In vivo and in vitro studies on sex steroid binding protein (SBP) regulation in rainbow trout (Oncorhynchus mykiss) : influence of sex steroid hormones and factors linked to growth and metabolism

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IN VIVO AND *IN VITRO* STUDIES ON SEX STEROID BINDING PROTEIN (SBP) REGULATION IN RAINBOW TROUT (*ONCORHYNCHUS MYKISS*): INFLUENCE OF SEX STEROID HORMONES AND OF FACTORS LINKED TO GROWTH AND METABOLISM

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Summary—The respective roles of sex steroids and hormones related to growth and metabolism, on SBP regulation have been studied in rainbow trout. *In vivo*, oestradiol (E2) supplementation induces a slow but significant increase of plasma SBP concentration. Testosterone or cortisol injections have no effect. *In vitro*, the steroid binding protein that accumulates in incubation medium of hepatic cell primary cultures has been characterized and found to be similar to blood SBP. Its production is increased by addition of E2 (maximum: +300%). This effect develops slowly over several days of culture and is dose dependent; as little as 1–10 nM E2 is effective.

Recombinant rainbow trout GH (rtGH)—0.01 to 1 μg/ml—also increases SBP accumulation as compared to control cells and seems to maintain SBP production over culture duration. In preliminary experiments, (1) insulin-like growth factor (IGF) and SBP concentrations were found to change inversely after a 4 days stimulation with increasing concentrations of GH; (2) recombinant human IGF_1 (250 ng/ml) tended to be inhibitory when SBP production was expressed per mg of total cellular protein, and a micromolar concentration of bovine insulin was clearly inhibitory.

Other hormones tested *in vitro*: triiodothyronine (10–1000 nM), thyroxine (100 nM), 17α,20β-dihydroprogesterone (10–2000 nM), and testosterone (1–1000 nM) did not influence SBP concentration in hepatic cells culture media.

INTRODUCTION

The presence of sex steroid binding protein (SBP) has been demonstrated in most mammals and also in lower vertebrates [1–4].

In mammals, SBP is synthesized in the liver and SBP mRNA has been demonstrated in cultured hepatoma cells [5]. This protein probably plays a role in the regulation of the action of steroid hormones and seems involved in certain mechanisms of action directly on steroid target cells [6, 7].

Numerous studies on the physiological variations of SBP have contributed to the idea that plasma SBP concentration reflects oestrogen/androgen balance, E2 increasing SBP concentration and T decreasing it [2, 7–10]. Clinical observations have reinforced this hypothesis. In women, oral administration (but not peritoneal injection) of E2 increases SBP concentration. Men with increased concentrations of circulating androgen have low levels of SBP [11, 12]. Inversely, androgen deficiency [12] or resistance to androgens [13] raises SBP concentrations.

However, *in vitro* or *in vivo* data have also provided evidence against the oestrogen/androgen balance hypothesis. For example, *in vitro*, E2 increases SBP production by hepatoma cells in culture, however, under the same conditions and at the same doses as E2, dihydrotestosterone increases SBP production slightly [14, 15]. Several authors have not demonstrated any significant variation of SBP

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Abbreviations: Oestradiol (E2): 1-3-5(10)-oestra-triene-3,17β-diol; Testosterone (T): 17β-hydroxy-4-androsten-3-one; 11-Ketotestosterone (11KT): 17β-hydroxy-4-androstene-3,11-dione; 17α,20β-dihydroprogesterone; (17α,20βDHP): 17α,20β-dihydroxy-4-pregnene-3-one; Cortisol (Col): 11β,17,21-trihydroxy-4-pregnene-3,20-dione; Thyroxine (T4): 3-(4-(4-hydroxy-3,5-diiodophenoxy)-3,5-diiodophenyl)-alanine; Triiodothyronine (T3): (*O*-(4-hydroxy-3-iodophenyl)-3,5-diiodo-tyrosine; growth hormone: GH.

after castration in human and monkeys [16]. In the female rhesus monkey, androgen supplementation decreases SBP concentration, but this effect is not suppressed by flutamide, a non-steroidal androgen antagonist [17]. In macaque, while androgens reduce SBP concentration, E2 has only a slight or no stimulatory effect [18]. Moreover, numerous factors other than steroid hormones could influence SBP concentration including growth factors, nutritional status and various factors and hormones implicated in general metabolism such as insulin, IGF, T3 and cortisol [5, 12, 14, 19–21]. While a direct effect of GH on SBP levels has never been demonstrated, a decreased SBP capacity is observed in acromegaly [22].

In the rainbow trout, physicochemical studies have shown that plasma SBP is a glycoprotein, migrating in polyacrylamide gels with an electrophoretic mobility similar to mammalian SBP. Salmonid SBP preferentially binds T with a high affinity (K_d T = 2.5 nM) and rapid kinetics of association and dissociation, but can bind other steroids with the following order of affinity: T > E2 > P ≫ col [23, 24]. In this species, the regulation of SBP production and the site of its production remain unclear. However, castration does not influence SBP levels in blood plasma while hypophysectomy decreases SBP in blood and liver cytosol but not in testis cytosol [24]. Moreover, a fast decrease of SBP during the spawning period was not found to be linked with an increase of androgen levels, but concomitant to an increase of GH circulating levels and reduced food intake. SBP decrease and GH elevation were attributed to a decreased hepatic function including a decrease of IGF production [24] and of GH receptors (Yao *et al.* unpublished data). However, treatment of trout with a dose of GH, which affected fish growth and circulating IGF levels, had no inhibitory effect on SBP [24].

In order to clarify the respective roles of sex steroid hormones and factors linked to growth and metabolism, the effects of treatment with E2, T, recombinant trout GH, and certain other hormones on SBP production in trout were studied both *in vivo* and/or *in vitro* using primary cultures of hepatocytes. In addition, vitellogenin, produced by the liver under the influence of E2 [25, 26], was also measured in some experiments for a comparative purpose.

EXPERIMENTAL

Animals

Rainbow trout were obtained from a fish farm and kept in a recirculating water system at 12–15°C under artificial light mimicking natural photoperiod.

Hormones

Recombinant trout GH (rtGH) was a gift from Eurogentec (Belgium), and recombinant human IGF_1 ($rhIGF_1$) was a gift from Ciba-Geigy (Switzerland). Unlabelled steroids were from Steraloids (U.S.A.), T3, T4 and cortisol were from Sigma (U.S.A.).

Steady state polyacrylamide gel electrophoresis (SS–PAGE)

SS–PAGE was performed as previously described [27]. Samples were preincubated with 2 nM [^{3}H]testosterone ([^{3}H]T). At the end of the electrophoretic migration in 6.5% acrylamide, 0.5% bis acrylamide gels containing 2 nM [^{3}H]T, the cylindrical gels were cut into 2 mm slices and the radioactivity of each slice counted.

Measurement of steroid binding protein

Steroid binding protein assays were performed as previously described [28, 29]. Aliquots (100 μl) of diluted blood plasma (1:150 in buffer A: 10 mM Tris–HCl, 1.5 mM EDTA, 12 mM monothioglycerol, 10% vol glycerol; pH 7.8) or undiluted culture medium were incubated for 30 min in glass assay tubes with 300 μl buffer A and 300 μl of DEAE bio gel (Biorad Richmond, U.S.A.) washed and diluted (1:2) in buffer A. After centrifugation, the supernatant was discarded and [^{3}H]T (generally 1 nM) with (NSB) or without (TB) an excess of unlabelled T was added to DEAE–protein complexes. Incubation was stopped after 2 h and DEAE binding-protein T complexes separated from free T by rapid filtration on Whatman GFA filters; the measurement of radioactivity on filters allowed the determination of total and non-specific binding of the [^{3}H]T.

IGF measurement

IGFs were measured in hepatocyte culture medium using a binding protein assay as described for human IGF measurement by Binoux *et al.* [30], and adapted for trout IGF by Niu *et al.* (unpublished data). Briefly, IGFs were separated from their binding proteins after dissociation in acidic conditions (NaCl 0.15 M,

Acetic acid 1 M, pH 3.3)—(Yield: 90%). Neutralized trout IGF samples were then incubated with an aliquot of a preparation enriched in trout IGF-binding-proteins, in competition with a tracer concentration of iodinated $rhIGF_1$ (^{125}I-$rhIGF_1$) in 200 mM Tris–HCl pH 9 and in the presence of 1% BSA (20 h at 4°C). At the end of incubation, bound and free ^{125}I-$rhIGF_1$ were separated by a charcoal treatment. Specific binding of ^{125}I-$rhIGF_1$ to the binding protein preparation measured for each sample was compared to a standard curve obtained with 0.1–25 ng of $rhIGF_1$. Serial dilutions of trout plasma provided a competition curve parallel to the standard curve. Human IGF_2 also cross reacts in this assay, thus the two growth factors cannot be differentiated by this means. However, pig and fish insulin, and several other peptidic hormones tested do not cross react in the IGF assay. The assay sensitivity was 0.4 ng/ml and the variation coefficients were as follows: intra assay: 13%; inter assay: 15%.

Vitellogenin ELISA

The wells of an ELISA plate were coated with purified vitellogenin (15 ng in 150 µl/well). After 5 washes, wells were treated with swine serum (2%) to reduce non-specific binding, washed again, and primary antiserum (rabbit antiserum raised against trout vitellogenin and diluted 1:100,000) and vitellogenin standards or samples were added to the wells (150 µl/well) and left to incubate for 16 h at room temperature. After 5 washes to eliminate the antiserum not fixed to the plate, the secondary antiserum (sheep anti-rabbit antiserum, 1:5000) was added for 2 h incubation at 37°C. Wells were washed with TBS, then binding of the second antibody was demonstrated with a peroxidase–anti-peroxidase complex (Dako; 1/5000) and revealed by a coloured reaction with *O*-phenylene-diamin (Sigma) in the presence of H_2O_2 (0.015%).

Serial dilutions of blood plasma or liver cytosol from an E2 treated male trout displaced the tracer in parallel to the standard curve while a control male trout serum or ram serum did not cross react in the assay. The lower limit of the assay was 2.5 ng/well (33 ng/ml) and the variation coefficients were as follows: inter assay: 9.8%, intra assay: 5.2%.

Oestradiol assay

Oestradiol was assayed as described by Fostier and Jalabert [31], by radioimmunoassay using rabbit anti-E2-17β-6-*O*-carboxymethoxyme-BSA as primary antibody, with a second incubation with 25% PEG for one night to precipitate the E2-antibody complexes.

Preparation of blood plasma

Blood samples were collected in heparinized tubes, from caudal vein on animals anesthesized with phenoxyethanol (3.5 ml/10 l). After centrifugation, (15 min, 4000 *g*), plasma samples were stored at −30°C.

Steroid removal

One volume of suspension of Dextran coated charcoal (1% charcoal 0.1% Dextran) was added to 9 vol of sample (blood plasma 1:10 in Tris buffer; undiluted or concentrated incubation media). Charcoal was eliminated by centrifugation (3700 *g*, 15 min, 4°C). Two successive charcoal treatments of 5 and 45 min were performed to allow efficient elimination of high concentrations of endogenous or exogenous steroids.

In vivo hormonal treatments

(a) Implantation of E2. Two groups of 16 immature trout were implanted with either 1.2 ml of cocoa butter alone (controls) or cocoa butter containing 5 mg E2/kg body weight (treated). Four animals from each group were sacrificed at various times after implantation and plasma levels of SBP, vitellogenin and E2 were determined. In addition, 4 untreated animals were sacrificed at the beginning of the experiment to establish initial levels of SBP, vitellogenine and E2.

(b) Injection of E2. Steroids were dissolved in 1 vol of ethanol then diluted with 9 vols of sterile 0.15 M NaCl. Adult males were injected intraperitoneally with the steroid suspension (0.5 and 1 mg E2/kg) or with only the ethanol–saline adjuvant at days 0 and 14 of the experiment. Blood was collected at different times between 0 and 21 days post-injection and assayed for E2 and SBP.

(c) Injection of testosterone and cortisol. Adult males were injected intraperitoneally with 1 mg/kg T, Col or with saline at days 0 and 21 of the experiment. Blood was collected and assayed for SBP at different times between 0 and 28 days.

In each experiment, all the fish were kept in the same tank and were fed throughout the experiment.

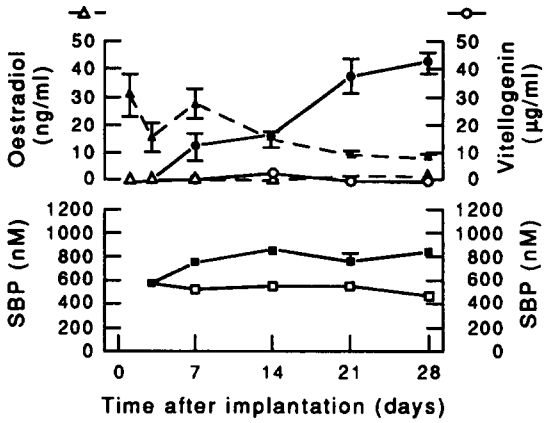

Fig. 1. SBP, vitellogenin and oestradiol concentrations in the blood of immature trout implanted with cocoa butter with the addition or not of E2 (5 mg/kg). Four animals from each group were sacrificed at different times after implantation. ○, △ and □: controls; ●, ▲ and ■: E2 implanted fish. Results are means ± SEM.

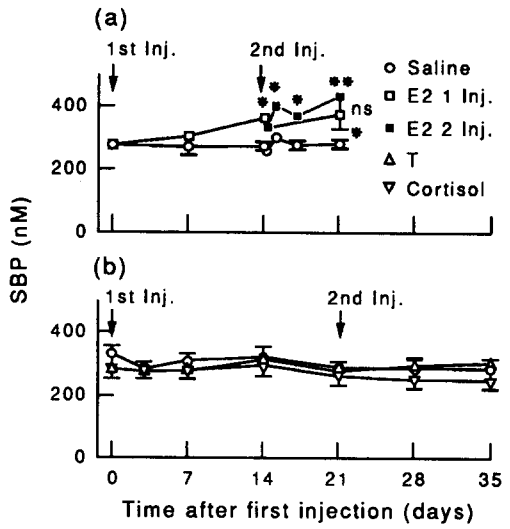

Fig. 2. Influence of steroid injection on SBP level in adult male trout in prespermiation stage. Arrows indicate the dates of injections. Results are means of five (a) or six (b) determinations ± SEM. (a) Injection of E2 or saline. The first E2 injection consisted of 0.5 mg/kg and the second one of 1 mg/kg. Full symbols indicates the values of SBP at days 14 and 21 in animals that were given 2 injections of E2. (b) Injection of T, cortisol or saline. Fish were given 1 mg/kg hormones at day 0 and 21 of the experiment.

Primary culture of trout hepatocytes

Hepatic cells were prepared from 300 to 800 g male trout, as previously described [32], with a perfusion of the liver for 30 min with 300 ml Hepes buffered saline containing 230 mg/l collagenase (Bohringer; Mannheim, Fed. Rep. Germany). The technique to prepare hepatocyte aggregates was first set up in the "Laboratoire de Biologie Moléculaire" in Rennes (France) by G. Flouriot (in preparation) and was adapted in our laboratory as follows: The cellular suspension obtained (1.5–2.10^6 cells/ml in Hepes buffered, pH 7.8, Dulbecco's modified Eagle's medium: DMEM, Sigma; 3 ml/well) was distributed into 6-well culture dishes (ϕ 3.5 cm). Plates were set on an orbital agitator (90 rev/min), at 18°C. After 5 days, aggregates of about 100 μm were formed, medium was renewed and hormones added to the wells. Following 48 h of stimulation, the media were collected, centrifuged (235 g, 10 min), aliquoted and frozen (−30°C). At the end of the experiment, aggregates from each well were collected and homogenized in 3 ml of buffer A. Homogenates were centrifuged at 2000 g (15 min) and 105,000 g (60 min) successively. Pellets were discarded and the resulting supernatant (cytosols) kept at −30°C until assayed.

In some experiments, cells aggregates were solubilized in NaOH (0.5 N) and, after dilution of NaOH, total cellular proteins were measured in a bicinchoninic (BCA) microassay (Pierce Rockford, U.S.A.).

RESULTS

In vivo hormonal supplementation

SBP, vitellogenin and E2 concentrations were measured in the plasma of immature trout at various times after their implantation with E2. 3 days after E2 implantation, SBP concentration remained at control values and plasma vitellogenin was not detectable whereas a large increase of blood E2 concentration was observed. At day 7 after implantation, SBP and vitellogenin concentrations were significantly increased while E2 remained high in blood of E2-implanted animals. At days 14, 21 and 28, SBP remained constant while vitellogenin kept increasing in the plasma of implanted fish. At the same time, E2 concentration fell progressively but remained significantly higher than in control animals until the end of the experiment (Fig. 1).

Implantation of immature trout with E2 induced a slow and delayed increase of SBP suggesting that a lag-time was necessary to increase the liver receptivity to E2 in male animals. We tried to get a faster response to the hormone by using two successive intraperitoneal injections. A first injection induced an increase of SBP concentration at day 7, this increase became statistically significant 14 days post injection. A second injection of E2 14 days

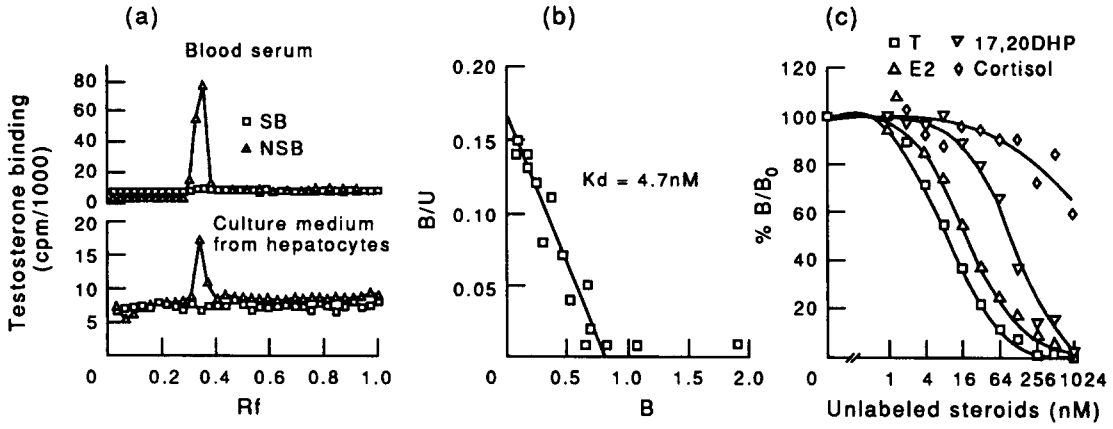

Fig. 3. Characterization of testosterone-binding in hepatocytes primary culture media. (a) Polyacrylamide gel electrophoresis of blood serum and culture media samples in gels containing 2 nM [^{3}H]T in the presence (non-specific binding: NSB) or not (specific binding: SB) of an excess of unlabelled T (800 nM). The rate front (R_f) of a slice is the ratio: (slice number)/(number of the slice containing bromophenol blue). (b) Scatchard plot from saturation analysis. Aliquots of culture medium were incubated with 1 nM [^{3}H]T plus increasing concentrations of unlabelled T. (c) Competition of various steroids with 1 nM [^{3}H]T for binding sites in concentrated culture media. Specific binding of [^{3}H]T in the absence of competitor is taken as 100%.

after the priming treatment, caused a steady increase in SBP concentration during the next 7 days while SBP levels remained constant in animals that had received only the first injection (Fig. 2a).

In a similar experiment, the effects of 2 successive injections of testosterone or cortisol was tested (Fig. 2b). No significative difference in SBP levels were observed in testosterone or cortisol injected fish compared to the controls.

Characterization of an SBP in trout liver cell culture medium

In order to demonstrate the similarity between the binding factor found in trout hepatocyte culture medium and blood SBP in the same species, partial characterization of its binding properties was undertaken on a pool of concentrated (×6) medium (Fig. 3a–c). Media obtained from repetitive 48 h incubations of liver cell aggregates in the presence of E2 (1 μM) were pooled and used after two successive dextran-charcoal strips.

Polyacrylamide gel electrophoresis at equilibrium (SS–PAGE), using [^{3}H]T in the presence or absence of a large excess of unlabelled T revealed a unique saturable testosterone binding site in trout hepatic cell culture media. In a 6.5% acrylamide gel, this binding factor had a mobility relative to bromophenol blue (R_f) of 0.44 (Fig. 3a). Under similar conditions, SBP from an adult male trout blood plasma migrated with the same R_f.

Further characterization of the binding was obtained by incubating samples with 1 nM of [^{3}H]T and increasing concentrations of unlabelled T (0–1024 nM). Scatchard plot analysis of saturation experiments revealed a unique class of high affinity binding sites ($K_d = 4.7$ nM; Fig. 3b).

Steroid hormone specificity of binding protein from hepatocyte culture media was determined by incubating samples with 1 nM of [^{3}H]T in competition with increasing concentrations of different unlabelled steroids (Fig. 3c). For each concentration of competitor, specific binding of tritiated steroid was determined and expressed as a percentage of specific binding in the absence of competitor (B/B_0 %). Apparent affinities obtained for the different competitors were in the following order: T > E2 > 17α,20βDHP. Cortisol was a poor competitor for [^{3}H]T binding. The association and dissociation kinetics were identical to those of blood SBP (data not shown).

All these characteristics were similar to those of blood SBP; so, in this report, binding protein measured in culture media from liver cells will be called SBP.

Time-course of SBP and vitellogenin productions by the liver cells under E2 and/or GH stimulation

In the first experiment (Fig. 4), after one 48 h period of stimulation, no effect of E2 (100 nM) or rtGH (1 μg/ml) on SBP production was recorded. At the end of a second period of stimulation (days 2–4), a moderate elevation of

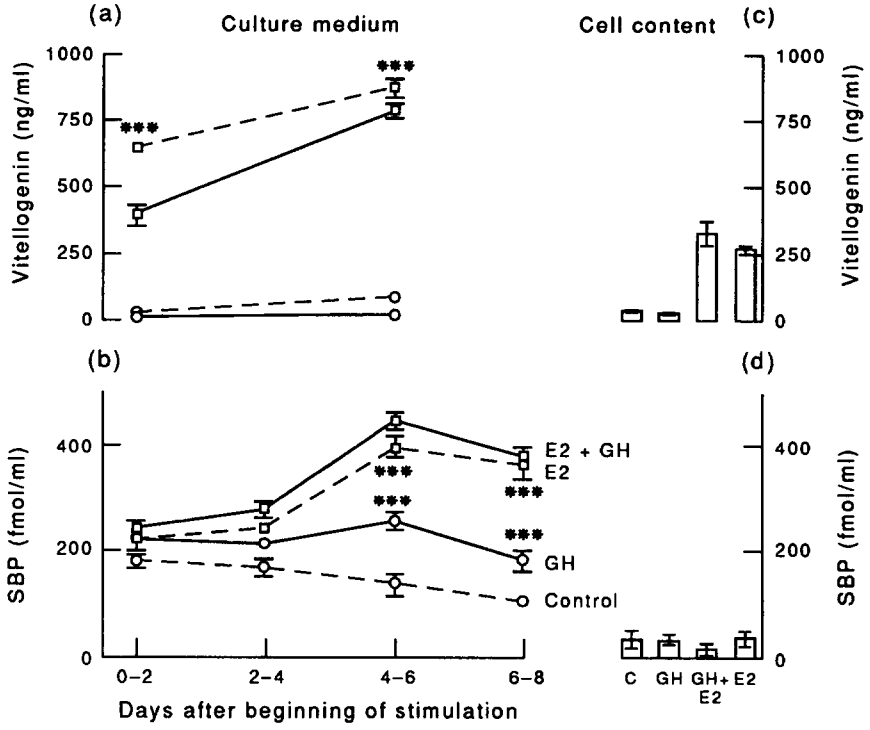

Fig. 4. Influence of E2 and GH on vitellogenin and SBP production in culture from trout hepatocytes. Results are means of 3 culture dishes ± SEM. □ and ○ respectively indicate E2 supplementation (100 nM) and absence of E2. — and - - - indicate respectively GH supplementation (1 μg/ml) and absence of GH in media. (a) Vitellogenin concentration in the media after the first and the third 48 h periods of hormonal stimulation. (b) SBP concentration in the media after each of the 4 consecutive 48 h hormonal stimulations. (c) and (d) Vitellogenin and SBP concentrations in cytosols prepared from the aggregates at the end of the 4th hormonal stimulation. ***$P < 0.001$, different from control value.

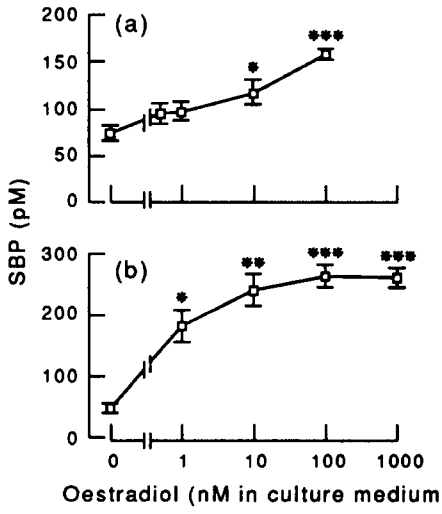

Fig. 5. (a) and (b). Two different experiments on the influence of increased concentrations of E2 on SBP accumulation in media from hepatocytes culture. E2 was added to the aggregates for three consecutive 48 h periods of hormonal stimulation. Figures represent SBP accumulation in media during the third period of incubation. Results are means ± SEM ($n = 3$). Asterisks indicate values significantly different from control values. *$P < 0.01$; **$P < 0.005$; ***$P < 0.001$—Student t-test.

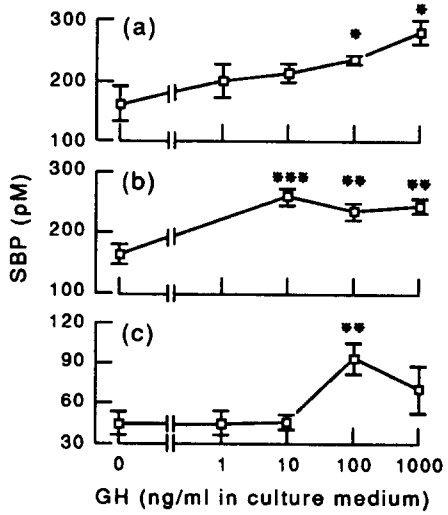

Fig. 6. Influence of increasing concentrations of recombinant trout GH on SBP level in the incubation media of three different hepatocyte cultures (a, b, c). Results are means ± SEM ($n = 3$ replicate dishes). (a) GH was added to the aggregates and media were collected 4 days later, without change of media. (b) GH was added to the aggregates and media were changed 4 days later for a second stimulation period of 4 days. Results are SBP concentration in media after this second incubation period. (c) Media were changed and GH was added to the aggregates every 48 h. (c) Presents SBP concentration in media at the end of the third period of stimulation (days 4–6).

SBP was noted with both hormones and after a third period of hormonal stimulation, SBP production was significantly increased in the presence of either rtGH (90% increase) or E2 (300% increase). Combined effects of the two hormones did not induce a significant increase over the E2 stimulated levels of SBP.

After a single 48 h period of stimulation, E2 (100 nM) induced a large production of vitellogenin by the hepatocytes, while vitellogenin was not detectable in control cultures. Presence of rtGH during E2 stimulation resulted in a lower production of vitellogenin. After the third incubation, the accumulation of vitellogenin in culture media was significantly higher compared with that measured after the first stimulation. The inhibitory effect of rtGH was no longer significant.

At the end of the experiment, T binding in cytosols was close to the limit of detection of the assay. Vitellogenin levels were also very low in cytosols of control or GH stimulated cells, but large amounts of vitellogenin were measured in cytosols from E2 stimulated cells (Fig. 4).

Dose–response of E2 stimulation of SBP production

In two different experiments (Fig. 5a, b), after repetitive incubations of hepatocyte aggregates in the presence of increasing concentrations of E2 (3 × 48 h incubation with change of culture medium and addition of hormone between each stimulation period), dose-related responses of SBP production were obtained during the third period of stimulation (days 4–6). The increase of SBP concentrations compared with those of controls were not always significant in the presence of 1 nM E2. However, 10 and 100 nM E2 were effective. In these two experiments, the increase of SBP concentration in media after the first or second incubations was not significant (results not shown).

GH stimulation of SBP production

The increase of SBP production after rtGH stimulation was recorded in three different experiments using different stimulation protocols (Fig. 6a–c):

(a) Continuous stimulation of hepatocytes with rtGH for 4 days (days 0–4 after the beginning of the experiment) caused a dose-related increase in SBP accumulation. The increase was significant ($P < 0.01$) in response to 100 and 1000 ng rtGH/ml.

(b) After a second period of stimulation (days 4–8 after the beginning of GH stimulation), a significant increase in SBP was obtained in response to 10, 100 and 1000 ng rtGH/ml. Differences between dishes supplemented with these rtGH concentrations were not significant.

(c) A significant 2-day accumulation of SBP in response to rtGH stimulation (days 4–6 after beginning of stimulation, with medium and hormone changes on days 2 and 4) was observed in dishes that received 100 ng rtGH/ml (in this experiment, little or no effect was observed on days 2–4).

In all the experiments, the magnitude of SBP accumulation due to GH stimulation was generally lower than that observed following E2 supplementation.

IGFs concentration in the culture media was also measured at the end of 2 or 4 days of GH stimulation (Fig. 7). After 2 days, a slight amount of IGF was measured in media, which tended to increase (not significantly) in the presence of increasing concentrations of GH. In the same media SBP concentrations were constant. However, after 4 days, IGF accumulation was clearly elevated; IGF production was then inversely correlated with the dose of GH added to the media whereas SBP concentration was increased as a function of growth hormone concentration.

Influence of other factors

The effect of rhIGF$_1$ and bovine insulin on SBP production was tested by addition of these hormones in the culture for three successive 48 h periods. During the first two 48 h stimulation, no effect of these hormones was observed while during the third period, 250 ng rhIGF$_1$/ml induced a significant increase of SBP accumulation per ml of incubation medium. IGF and insulin are believed to induce cell growth, so, results were corrected for total cellular protein content in cells and expressed per mg protein. In this way, we were able to demonstrate a non-significant decrease of SBP production in the presence of rhIGF$_1$ (250 ng/ml), and a significant decrease of SBP production in the presence of insulin (2000 ng/ml). Correction for protein concentration did not affect significantly the expression of the increase of SBP concentration in the presence of E2 or GH (Fig. 8).

Other hormones tested in culture: T3 (10–1000 nM), T4 (100 ng/ml), T (1–1000 nM), 17α,20βDHP (10–2000 nM), did not exhibit any

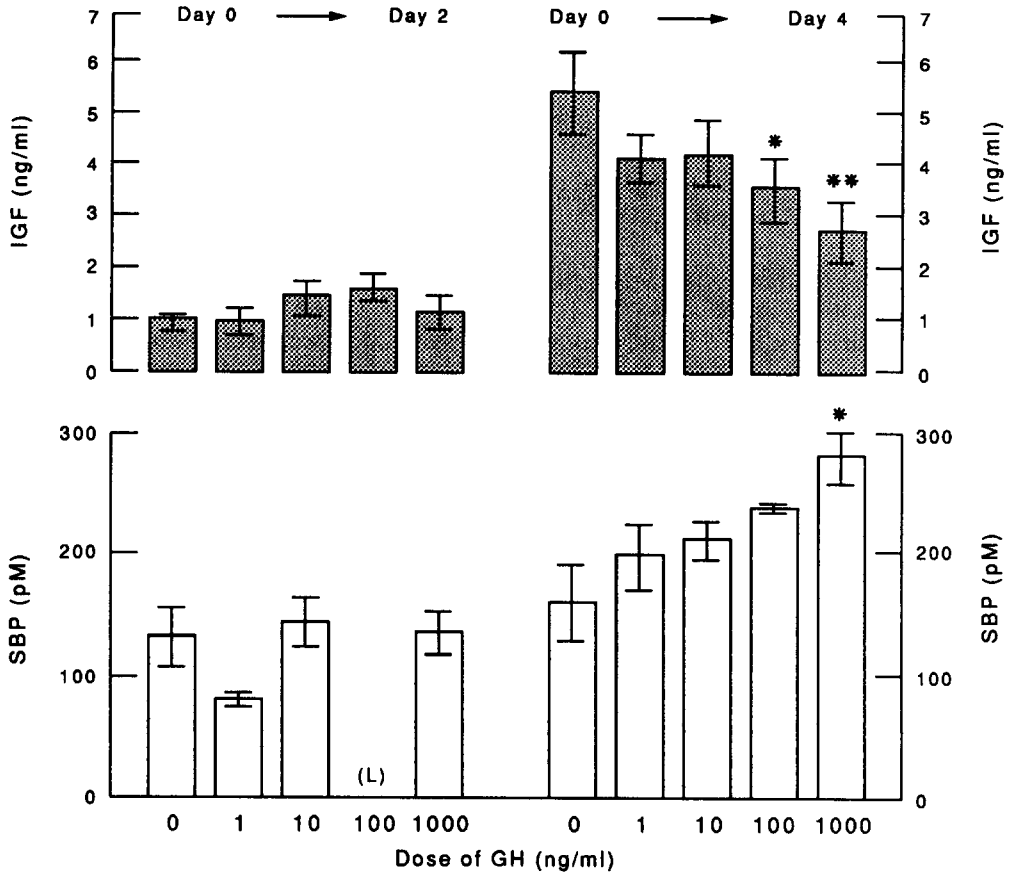

Fig. 7. SBP and IGF accumulated in hepatocyte culture media, between days 0 and 2 or days 0 and 4 after the beginning of stimulation with increasing concentrations of recombinant trout GH. Results are means of 3 determinants (±SEM). "L" indicates a lack of sample.

significant effect on SBP levels even after 3 repeated 48 h incubations (see Table 1).

DISCUSSION

We have shown that in trout, liver cells are the site of SBP production. To our knowledge, this is the first time that SBP has been studied in hepatocyte primary cultures. When trout hepatocytes were cultured using a monolayer technique, both the cell survival and response to hormones were highly variable (data not shown) and this is why the use of cultured aggregates was adopted.

Oestradiol, either *in vivo* or *in vitro*, induced a delayed and slow increase of SBP binding capacity in blood plasma and culture media, respectively. The amplitude of the response to high E2 concentrations was large *in vitro* (+150 to +300%) and in fact was considerably higher than responses previously obtained with similar E2 concentration on hepatoma cell lines of human origin (HepG2; 14, 15, 20). Furthermore, a trophic effect was also obtained *in vitro* with 1–10 nM E2, a concentration in the same order of magnitude as the circulating E2 levels in male salmonids (0.2–1 ng/ml), suggesting the physiological relevance of these results.

In contrast to a previous report on E2 regulation of vitellogenin in male trout [25], we found that a second injection of E2, 2 weeks after a priming treatment did not induce a greater or more immediate response to this hormone. *In vitro*, the vitellogenin response to E2 was larger and faster than the SBP response, suggesting that low E2 receptivity of male hepatocytes is not the only reason for the slow SBP response to E2. Such delays were also shown both *in vivo* in women, and *in vitro* using hepatomas from human origin [14, 33]. Whether E2 acts at the SBP gene expression level remains in question since Mercier-Bodard *et al.* [14] found a 50% stimulation of the specific SBP mRNA by E2 while, using similar hepatoma cell lines (HepG2), Hoop *et al.* [34] found no effect of the sex steroids. In the present report, a

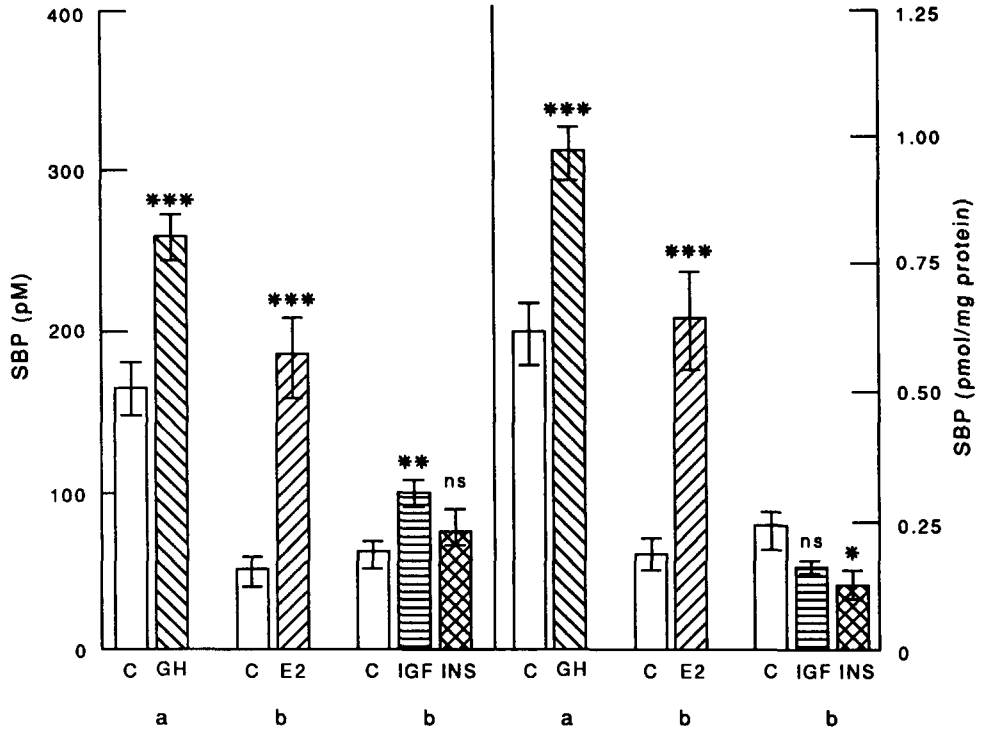

Fig. 8. Influence of E2 (100 nM), GH (1000 ng/ml), IGF (250 ng/ml) and insulin (2000 ng/ml) on SBP accumulation in culture media. (C: control). SBP concentration is expressed in pM (left side) or corrected for total cell protein content (right side of the figure). (a) Accumulation of SBP in culture media over a 4-day period (days 4–8 after the beginning of stimulation). (b) Accumulation of SBP in culture media over a 2-day period (days 4–6 after the beginning of stimulation). Results are means of 3 (E2 and GH) or 4 (IGF and insulin) determinations ± SEM.

late and slow response to E2 may reflect a mechanism different from the transcriptional mechanism involved in the response of vitellogenin to E2.

Testosterone administered under the same conditions as E2, had no effect, either *in vivo* or *in vitro*, on SBP levels. The fact that we used an aromatizable androgen for our study could have obscured the inhibitory effect of T. However, in our hands *in vivo*, large variations of 11KT, an active and non-aromatizable androgen, were not associated with modifications of SBP circulating levels. This absence of effect of T supplementation (this study) or of castration [24] on SBP is in contradiction with the inhibitory influence generally attributed to androgens in physiological or clinical studies on human [10, 12, 35]. However, Mercier-Bodard *et al.* [36] and Hoop *et al.* [34] respectively found a very moderate and non-inhibitory effect of androgens on SBP mRNA. Furthermore, the pubertal decline of SBP is now considered by certain authors to be independent of the androgen rise that occurs at puberty [37, 38].

The absence of a T3 effect is more surprising since in cultured hepatoma cells T3 induces an increase of SBP production greater than that obtained with E2 [14]. In primate, in hyperthyroidism or after thyroid hormone treatment a large increase of SBP has been reported [39].

Table 1. Effect of different hormones on SBP production by trout hepatic cells in culture

Hormones tested	Dose	SBP (% of control)	Significance
Oestradiol	100 nM	250–400	$P < 0.001$
Testosterone	1000 nM[a]	98	NS
17α,20β-DHP	2000 nM[a]	100	NS
Cortisol	1000 nM[a]	100	NS
GH	100 nM	150–220	$P < 0.001$
IGF_1	250 ng/ml[a]	75*	NS
Insulin	2000 ng/ml[a]	55*	$P < 0.01$
T3	100 nM	100	NS
T4	100 nM	100	NS

[a]The maximum hormone concentration tested is indicated.
*SBP from data expressed in pmol/mg total cellular protein at the end of the experiment.

In contrast, decreased thyroid hormone concentrations, in hypothyroidism [2, 12, 16] or during fasting [40], are not related to an important decrease of SBP, suggesting that the role of T3/T4 in the regulation of SBP may be modulatory only in certain circumstances.

Our previous results obtained *in vivo* (after hypophysectomy, during fasting or spermiation and after Gh injections) [24] suggested that GH might be involved in SBP regulation and that, in several circumstances, decreased levels of SBP were related to low levels of circulating IGF and/or to impaired liver GH receptivity. The present study demonstrates that, *in vitro*, GH has a trophic effect on SBP production. In some experiments, a significant effect was obtained with GH concentrations as low as 10 ng/ml, which is consistent with circulating levels of GH in trout [41]. This GH action, moderate and slow to develop, seems to maintain SBP production over culture duration. No synergy was found between GH and E2 *in vitro*. Together with our previous *in vivo* data, these results suggest that GH plays an important role in SBP basal production by the liver.

The administration of GH to children with growth hormone deficiency induces a decrease of SBP concentration inversely correlated with an increase of IGF_1 level [42]. Furthermore, in short-term fasting or reduced diet in human, SBP concentration increases whereas IGF_1 concentration decreases in blood plasma [43]. In two preliminary studies, we attempted to find whether IGF was involved in the hepatic response to GH. We found that long-term GH stimulation results in an SBP increase related to a decrease of IGF accumulation in the media.

Our assay for insulin growth factors measures both hIGF1 and hIGF2 and we cannot exclude that the variation observed is the result of 2 different effects on the 2 factors in trout. However, a biphasic effect of GH on IGF1 has been described previously in rat, involving a transient short-term (2 h) elevation of IGF_1 mRNA levels followed by an inhibition of IGF_1 mRNA levels in liver cells after GH treatment [44].

Furthermore, in this study, a treatment with $rhIGF_1$ was found to be inhibitory when the results were expressed per mg total cellular protein. Therefore, these preliminary *in vitro* results are in accordance with the recent data of Hoop *et al.* [34] demonstrating an inhibitory action of IGF_1 (1 nM) on SBP protein secretion and on specific SBP mRNA (relative to internal control of mRNA) in hepatoma cell (HepG2) cultures.

The action of a high concentration of insulin on SBP production by trout hepatocyte primary culture was inhibitory as was previously found in hepatoma cells in culture [20]. Whether the effect obtained with high insulin concentrations is a true insulin effect or due to an interaction with IGF receptor is not known.

In conclusion, the liver appears to be the production site for SBP in trout and while androgens do not seem to influence this production, oestrogens are important potential regulators. Different experimental conditions reveal that GH also plays a role in SBP regulation. The importance of insulin/IGF family factors is suggested but remains to be clarified.

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