Hormonal mechanisms regulating hepatic vitellogenin synthesis in the gilthead sea bream, Sparus aurata

G. MOSCONI, O. CARNEVALI, H. R. HABIBI, R. SANYAL, and A. M. POLZONETTI-MAGNI

1Dipartimento di Scienze Morfologiche e Biochimiche Comparate, Università degli Studi di Camerino, 62032 Camerino (MC) Italy; 2Istituto di Scienze del Mare, Università di Ancona, 60131 Ancona, Italy; and 3Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4

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Mosconi, G., O. Carnevali, H. R. Habibi, R. Sanyal, and A. M. Polzonetti-Magni. Hormonal mechanisms regulating hepatic vitellogenin synthesis in the gilthead sea bream, Sparus aurata. Am J Physiol Cell Physiol 283: C673–C678, 2002; 10.1152/ajpcell.00411.2001.—Experiments were carried out to study in vitro the effects of 17β-estradiol (E2), homologous pituitary homogenate (HPH), and recombinant red sea bream growth hormone (sbGH) on vitellogenin (VTG) secretion from cultured sea bream liver fragments. Basal secretion of VTG was found to be significantly higher in the prespawning period, compared with sea bream liver in the spawning and postspawning periods. Similarly, the sea bream liver obtained during the prespawning period responded more significantly to treatments with E2, HPH, or sbGH compared with sea bream liver during spawning. In the postspawning period, treatments with E2, HPH, or sbGH were without significant effect on VTG secretion level in sea bream liver. The level of E2 receptors was also analyzed by Western blot analysis. The result demonstrates a significantly higher level of E2 receptors in the sea bream liver at the prespawning stage compared with those at the spawning and postspawning stages. The findings support the hypothesis that homologous upregulation of estrogen receptors plays an important role in the estrogen-sensitive control of VTG synthesis in the sea bream liver.

vitellogenin induction; hormones; estradiol receptor

THE GILTHEAD SEA BREAM, Sparus aurata, is an economically important species because of high demand and the high quality of its meat. Recent studies have provided information on reproductive endocrinology of sea bream (3), and there is evidence for the presence of three forms of gonadotropin-releasing hormones (GnRH) in the brain of this species (4, 11). GnRH in turn stimulates gonadotropin production, which is the main regulator of gonadal development, steroidogenesis, and ovulation. In female fish, the accumulation of yolk proteins, i.e., vitellogenesis, is crucial for oocyte development and spawning. As in a number of oviparous species, production of vitellogenin (VTG) in the liver is controlled by a number of gonadal and pituitary hormones (6, 12, 14). S. aurata is a true hermaphrodite and functions as a male during the first 2 years of life. In the third year, there is testicular degeneration and ovary development, resulting in transformation of the fish to functional females. Sea bream is classified as a “group synchronous” spawner (15) with short and regular spawning cycles. Because spawning may be prolonged over months, vitellogenesis, which is part of yolk protein formation, is continuous throughout the breeding season (1). However, apart from the work by Mosconi et al. (7), little information is available on hormonal control of hepatic VTG synthesis in sea bream maintained under intensive culture conditions. Recent studies characterized the S. aurata VTG molecule and developed an enzyme-linked immunosorbent assay (ELISA) method for its measurement (7). Changes in plasma VTG were shown to be correlated with those of 17β-estradiol (E2) in both prespawning and spawning female sea bream. In the postspawning period, however, VTG was found to be undetectable when the circulating level of E2 was the same as that in the spawning period. This may, therefore, raise questions about the role of estradiol in triggering and terminating VTG synthesis in sea bream. There is also evidence that estrogen-induced vitellogenesis is influenced by growth hormone (GH) in silver eel (9) and by other pituitary hormones in certain oviparous species (10).

In the present study, sea bream liver was cultured in the presence of E2, homologous pituitary homogenate (HPH), and recombinant red sea bream growth hormone (sbGH), followed by measurement of VTG in the culture media (CM). We also measured the estradiol receptor level in the same hepatic tissue as that used in incubation experiments by Western blot analysis.

MATERIALS AND METHODS

Animals. Gilthead sea bream (S. aurata) living in the Mediterranean area naturally spawn from November until February. In the present study, adult females (between 1.5 and 2 kg body wt) were obtained from a commercial fish farm...
(La Rosa, Orbetello, west coast of Italy; 42°28’ N, 11°12’ E).
The animals were rapidly anesthetized in a tank containing
water plus 100 mg/l of MS222 (Sigma), and blood samples
were collected from the caudal vein with a syringe. The blood
samples were placed in heparinized tubes containing 1 mM
phenylmethylsulfonyl fluoride (PMSF) and centrifuged
(1,500 g for 15 min at 4°C), and plasma samples were stored
at -20°C. The liver was removed and processed for in vitro
incubation and Western blot analysis.

Hormones. HPH was prepared by homogenizing homolo-
gous total female pituitary glands in CM. E2 and sbGH were
purchased from Sigma (St. Louis, MO) and GroPrep (Ad-
elaide, Australia), respectively.

Liver incubation. Sixty milligrams of liver (~6–7 pieces)
were weighed, placed in a Falcon culture dish well, and
covered with 1 ml of medium 199 (Sigma) with Hanks’
salts and l-glutamine and without NaHCO3, and the salinity was
adjusted with NaCl and NaHCO3. The medium, enriched
with oxygen, was supplemented with 1% (vol/vol) Ultraser
(IBF Biotechnics), 100 U/ml penicillin, and 100 mg/ml strep-
tomycin. The pH was adjusted to 7.4. Liver fragments were
incubated at 18°C on a rocking table (4 cycles/min) for up to
8 days. E2 (10^-6 M), HPH (1/20 eq gland^-1 ml^-1), and/or
sbGH (1 μg/ml) were added to each well. In this study, five
wells were prepared for each treatment. The media contain-
ning appropriate hormones were oxygen enriched and changed
daily. The control wells contained CM plus vehicle only. At
the end of incubation, tissue was examined under light mi-
croscopy to be sure of its preservation. The liver incubations
were performed during prespawning, spawning, and post-
spawning periods.

ELISA for VTG in CM. The VTG concentration in the CM
was assayed with an ELISA method previously validated and
described by Mosconi et al. (7). The sensitivity (amount of
VTG that gave 90% of binding) was ~8 ng/ml, with an
intra-assay variation of 4.8% (n = 16) and an interassay
variation of 7.2% (n = 12) around 50% of binding. VTG
content was normalized against the tissue total protein con-
tent of each sample. The results are expressed as accumu-
lated levels of VTG.

E2 assay in plasma. Plasma E2 levels were analyzed by an
enzyme immunoassay (EIA) method (estradiol EIA kit, Cay-
man) with a sensitivity of 8 pg/ml.

In vivo experiments. Adult female sea bream (n = 6) were
injected during the postspawning period with 5 mg/kg body
wt of E2 dissolved in peanut oil (5 mg/ml); after 5 days, both
E2-treated and control animals (6 females injected with
vehicle only) were rapidly anesthetized as described in Ani-

Fig. 1. Dose-response curves, obtained
during spawning period, of sea bream
growth hormone (sbGH; A) and 17β-
estradiol (E2; B) in inducing vitelloge-
nin (VTG) synthesis and release in the
culture medium (CM). Results are ex-
pressed as means ± SD (n = 5).
Western blot analysis for estradiol receptor evaluation. Total protein extraction was carried out with RIPA buffer (25 mM Tris pH 7.5, 0.1% SDS, 1% Triton X-100) to homogenize the liver; then 10 μl of PMSF (10 mg/ml) was added before centrifugation (30 min at 12,000 rpm); the supernatant was quantified with a Hitachi U2000 spectrophotometer (Tokyo, Japan), and the bicinchoninic acid (BCA) method for protein quantification (Pierce, Rockford, IL) was applied. For the protein gels, 10 μg of total protein was loaded per lane of gel. The protein samples were resolved on a 10% resolving gel and a 3.75% stacking gel. Samples were electrophoresed at constant voltage (150 V) until the bromophenol blue dye front reached the bottom of the gel. Gels used for Western blot transfer were run with Amersham molecular weight markers (rainbow markers) and used for transfer immediately without staining. Western blotting proteins were transferred to polyvinylidene difluoride (PVDF) plus membrane (Micron Separations) in a Bio-Rad Mini-Protean 2 cell apparatus at 100 V for 1 h. The ECL Western blotting system (Amerham Life Sciences) was used for detection purposes. The primary antibody used was polyclonal antibody raised against human estrogen receptor (ER)-α in rabbit (Sigma) at a dilution of 1:1,000 for a minimum time of 1 h overnight. Horseradish peroxidase-labeled secondary antibody (conjugated anti-rabbit) was used at a dilution of 1:3,000 for 1 h. Blots were exposed on Kodak autoradiography-sensitive film for as little as 40 s, and a clear signal was detected. The dilution of the second antibody was optimized to give a clear background.

Statistical analysis. The results were analyzed by ANOVA with Stat View 512+ (Brain Power) statistical software package. A probability level of 0.05 was taken to indicate a statistical difference between means. Results are expressed as means ± SD (n = 5).

### RESULTS

VTG secretion from cultured sea bream liver fragments in vitro. Experiments were carried out to study the effects of E2, HPH, and recombinant sbGH on VTG secretion on cultured sea bream fragments in vitro. Cultured sea bream liver was found to produce and spontaneously release VTG in the CM in a time-related manner. Treatment with sbGH resulted in a dose-related increase in secretion of VTG compared with controls (Fig. 1A). The dose-related effect of sbGH became apparent after 6 days of culture in vitro. In a similar experiment, we also tested the effect of various concentrations of E2 on cultured sea bream liver. Treatment with E2 also resulted in a dose-related increase in VTG secretion in vitro, and the steroid action became apparent after 7 days of treatment (Fig. 1B). In these experiments, the most effective doses were found to be 10⁻⁶ M and 1 μg/ml for E2 and sbGH, respectively.

Further experiments were carried out to test the effects of E2, HPH, and sbGH on sea bream liver fragments obtained from animals at different stages of the reproductive cycle. Our initial observation indicated significant differences in the basal secretion rate of VTG, which was found to be significantly (P < 0.05) higher in the prespawning period (20 ± 3.5 μg/ml) than in the spawning (8.5 ± 3.1 μg/ml) and postspawning periods.

**Table 1. Plasma levels of estradiol-17β and vitellogenin**

<table>
<thead>
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<th>Prespawning</th>
<th>Spawning</th>
<th>Postspawning</th>
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<tr>
<td>Estradiol-17β, ng/ml</td>
<td>2,188 ± 158</td>
<td>1,010 ± 95</td>
<td>1,100 ± 90</td>
</tr>
<tr>
<td>VTG, mg/ml</td>
<td>1.5 ± 0.16</td>
<td>0.7 ± 0.08</td>
<td>0.01 ± 0.001</td>
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Results are expressed as means ± SD (n = 5) of plasma estradiol and vitellogenin (VTG) levels in animals in the in vitro experiments.
Fig. 4. Western blot analyses of E2 receptors in liver samples from animals at prespawning, spawning, and postspawning periods. A: representative profiles of Western blot analyses. B: densitometric analysis of the bands was performed to obtain the relative density of ER. Results are expressed as means ± SE of 3 independent blots.

(1.2 ± 0.4 μg/ml) periods after 8 days of culture in vitro (Fig. 2). Incubation of sea bream liver with E2, HPH, or sbGH significantly (P < 0.05) increased VTG secretion during both prespawning and spawning periods. In the prespawning period (Fig. 2A), treatment with E2, HPH, or sbGH significantly (P < 0.05) increased VTG secretion from day 2 until day 8. The positive effect of E2 at day 6 was more pronounced than that of pituitary hormones (P < 0.05). In the spawning period (Fig. 2B), although HPH resulted in significant increase in VTG secretion from day 2 to day 8 of culture, the differences for E2 and sbGH became apparent only after 6–8 days of culture. In the postspawning period, treatments with E2, HPH, or sbGH were without significant effect on the VTG secretion level (Fig. 2C).

Plasma E2, VTG, and liver ER levels at different reproductive stages in sea bream. The levels of plasma E2 show seasonal changes, with the highest levels found during prespawning (2,188 ± 158 pg/ml), compared with levels at spawning (1,010 ± 95 pg/ml) and postspawning (1,100 ± 90 pg/ml) periods (Table 1). The highest VTG plasma levels were found during prespawning (1.5 ± 0.16 mg/ml), significantly decreasing during spawning (0.7 ± 0.08 mg/ml) and reaching a

Fig. 5. Western blot analyses of E2 receptors in liver samples from postspawning animals injected with E2 and animals injected with saline (control). A: representative profiles of Western blot analyses. B: densitometric analysis of the bands was performed to obtain the relative density of ER. Results are expressed as means ± SE of 6 independent blots.
minimum (0.01 ± 0.001 mg/ml) at the postspawning period (Table 1).

Immunoblotting analysis with the heterologous antibody against human ER on sea bream liver shows two bands at 65 and 52.5 kDa, whereas the immunoblot of protein obtained from a human breast cancer cell line (MCF-7) with this antibody gives a single band of ~66 kDa corresponding to human ER-α (Fig. 3). The larger band obtained in liver sea bream is likely to be the 64.9-kDa sea bream ER-α previously described by Munoz-Cueto et al. (8, 13).

Liver samples from the same animals used for the E2 and VTG determinations during prespawning, spawning, and postspawning periods were analyzed to determine the level of E2 receptors by Western blot analyses. In this experiment, significantly (P < 0.05) higher levels of E2 receptor were obtained in the sea bream liver at the prespawning stage, compared with those at the spawning and postspawning stages (Fig. 4). The highest level of E2 receptor corresponded with the highest basal circulating E2 level in prespawning sea bream. The injection of females with E2 during postspawning induced the highest level of E2 receptor density (Fig. 5).

DISCUSSION

The present study provides information on VTG secretion level in sea bream liver in response to in vitro treatments with E2, HPH, and sbGH; moreover, it gives novel information on in vivo ER level in the sea bream liver at different stages of reproduction, demonstrating a positive correlation between E2 receptor level and liver responsiveness to hormonal treatments. The findings provide an insight into the mechanism by which estradiol may effect hepatic VTG synthesis in sea bream liver and its release. Our results demonstrate for the first time in fish that E2 is not the only hormone that exerts direct stimulatory action on hepatic VTG secretion. In this context, treatments with HPH and sbGH stimulated VTG synthesis in in vitro cultured sea bream liver. The involvement of pituitary hormones such as GH and prolactin in induction of VTG synthesis was previously demonstrated in Anguilla japonica (5) and A. anguilla (9). In female silver eel, GH treatment was shown to stimulate VTG synthesis in cultured hepatocytes (9). The multihormonal stimulation of GH and/or prolactin as well as E2 was found to be essential for the active synthesis of VTG in A. japonica (5). In S. aurata, which is a marine teleost with a reproductive strategy different from that of eels, the liver responds directly to GH stimulation and produces VTG in concentrations higher than those in eels. There appears to be a specific liver sensitivity to estrogenic compounds (estradiol) and pituitary hormones (GH), depending on the reproductive phase. In this context, although estradiol was found to be more potent than sbGH in the prespawning period, pituitary hormones induced more significant stimulatory effect on VTG secretion than did estradiol during spawning period. The present results clearly demonstrate that basal VTG production has seasonal changes and that the responsiveness to E2 and pituitary hormones of sea bream liver varies during the reproductive cycle. The stimulatory action of pituitary hormones, as well as estradiol, on liver VTG production, was also previously demonstrated in other oviparous vertebrates (10). GH was shown to act as a coregulator of VTG synthesis in fish (12), and there is evidence for the involvement of GH via production of insulin-like growth factor I (IGF-I) activating second messenger system (2, 16).

In this study, a positive correlation between E2 receptor level and liver responsiveness to E2 was clearly observed. During prespawning, when E2 receptor levels are highest, the liver produces the highest levels of VTG in response to E2. These data are supported by the in vivo results showing the band of ~66 kDa, corresponding to human ER-α, suggesting positive response to E2 treatment.

In this study, our findings suggest that estradiol upregulates ERs that may, in part, be responsible for changes in seasonally dependent sensitivity to circulating estrogen level in sea bream.

In summary, the present results demonstrate a direct action of GH and estradiol on sea bream liver VTG secretion in vitro. Furthermore, the findings demonstrate a seasonal variation in hormone-induced VTG secretion response correlated with the E2 receptor level in sea bream liver.

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REFERENCES


