INTRODUCTION

Vitellogenin (VTG) is one of the female-specific proteins that appear in the circulation of female fish during vitellogenesis, and also of male or immature fish treated with estradiol-17β (E2).\(^1\,^2\) This protein is a precursor form of egg yolk and is synthesized in the liver under the influence of E2.\(^3\,^4\) It is generally accepted that E2 is the most important steroid hormone to induce VTG in fish hepatocytes. A direct or indirect role for other hormones on inducing VTG synthesis in the tilapia hepatocytes has been suggested in rainbow trout\(^5\) and eels.\(^6,^7\) Although similar in vitro studies have not been conducted in tilapia, in vivo experiments revealed that cortisol induces rapid and transient transcription of VTG mRNA in Oreochromis aureus,\(^8\) and that 17α-methyltestosterone inhibits VTG gene expression in O. niloticus.\(^9\) Therefore, it is considered that several hormones are involved in the regulation of VTG synthesis in tilapias also.

In the present study, we developed a primary culture system of tilapia (O. mossambicus) hepatocytes and investigated effects of E2, insulin, and growth hormone on the in vitro production of female-specific proteins (vitellogenin; VTG) in fish hepatocytes. Absence of insulin in the culture media did not affect either cell attachment or monolayer formation. When VTG levels in the culture media were measured by an enzyme-linked immunosorbent assay (ELISA) using antibody against tilapia egg homogenate (a-E), VTG was detected in the medium for at least 8 days from hepatocytes isolated from a vitellogenic female but not from a male fish. However, the addition of estradiol-17β (E2) to the media of hepatocyte cultures from the male tilapia resulted in the elevation of VTG. Insulin and ovine growth hormone treatments did not have a significant effect on VTG induction. These results suggest that E2 is a major steroid hormone to induce VTG synthesis in the tilapia hepatocytes and that involvement of other hormones in the vitellogenic process is different among teleost species.

MATERIALS AND METHODS

Fish

Tilapia Oreochromis mossambicus used in the present study were collected from rivers in the Okinawa prefecture using a casting net. The fish were maintained in concrete tanks (2 metric ton capacity), with aeration and a filtering system for
water recirculation under ambient temperature at Sesoko Station, Tropical Biosphere Research Center, University of the Ryukyus, Okinawa, Japan. Fish body mass ranged from 100 g to 500 g and they were fed daily with a commercial tilapia food (41M; Zen-no, Tokyo, Japan).

Blood and ovarian samples were taken from several fish. After anesthetizing the fish in 0.05% 2-phenoxyethanol (Kanto Chemical, Tokyo, Japan), blood was collected from the caudal vein/artery vessel using a heparinized syringe containing aprotinin (Wako Pure Chemicals, Osaka, Japan) at 500 KIU/mL blood, and centrifuged at 2000×g for 15 min at 4°C. The plasma obtained was stored at −80°C (for absorption of antiserum) or used immediately (for electrophoresis and Western blot analyses). Ovaries were removed from the abdominal cavity and rinsed with 0.01 M phosphate-buffered saline (PBS), pH 7.4. Ovaries with yolky oocytes were homogenized 10 times (w/v) in PBS, and centrifuged at 9600×g for 20 min at 4°C. The supernatant (egg homogenate) was stored at −80°C. The protein concentrations of plasma and egg homogenate were estimated with protein assay reagent (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) fraction V (Sigma Chemical Co., St Louis, MO, USA) as the standard.

**Induction of vitellogenin by estrogen injection**

Estradiol-17β (E2; Sigma Chemical Co.) was dissolved in absolute ethanol (Wako Pure Chemicals) and diluted with an equal volume of physiological saline. E2 was injected into males intraperitoneally at a concentration of 5 μg/g bodyweight. After 5 days, blood collection and plasma separation were done as mentioned earlier. The plasma was used immediately for electrophoresis and Western blot analyses after measuring protein concentration.

**Hepatocyte isolation and culture**

Hepatocyte isolation was performed according to the modified method of Mommsen et al. The liver was carefully removed from the abdominal cavity of the anesthetized fish and transferred onto a watch glass and perfused with Ca2+-free saline solution (hepatocytes buffer: 136.9 mM NaCl, 5.4 mM KCl, 0.81 mM MgSO4, 0.44 mM KH2PO4, 0.33 mM Na2HPO4, 5.0 mM NaHCO3, pH 7.6) for 10 min at room temperature. Flow rate of a peristaltic pump (ATTO, Osaka, Japan) was set at 2.5 mL/min. After clearing blood from the liver, the tissue was digested for 15–20 min at room temperature with the hepatocytes buffer containing collagenase (Wako Pure Chemicals) at a concentration of 0.3 mg/mL. The softened liver was then minced with a razor blade and filtered through a 200 μm and then 50 μm nylon meshes, respectively. Cell suspension was transferred to a sterilized centrifuge tube (Falcon, Franklin Lakes, NJ, USA), and centrifuged four times at 50×g for 90 s at 10°C with hepatocytes buffer containing 1.5 mM CaCl2. After the final rinse, the cell pellet was resuspended in Leibovitz-15 (L-15) medium (Sigma Chemical Co.) containing 5 mM NaHCO3, penicillin (70 μg/mL) and polymyxin B (10 μg/mL). Cells were counted using a hemocytometer and cell viability was determined using the trypan blue exclusion method.

Isolated hepatocytes were seeded at a density of 1×106/mL (approximately 2.1×104/cm2) in ‘Primaria’ plates (Falcon) and incubated at 25°C in air with saturated humidity. The media was changed every two days. E2 and ovine growth hormone (oGH; Sigma Chemical Co.) were dissolved in ethanol and saline, respectively, while bovine insulin (Sigma Chemical Co.) was dissolved in L-15 medium. They were added to the cells after two days in culture. The final concentration of ethanol in the media never exceeded 0.1%. The media were collected from the plates and used immediately for measurement of total VTG level using an enzyme-linked immunosorbent assay (ELISA) (discussed later).

**Preparation of antiserum**

Antiserum against tilapia egg homogenate was prepared according to Takemura et al. An emulsion of tilapia egg homogenate (400 μg/mL) and an equal volume of Freund’s complete adjuvant were injected intradermally into the back of rabbits once a week for 4–5 weeks. One week after the final injection, blood was drawn from an ear artery, and allowed to clot at room temperature for 1 h. Serum was separated by centrifugation at 2000×g at 4°C for 15 min. To remove residual components common to both sexes, four parts of antiserum were absorbed with one part of pooled normal male serum. This absorbed antiserum was purified to the IgG fraction (a-E) with a column (1 cm×2 cm) of Protein A agarose 4FF (Pharmacia-LKB, Uppsala, Sweden) according to the manufacturer’s instruction.

**Electrophoresis and Western blots**

Sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 7.5% non-
gradient gel under reducing conditions according to the method of Laemmli. Electrophoresis was carried out at room temperature. Gels were stained for detection of total proteins with Coomassie brilliant blue R-250 (CBB R250; Nakarai tesque, Osaka, Japan). The molecular mass markers used were myosin (212 kDa), α2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa) (Pharmacia-LKB).

Separated proteins with SDS-PAGE were transferred onto a nitrocellulose membrane (Bio-Rad) in 25 mM Tris, 192 mM glycine, containing 20% (v/v) methanol for 1 h. After rinsing the membrane with PBS, protein-binding sites on the membrane were blocked in 25 mM Tris-buffered saline (TBS) containing 5% skim milk for 1 h at room temperature. The membrane was incubated with a-E (1 : 5000) diluted in TBS buffer for 1 h. After rinsing three times with TBS, the membrane was transferred to TBS containing goat antirabbit IgG antibody conjugated with alkaline phosphatase (1 : 10000; Sigma Chemical Co.). After washing three times with TBS, visualization of the immunoreaction was performed by incubating the membrane in a substrate solution of 330 mg/mL nitroblue tetrazolium (NBT; Kanto Chemical) and 165 mg/mL 5-bromo-4-chloro-3-indolyl phosphate (BCIP; Sigma Chemical Co.) in 0.1 M Tris-HCl buffer (pH 9.5) containing 100 mM NaCl and 5 mM MgCl₂. The color reaction was stopped by transferring the membrane to water.

Enzyme-linked immunosorbent assay

In the present study we carried out a sandwich ELISA method according to the modified method of Takemura. Conjugation of horseradish peroxidase (HRP; Sigma Chemical Co.) to a-E was performed in accordance with the method of Nakane and Kawaoi. Each well of a 96-well microtiter plate (Iwaki, Chiba, Japan) was coated with 100 µL of 50 mM carbonate buffer, pH 9.6, containing a-E at a concentration of 10 µg/mL for 2 h at room temperature or overnight at 4°C. After washing the plate with PBS containing 0.05% Tween 20 (PBS-T) using a plate washer type 1575 (Bio-Rad), residual protein-binding sites on each well were blocked by adding 200 µL of PBS-T containing 1% BSA for 1 h at room temperature. After washing the plate three times with PBS-T, standards (serial dilution of a mature female plasma) and culture media were dispensed in duplicate (100 µL per well) into the plate, which was incubated for 2 h at room temperature. After washing, 100 µL of HRP-conjugated a-E, which was diluted with PBS-T (1 : 4000), was added to each well of the plate. Incubation was done for 2 h at room temperature. Following three washes with PBS-T, 100 µL of 0.1 M citrate buffer, pH 4.5, containing 1 mg/mL of o-phenylenediamine dihydrochloride (Sigma Chemical Co.) and 0.04% H₂O₂ was added to the wells. Color development proceeded for 30 min at room temperature and was then stopped by the addition of 25 µL of 2 M sulfuric acid to the wells. Absorbance of each well was measured at 492 nm using a plate reader (Model 550; Bio-Rad).

The VTG concentration in the culture media was represented as a relative value to the VTG present in the plasma of a vitellogenic female. The plasma, diluted 5000 times, was expressed as 1000. The intra-assay coefficient of variation (n = 6) at high (1 ¥ 10⁴ times dilution), middle (8 ¥ 10⁴), and low (32 ¥ 10⁴) was less than 7.3%, whereas the interassay coefficient of variation was less than 5.5%.

Statistical analysis

The VTG concentrations in the culture media were expressed as mean ± SEM for three dishes. The data were analyzed using two-way ANOVA. Significance was accepted at P < 0.05.

RESULTS

Electrophoresis and Western blot of tilapia plasma

Figure 1 shows a SDS-PAGE pattern of plasma proteins from E₂-treated and untreated male tilapia. Two new proteins appeared after E₂ treatment and had apparent molecular masses of 210 kDa and 140 kDa. Similar proteins were detected also in the plasma samples of vitellogenic females (data not shown).

The plasma proteins separated with SDS-PAGE were then transferred onto the nitrocellulose membrane and subjected to Western blot analysis. Two proteins were clearly immunostained with a-E in the plasma of E₂-treated males, but not those males that did not undergo E₂ treatment (control). The molecular masses of these proteins were 210 kDa and 140 kDa (Fig. 2).

Isolation of tilapia hepatocytes

The yield of tilapia hepatocytes ranged from 34 ¥ 10⁶ cells/g to 88 ¥ 10⁶ cells/g liver weight. Cell
viability, as determined by the trypan blue exclusion test, was greater than 92%.

The freshly isolated hepatocytes appeared round and showed cytoplasm with lipid granules (Fig. 3a). At 2 days after culture, the hepatocytes fused together and formed chain-like structures (Fig. 3b). At 4 days after culture, the hepatocytes jointed together and formed a monolayer (Fig. 3c), while 6 days after culture, the hepatocytes appeared confluent (Fig. 3d). Thereafter, the same
morphological features were maintained for more than 2 weeks, although cell numbers on the plate decreased gradually.

The effect of insulin on the monolayer formation of tilapia hepatocytes was examined. In the culture condition with or without bovine insulin (0.2 μM/mL), there were no differences in morphological phases of cell conjugation and attachment (Fig. 4a,b), and monolayer formation (Figs 4c,d).

Enzyme-linked immunosorbent assay

To estimate total VTG level (two distinct types of VTG) in the culture media, a-E was used to recognize both types simultaneously. Total VTG levels of the medium were represented as a relative value to that of a vitellogenic female plasma used as the standard. Figure 5 shows a typical standard curve and cross-reactivity of a-E against the egg homogenate, E2-treated and untreated male plasma, and culture media. The egg homogenate (serial dilutions from 20,000 times) and the plasma from E2-treated male plasma (serial dilutions from 5000 times) reacted with a-E and were parallel to the standard curve (Fig. 5b). The same parallelism was observed between culture media (serial dilutions from 1 time) from female and E2-treated hepatocytes (Fig. 5c). The male plasma and medium from male hepatocytes did not show any cross-reactivity against the antibody (Figs 5b,c).

Synthesis of vitellogenin by tilapia hepatocytes

Changes in VTG production in the media from cultured tilapia hepatocytes during the course of the
experiment were determined using ELISA. Hepatocytes prepared from a vitellogenic female secreted VTG into the media even in the absence of any hormone treatment. The VTG levels in the medium gradually decreased over time in these cultures. Conversely, VTG was not detected in the media of cultured hepatocytes isolated from male fish (Fig. 6).

**Induction of vitellogenin synthesis by E2**

Figure 7 shows the effects of different E2 concentrations (1 × 10⁻⁷ – 1 × 10⁻⁵ M) on VTG synthesis by the cultured male hepatocytes. Two days after E2 treatment, VTG was detected in the media at E2 concentrations of 1 × 10⁻⁶ – 1 × 10⁻⁵ M. These VTG levels were significantly increased at 4 and 6 days after the addition of E2. Vitellogenin was first detected at 6 days after E2 treatment at 1 × 10⁻⁷ M. This protein was not detected in E2-untreated media (control).

**Effect of insulin and ovine growth hormone on vitellogenin production**

The effect of bovine insulin on VTG production in the tilapia hepatocytes was examined (Fig. 8). This protein was induced in the medium with or without insulin at 2, 4 and 6 days after E2 treatment.
The VTG levels increased gradually from day 2 to day 6 in the media with and without insulin. There were no significant differences in these VTG levels. The effect of oGH on VTG synthesis by tilapia hepatocytes was also examined (Fig. 9). The VTG levels were measured using ELISA at 2, 4 and 6 days after hormone treatment. When the hepatocytes were cultured with oGH only (100 ng/mL), VTG was not detected in the media. Treatment of the cells with E2 induced VTG at 2, 4 and 6 days after hormone treatment. However, there was no significant increase in VTG levels with the cotreatment of oGH and E2.

**DISCUSSION**

In the present study, E2 treatment induced two proteins in the plasma of male *O. mossambicus*. These two proteins had apparent molecular masses of 210 kDa and 140 kDa with 7.5% SDS-PAGE under reducing conditions and reacted with rabbit anti-tilapia egg homogenate antibody (a-E). Previous studies have identified similar proteins in the plasma of vitellogenic female or E2-treated male *O. aureus*, *O. mossambicus*, *O. niloticus*, and the black-chinned tilapia *Sarotherodon melanotheron*. Kishida and Specker purified two proteins from the E2-treated male *O. mossambicus* plasma with a combination of DEAE–agarose ion-exchange chromatography and gel filtration. They had molecular masses of 200 kDa (tVTG200) and 130 kDa (tVTG130) by SDS-PAGE. Buerano et al. also separated two E2-inducible proteins from the plasma of *O. niloticus* by precipitation with Mg2+ ions in the presence of ethylenediamine tetraacetic acid (EDTA). These proteins had molecular masses of 185 kDa (EIP1) and 120 kDa (EIP2) by SDS-PAGE, and they were both phosphoglycolipoproteins and immunoreacted with the antiserum raised against egg proteins. It has been reported that precursor forms of vitelline envelope (choriogenin) are also induced in the plasma of several fishes by the influence of E2. Their molecular masses have been estimated to be 49 kDa (L-SF) and 74–76 kDa (H-SF) for medaka, *Oryzias latipes*, 78 kDa (α), 54 kDa (β) and 47 kDa (γ) for cod *Gadus morhua*, 50 kDa (α), 55 kDa (β) and 60 kDa (γ) for rainbow trout, and 48/54 kDa (hVERP) and 42/46 kDa (lVERP) for Sakhalin taimen *Hucho perryi*. Their molecular masses were markedly lower than the two proteins (210 kDa and 140 kDa) induced in the present study. Additionally, immunohistochemical observations revealed that antibodies raised against each purified protein did not react with the egg envelope of tilapia oocyte (data not shown). These results indicate that the plasma proteins detected in the present study are VTG but not choriogenin of *O. mossambicus*.

Primary culture was used in the present study to clarify the effects of certain hormones on VTG induction in tilapia hepatocytes. In primary culture of eel hepatocytes, insulin or fetal calf serum was an essential factor for the attachment of hepatocytes to the plate and the maintenance of cell viability in culture. In the tilapia hepatocytes, however, the absence of insulin did not affect the morphological characteristics of the cells. These results suggest that demands of hormones for the maintenance of cell viability and responses of hormones to VTG synthesis are different among fish species.

When the hepatocytes from sexually mature female and male tilapia were cultured, VTG was detected in media (L-15) obtained from female but not male hepatocytes. This is perhaps due to the expressions of E2 receptor and VTG genes by E2 in the maturing female prior to cell isolation and, as a result, the effect of *in vitro* E2 in culture decreases gradually over time. Male hepatocytes had already induced VTG 48 h after exposure to E2. Additionally, there was a dose-dependent effect of E2 on VTG
induction, suggesting that E₂ is a potent hormone to inducing VTG synthesis in tilapia hepatocytes. Physiological doses of E₂ (10⁻⁷ – 10⁻⁶ M) induce VTG protein expression in the culture media and/or the hepatocytes from rainbow trout,2⁹,3¹ Japanese eel,5 and European eel2,3⁰ and VTG mRNA expression in rainbow trout hepatocytes.5,3² In the case of eels, pituitary hormones such as growth hormone6,7 and prolactin⁸ are required for the in vitro induction of VTG by physiological doses of E₂. A pharmacological dose of E₂ (10⁻⁴ M) was needed to induce VTG synthesis in Japanese eel hepatocytes in the absence of pituitary hormones.3³ Peyon et al. have suggested that growth hormone alone or in synergism with E₂ exerts transcriptional and post-transcriptional effects on the expression of specific genes, such as the E₂ receptor and VTG genes.⁷ Conversely, VTG production after cotreatment with E₂ and insulin was drastically reduced in hepatocytes from male rainbow trout.¹¹ In the present study, insulin and oGH did not show any significant effect on VTG induction in tilapia hepatocytes. Involvement of the pituitary hormones and insulin on in vitro VTG synthesis in hepatocytes may be different among teleost species.

The antibody (a-E) used in the present study recognized two types of VTG and, therefore, the total VTG level (i.e. VTG 210 + VTG 140) was measured in the media by ELISA. The effect of E₂ on the synthesis of each VTG could not be assessed in the present study. However, it is possible that the hormonal effects inducing each type of VTG are different. It is necessary to purify the different types of VTG separately, raise specific antisera, and develop ELISA for the respective VTG in order to clarify the hormonal regulation of the two distinct types of VTG using primary culture from tilapia hepatocytes.

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