=10) (Yeo and Mugiya, 1997).

Statistical Analysis

Data were analyzed by an one-way ANOVA followed by Fisher PLSD test or Student's t-test for unpaired observations. Significance was accepted at $P<0.01$. Percent data were statistically analyzed after being arcsine transformed.

Results

The present collagenase perfusion method yielded about 3×10^8 hepatocytes per fish. Cell viability was estimated at over 90% by Trypan Blue staining. After 2 days precultures, the amount of DNA per dish of hepatocytes on Day 7 in cultures decreased to 13.7% and 14.0% with and without E_2, respectively (data not shown). There ware no differences in DNA content between the control and E_2-treated cultures. The rates of VTG to total protein concentrations in serum and the incubation medium with or without E_2 were 26.2% and 11.0%, respectively (Fig. 1). The degree of VTG production *in vivo* was relatively higher than that *in vitro*.

Calcium Fractions in Serum and Incubation Medium

Figure 2 shows total and diffusible calcium concentrations in serum (A) and the incubation

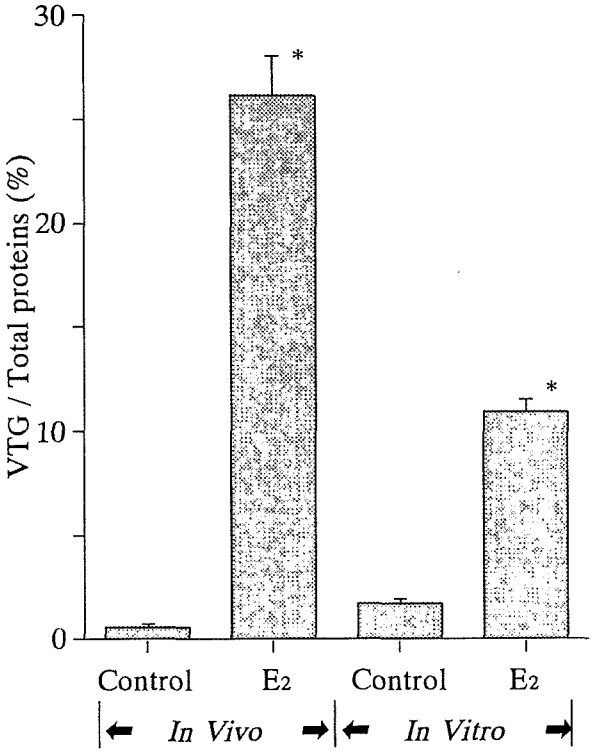

Fig. 1. Effects of E_2 on the VTG synthesis by hepatocytes *in vivo* and *in vitro*. Vertical bars represent the average (mean ± SE) percentage of three experiments. * $P<0.01$, E_2 vs control.

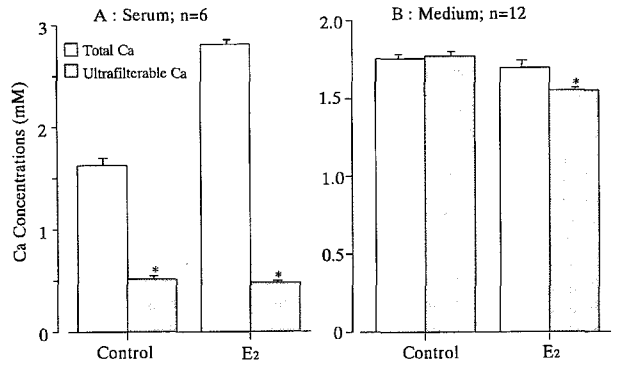

Fig. 2. Total and ultrafilterable calcium concentrations in serum (A) and the incubation medium (B) during vitellogenesis.
* $P<0.01$, Total Ca vs ultrafilterable Ca.

medium (B) with or without E_2. Total calcium was 1.62 mM and 2.81 mM in the control and estrogenized sera, respectively (Fig. 2A). Total calcium in estrogenized sera significantly increased, compared with the control ($P<0.01$), while diffusible calcium was insignificant. On the other hand, total calcium in the incubation medium was 1.75 mM and 1.70 mM in the control and with E_2,

respectively, which were insignificant in both (Fig. 2B). However, diffusible calcium in the incubation medium with E_2 was significantly reduced to 1.55 mM ($P<0.01$).

Calcium uptake by Estrogenized Hepatocytes

Figure 3 shows the effect of E_2 on calcium uptake by hepatocytes 30, 60, 90, 120, and 180 min after addition of E_2 and ^{45}Ca. The uptake of calcium by hepatocytes significantly increased 90 min after addition of E_2. Then, the uptake decreased to the control level after 120 min.

Furthermore, the accumulation of intracellular calcium during VTG production was examined from 7 to 9 days after E_2 addition. The accumulation of intracellular calcium increased in the cultures with E_2, regardless of the calcium concentrations in the incubation media (Fig. 4). These results suggest that the entry of extracellular calcium into the cytoplasm is an important step for VTG production in hepatocytes.

Effects of Calcium on E_2-primed VTG production

E_2-primed VTG production significantly decreased by removing E_2 or calcium from the media (Fig. 5). The production decreased to 87% ($P<0.01$) and 70 % ($P<0.01$) of the control at 1.8 mM Ca without

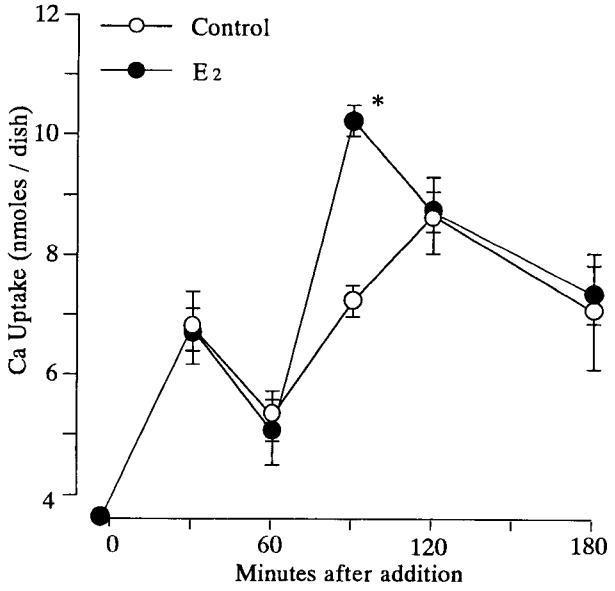

Fig. 3. Time course of ^{45}Ca uptake by hepatocytes in culture with E_2. Hepatocytes were precultured for 2 days and then E_2 (2×10^{-6} M) and ^{45}Ca (0.1 Mbq/mℓ) was added to the medium. Vertical bars represent the average (mean ± SE) nmoles/dish of triplicate incubations.
* $P<0.01$, E_2 vs control.

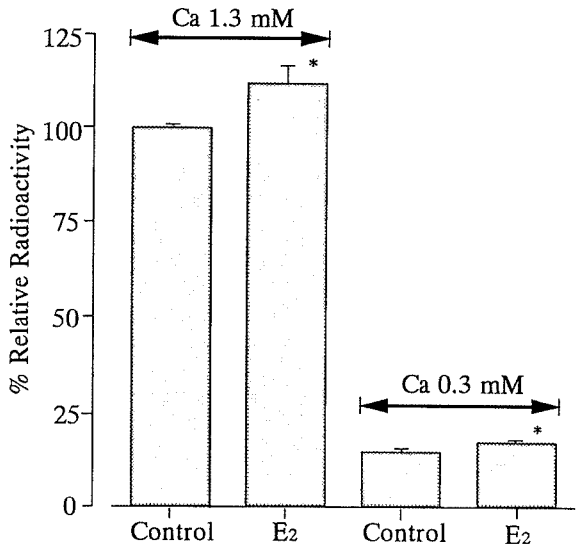

Fig. 4. Effects of calcium concentrations in the incubation medium on the accumulation of intracellular calcium during VTG production by cultured hepatocytes. Hepatocytes were precultured in the media containing E_2 (2×10^{-6}M) for 5 days and then were loaded with ^{45}Ca (0.1 Mbq/mℓ) for 2 days. Radioactivity was expressed as the percentage of the experimental to control values (Ca 1.3 mM). Vretical bars represent the average (mean ± SE) percentage of seven experiments.
* $P<0.01$, E_2 vs control.

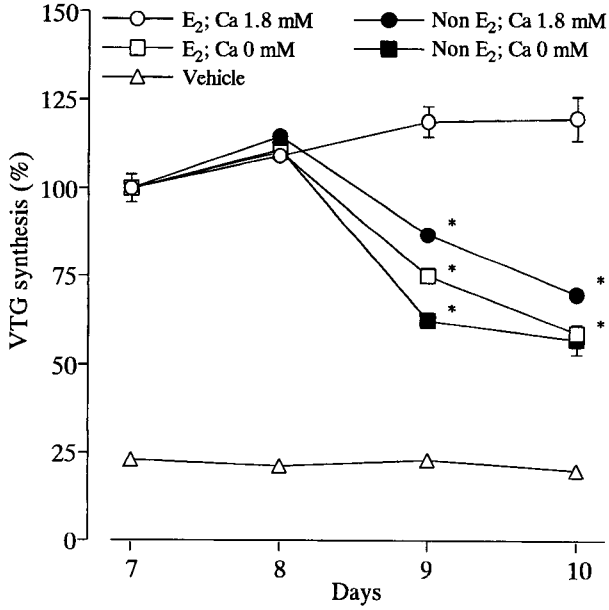

Fig. 5. Effects of removal of E_2 and/or Ca on E_2-primed VTG production. Hepatocytes were cultured in the medium containg E_2 and Ca (1.8 mM) for 7 days and then E_2 and/or Ca were removed. Vretical bars represent the average (mean ± SE) percentage of triplicate experiments.
* $P<0.01$, E_2 vs control.

E_2 on days 9 and 10, respectively. VTG production also decreased at a calcium concentration of 0 mM with E_2. Ca-free and E_2-free incubation further decreased VTG production to 62% and 57 % on days 9 and 10, respectively. These results suggest that calcium in the incubation medium is directly concerned with VTG production even in the primed-synthesis of VTG.

Discussion

Hepatocyte culture provides an excellent system to elucidate mechanisms involved in vitellogenesis. In fish, however, success in hepatocyte culture largely depends on fish species. Although improvements have been developed (Lipsky *et al.*, 1986; Kocal *et al.*, 1988), trout hepatocytes still have some difficulties in attaching to a dish or substrate and in forming a monolayer (Kwon *et al.*, 1993). The present study used positively charged dishes to which hepatocytes attached well. High DNA content was also maintained during culture.

In oviparous species including fish, vitellogenesis is an important step for ovarian development on which further embryonic development depends. One of features during vitellogenesis is the increase of plasma calcium levels (Fleming *et al.*, 1964). Björnsson and Haux (1985) reported that this increase is exclusively due to an increase in protein-bound calcium fraction and that ionized or diffusible calcium remains unchanged. The present results also showed the similar results. It was suggested that calcium in the ambient water and in internal stores such as scales and bone was available for the increased demand of calcium (Björnsson and Haux, 1985). Persson *et al.* (1994) also demonstrated that E_2-treated rainbow trout showed the increased uptake of calcium from the external environment.

Morley *et al.* (1992) reported that intracellular calcium increased by 4~8 folds immediately after addition of E_2 in chicken granulosa cell culture. The present study showed that the uptake of calcium by hepatocytes significantly increased 90 min after E_2 addition. Total intracellular calcium concentrations was also higher in estrogenized hepatocytes than in the control. These results suggest that the entry of extracellular calcium into the cytoplasm is an important step for VTG production in hepatocytes. Additionally, it is possible that the influx of calcium is directly related to the synthesis of VTG mRNA and/or influx of E_2. However, the specific mechanisms whereby intracellular calcium increased 90 min after E_2 addition are not well understood. Mechanisms in which E_2 is involved in the influx of extracellular calcium remain to be studied.

Calcium is required for the maintenance of optimal rates of protein production in a variety of eukaryotic cell types including isolated rat hepatocytes (Brostrom *et al.*, 1986; Chin *et al.*, 1988). Hepatocytes incubated in an EGTA-containing solution incorporated amino acids into a protein at a 5-fold slower rate than the control cells (Brostrom *et al.*, 1983). The production of VTG was affected by extracellular calcium in a concentration-dependent way in primary cultures of rainbow trout hepatocytes (Yeo and Mugiya, 1997). Therefore, VTG production may be highly dependent on cytozolic calcium states.

The present study found that E_2-primed VTG production was significantly decreased by withdrawal of E_2 from the incubation medium. Therefore, high VTG production requires the continuous presence of E_2. Moreover, VTG production by E_2-primed hepatocytes decreased when calcium was removed from the incubation medium with or without E_2. The magnitude of reduction in E_2-primed VTG production was larger at 0 mM Ca with E_2 than at 1.8 mM Ca without E_2, which suggests that calcium affected VTG production after the transcription of VTG mRNA. It is well known that the depletion of intracellular calcium inhibits the calcium-dependent translational initiation of protein production through the reduction of activated eIF-2 and the phosphorylation of the α-subunit of eIF-2 (Brostrom *et al.*, 1989; Kimball and Jefferson, 1992). Therefore, these results suggest that extracellular calcium is necessary for the synthetic pathway of VTG molecules, probably at a final stage of VTG synthesis, such as the translational and/or posttranslational stage. It would be desirable to examine the incubation of VTG mRNA at calcium-deficient conditions.

In conclusion, VTG production by E_2-primed hepatocytes decreased by removing calcium from the incubation medium. Therefore, extracellular calcium is necessary for VTG production probably at a level of translational and/or posttranslational level of VTG synthesis. Moreover, the entry of extracellular calcium into the cytoplasm is an important step for VTG production in primary hepatocyte cultures in rainbow trout.

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