Determination of circulating maturational gonadotropin in rainbow trout (*Oncorhynchus mykiss*) using a heterologous radioimmunoassay

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A radioimmunoassay (RIA) for the measurement of maturational gonadotropin (GtH II) in the plasma of rainbow trout was developed using a rabbit antiserum to b subunit of chum salmon GtH II. Intact GtH II was used as standard and radioactive competitor. RIA was performed using a double-antibody method under non-equilibrium conditions, and was used to evaluate plasma GtH II release stimulated by a gonadotropin releasing-hormone (GnRH) analogue: des-Gly-GnRH (1-9) ethylamide. The GnRH analogue was administrated during 6 days either orally or parenterally (one single ip injection or two ip injections with a 6-day interval). Plasma concentrations of GtH II in control (without hormone induction) and with oral GnRH were low (less than 2 ng/ml) in spite of a higher ovulatory rate. In contrast, both injections of GnRH (15 μg/kg body weight) induced production and secretion of GtH II (16.6 ± 9.06 ng/ml). These data suggest that GnRH injection, but not oral administration, is an appropriate method to induce GtH II and higher ovulation rates in rainbow trout. The heterologous RIA was effective for detecting plasma GtH II from rainbow trout at concentrations higher than 2 ng/ml.

Key terms: *GnRH, gonadotropin releasing-hormone, gonadotropins, GtH II,* ***Oncorhynchus mykiss****, radioimmunoassay, rainbow trout.*

INTRODUCTION

Control of gonadal function by two pituitary gonadotropins (GtHs) is a generalized feature of vertebrate reproduction (Idler and Ng, 1983). GtHs are released in response to gonadotropin releasing-hormone (GnRH), a decapeptide produced by the hypothalamus which stimulates the release of gonadotropins from the pituitary glands of diverse vertebrate species (McKenzie *et al*, 1984).

Both gonadotropins consist of two non-covalently bound, chemically distinct subunits, designated a and b. In mammals, the a subunit is common among gonadotropins within a species, whereas the b subunit is distinct for each hormone.

Whether fish reproduction is regulated by one or two pituitary GtHs has been controversial for two decades (Kawauchi *et al*, 1989). Recently, two distinct forms of GtH have been isolated and characterized (Suzuki *et al*, 1988 a). GtH I is the predominant form

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both in the pituitary and plasma of salmon during vitellogenesis and spermatogenesis, whereas GtH II levels exceed GtH I at the times of ovulation and sperm release (Nozaki *et al*, 1990). GtH I and GtH II secretion varies during reproductive development (Fontaine and Dufour, 1987). Comparative studies have revealed that two GtHs, which are homologous to LH and FSH, exist in most species of tetrapods (Licht *et al*, 1977).

The use of GnRH to induce ovulation through an increase of plasma GtH has been shown to be an effective method to synchronize final stages of oocyte maturation in salmonid fish farming (Breton *et al*, 1990). However, the conditions for hormone administration, and the effectiveness of GtH stimulation and ovulation induction are still under study.

In the present study we describe a heterologous radioimmunoassay (RIA) for rainbow trout (*Oncorhynchus mykiss*) GtH II using antibodies raised against chum salmon (*Oncorhynchus keta*). The structure of GtH II purified from salmon chum is similar to that of rainbow trout GtH II. Therefore, chum salmon GtH II was used as the tracer and standard in the RIA. Using the heterologous RIA we determined plasma GtH II levels in preovulatory female rainbow trout treated with a GnRH analogue. One group received an oral administration of the hormone, and the other group received two injections of the same GnRH analogue with a six-day interval. Results demonstrate that oral GnRH administration failed to stimulate GtH II release from pituitary; on the contrary, increased plasma GtH II levels were detected after parenteral GnRH administration. The use of salmon chum GtH II antibodies were effective for rainbow trout GtH II detection.

MATERIALS AND METHODS

Reagents

GtH II and rabbit antisera against chum salmon GtH II b were a kind gift of Dr H Kawauchi, Kitasato University, Japan. The purification protocols for GtH II b subunit and immunization for production of rabbit anti chum salmon GtH II were as reported elsewhere (Suzuki *et al*, 1988b). The GnRH analogue used was D-ala, des-Gly10-GnRH (1-9) ethylamide (GnRHa D-Ala) (Syndel Laboratories, Canada).

Iodination of GtH II

Iodination of GtH II was carried out according to the method of Salacinski *et al* (1981) using Iodogen (1,3,4,6-tetrachloro-3a, 6a-diphenylglycouril, Pierce Chemical Co.). GtH II (2.5 µg) dissolved in 50 µl of 50 mM sodium phosphate buffer, pH 7.4 and 0.2 mCi of carrier free ^{125}INa in 2 µl of NaOH (C.CH.E.N.) were mixed in a polypropylene tube coated with 24 µg of Iodogen. After 10 min of incubation at 0 °C, the reaction was stopped by removing the reaction mixture into a vial containing 150 µl cold 10 mM Tris-HCl buffer, pH 7.5 containing 0.1% bovine serum albumin (BSA) and 0.1% NaN_3 (Tris-BSA buffer) (Kanamori *et al*, 1987). Unreacted iodine was separated from the labeled hormone by gel filtration on a Sephadex G-50 column (20 x 0.6 cm), which had previously been saturated with Tris-BSA buffer. The iodination reactants were eluted with Tris-BSA buffer. Fractions (1 ml) were collected, and 5 µl of each eluted fraction was counted in a gamma counter. Radioactive peaks were assayed for determination of immunoprecipitable material. Fractions containing radioiodinated protein were combined and stored frozen at -20 °C until radioimmunoassay. Using the iodination procedure GtH II was labeled to a specific activity of 16.1 µCi/mg.

Radioimmunoassay

GtH II RIA was performed using a double-antibody method under non-equilibrium conditions. The assay buffer was 0.01 M phosphate buffer, pH 7.2, containing 0.14 M NaCl (PBS), 1% bovine serum albumin (BSA), 0.1% Triton X-100, and 0.1% NaN_3. Standards and plasma samples were serially diluted in assay buffer. 50 µl of each plasma sample were added to each tube and diluted to a final volume of 100 µl/tube. Samples were assayed in duplicate. Anti GtH II b antisera was diluted 1:8000 with assay buffer, containing 0.05 M EDTA (100 µl/

tube). After incubation for 12 h at 4 °C, 100 µl of iodinated GtH II (20,000 cpm) was added to each tube and incubated for 12 h at 4 °C. The antibody-bound hormone was precipitated by addition of 200 µl of goat anti-rabbit gamma-globulin (diluted 1:30 in assay buffer containing 0.05 M EDTA, 1% normal rabbit serum and 10% polyethylene glycol 6000). After incubation for 2 h at room temperature, the tubes were centrifuged for 60 min at 4,000 g and 4 °C. The supernatant was aspirated and radioactivity in the precipitated bound fraction was determined in a gamma counter. The standard curve included tubes containing 0.19, 0.39, 0.78, 1.56 and 3.13 ng /tube.

Animals

Female rainbow trout were selected from the hatchery at Río Blanco (Catholic University, Valparaíso) located in Río Blanco (32 54' Lat. S; 70 18' Long. W), 1,500 m overseas, V Region of Chile. Seventy one fish (6 years-old) in their third ovulatory period were divided into two groups according to the form of GnRH analogue administration. Oral (Group 1) and intraperitoneal (Group 2) hormone administration was used to induce ovulation. The selected animals were maintained in flow-through stock two weeks before the induction. The mean temperature during experimental conditions was 6 ± 1 °C. Animals were anesthetized by immersion in 0.05% tricaine methanesulphonate (MS-222) during 3 minutes followed by the appropriate treatment. Group 1 (n=25) received the hormone mixed in the food in a concentration of 1.25 mg/kg food. The hormone was diluted in 0.9% saline and incorporated to cold food extended in a monolayer by atomization. Each fish received a mean dose of 10 µg hormone/kg body weight/day during five days. Control fish (n=25) animals received the same standard diet but without the hormone. Group 2 (n=50) was injected with two intraperitoneal injections of the GnRH analogue (15 µg/kg body weight) with an interval of 6 days. Controls (n=21) received 0.9% saline instead of the hormone. Plasma samples from 9 randomly selected animals of each group (group 1 and 2 and their respective controls) were obtained from the caudal peduncle 6 or 9 h after the administration of the hormone for group 2 and 1, respectively. Samples were frozen until hormone determination by RIA.

RESULTS

Two peaks were obtained from gel filtration on Sephadex G-50: one containing unreacted iodine, and one with the labeled GtH II (Fig. 1). Aliquots of peak fractions containing

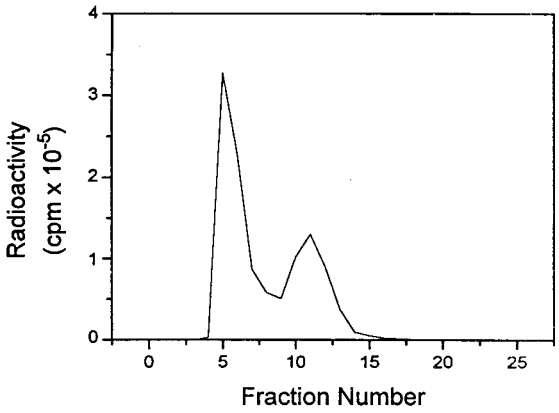

Fig 1. Chromatographic separation of labeled ^{125}I-GtH II on Sephadex G-50. GtH II iodinated with IODOGEN as described in Methods. Radioactive peaks represent iodinated GtH II, and free iodine. Radioactivity expressed as counts per minute (cpm) in 5 ml from each eluted fraction.

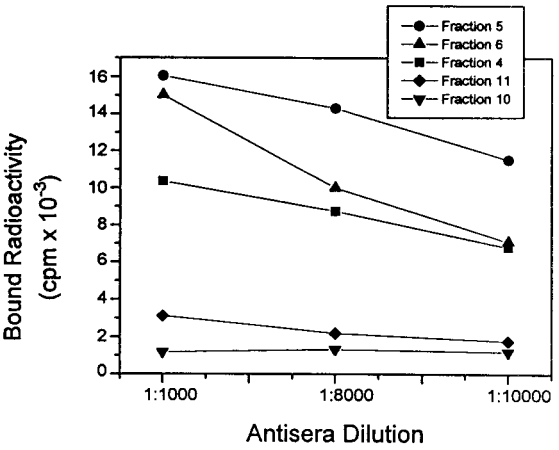

Fig 2. Specific binding of fractions (1 ml) obtained from chromatographic separation of iodinated GtH II. Radioactivity in each fraction was immunoprecipitated with serial dilutions of the rabbit anti chum salmon immunoglobulin. Fraction 5 selected for use in sequential determinations of plasma GtH II levels. Bound radioactivity expressed in counts per minute (cpm) in each assay tube.

20,000 cpm were incubated with different dilutions of the antisera as described in methods. Bound radioactivity is depicted in Fig. 2 showing that the labeled hormone was present in the first peak (fraction 5). Successive experiments were performed with aliquots of ^{125}I-GtH II contained in fraction 5 diluted 1:8000.

The RIA competitive inhibition curves were plotted as percent bound (logit scale) versus hormone concentration (ng/tube) (log scale) (Fig. 3) Using GtH II extracted from salmon chum pituitaries as standard and tracer, the double-antibody immunoassay had a minimum detection limit of 97 pg/tube at 84% binding, and an effective dose (ED_{50}) of 390 pg. There was a linear displacement between 0.097 and 1.56 ng/tube (Fig. 3).

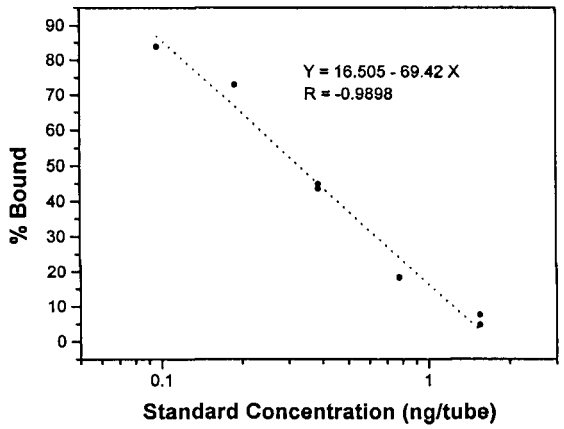

Fig 3. Competitive inhibition curve for GtH II using rabbit anti chum salmon immunoglobulin. Standard GtH II concentrations were 0.19, 0.39, 0.78, 1.56 and 3.13 ng /tube. Values plotted are % Bound *vs* Standard concentration (log scale).

With the purpose of detecting the circulating GtH secreted in response to oral and injected GnRH, plasma from the two experimental groups were collected. Plasma GtH II concentration from both treated groups and their respective controls are shown in Table I. Oral administration failed to increase GtH II secretion from pituitary over the control group (Table I).

In the control population, circulating GtH II levels were not detected by our RIA; only one animal showed circulating GtH II concentrations within the detection range (2.52 ng/ml) (Tables I and II). Injection of

TABLE I

Circulating GtH II concentrations in rainbow trout after oral administration, or single injection (ip) of the GnRH analogue GnRHa D-Ala

Sample Number	GtH II (ng/ml)		
	Control	Oral	Injected
1	ND	ND	12.10
2	ND	ND	10.84
3	ND	ND	ND
4	ND	ND	16.02
5	ND	ND	20.80
6	ND	ND	11.34
7	ND	ND	ND
8	ND	ND	ND
9	ND	ND	17.00

Plasma GtH II concentrations determined by a heterologous RIA. Oral administration: 10 mg/kg BW/day during 5 days. Single injection: 15 mg/kg BW. Plasma samples extracted 9 h after final oral administration, or 6 h after injection of GnRH analogue.

ND = non detectable.

TABLE II

Circulating GtH II concentrations in rainbow trout after two injections (ip) of the GnRH analogue GnRHa D-Ala

Sample Number	GtH II (ng/ml)	
	Control	Injected
1	ND	22.00
2	ND	13.32
3	2.52	18.90
4	ND	17.18
5	ND	16.46
6	ND	20.40
7	ND	12.94
8	ND	13.42
9	ND	14.78

Plasma GtH II concentrations determined by a heterologous RIA. Injections of GnRH analogue 15 mg/kg BW, with 6-day interval between injections. Plasma samples extracted 6 h after the injection of GnRH analogue.

ND = non detectable.

the GnRH analogue induced a significant increase in circulating GtH II (Table I). Although the first injection failed to increase the GtH II levels in three animals (Table I), the second injection of the hormone stimulated GtH II secretion in all of the animals studied (Table II). GtH II production was in a range between 12 and 22 ng/ml with a mean of 16.6 ± 9.06 ng/ml.

In addition to detection of plasma GtH II, female fish were examined by abdominal palpation every 3 days to detect ovulation (data not shown). It is noteworthy that although oral administration of the hormone failed to increase GtH II secretion, induction of ovulation in the same group was significantly higher than in control groups. The administration of GnRH analogue by parenteral injection significantly increased plasma GtH II concentration, and all stimulated animals showed a high rate of ovulation necessary for the fertilization process.

DISCUSSION

During the last decade, attempts have been made to use GnRH to induce and synchronize spawning in various farmed fish species (Van Der Kraak *et al*, 1987; Weil *et al*, 1992). Gonadal ovulation is activated by the GtH secreted from the pituitary stimulated by GnRH. LHRH or its analogues are being used for oral delivery instead of GtH in species which have a stomach, because the presence of a gastric phase of digestion severely limits the uptake of peptides by the fish gut (McLean and Donaldson, 1990; Suzuki *et al*, 1988c). Recently, Thomas and Boyd (1989) and McLean at al (1991) reported that the administration of synthetic LHRH and a GnRH analogue respectively in the diet (oral administration), induced ovulation in two different teleost species. These authors also reported that oral delivery of LHRH or an analogue to 17b-oestradiol-primed coho salmon (*Oncorhynchus kisutch*) resulted in a significant release of GtH II. In our studies, oral administration of the GnRH analog was ineffective in increasing circulating GtH II levels in *Oncorhynchus mykiss*, but had a minor positive effect in increasing ovulatory rate (data not shown) These differences could be explained by larger doses of LHRH analogue used in the earlier studies (20 µg/ g body weight) and a different delivery route for the hormone preparations (intubation). The slight increase in ovulatory rates without changes in circulating GtH II levels could be explained by the fact that the blood samples for GtH II screening were taken 9 h after the final feeding period. Donaldson and Hunter (1983) demonstrated that plasma gonadotropin levels decreased to basal levels after 24 h of a single injection with a dosage of LHRH that was 6-fold higher than that used in our studies. Therefore it is probable that by the time that we collected the blood samples, GtH II levels were already down to baseline level. However, the GnRH analog could have positively induced ovulation by increasing GtH II at times prior to collection of the samples, indicating that peptide was partially absorbed at the intestinal level.

Circulating GtH II levels in control fish were below the detection limit of our RIA. Different authors have detected less than 2 ng/ml of plasma GtH concentrations without hormone induction (Breton *et al*, 1990; Crim *et al*, 1988) or below 5 ng/ml (Salbert *et al*, 1990). Our findings confirm this previous data showing the low levels of circulating GtH II in preovulatory female fish.

After two injections of the GnRH analog there was a marked increase in GtH II levels in all animals screened. These results are similar to those reported by Salbert *et al* (1990) who detected 25 ± 3 ng/ml in similar reproductive periods of rainbow trout, but using higher doses of LHRH (20 µg/kg body weight of [D-Trp^6] LHRH). Crim *et al* (1988) detected 14 ± 4 ng/ml after 5 h of an injection of 100 µg/kg of the same GnRH analogue. In coho salmon (*Oncorhynchus kisutch*), a single injection of different GnRH analogues induced a concentration between 14.2 ± 0.9 and 21.3 ± 1.5 ng/ml of plasma gonadotropin levels (Van Der Kraak *et al*, 1988). The mean GtH II levels after the first injection was not different than that observed after a second injection with the analog. After the first injection with GnRH three animals failed to show an increase in GtH II levels whereas after the second injection all

animals showed an increase in GtH II levels. An explanation for this difference raises from the possibility that LHRH increases the responsiveness of the pituitary gland to itself, similar to the priming effect of LHRH observed in mammals (Aiyer *et al* 1974).

The heterologous RIA used in these experiments allowed the detection of plasma GtH II concentration in rainbow trout at concentrations above 2 ng/ml. This could be a useful method to detect the peak of GtH II that precedes ovulation and become a common necessary practice for increasing productivity in fish farming.

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