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GnRH Analog Stimulates Gonadotropin II Gene Expression in Maturing Sockeye Salmon

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ABSTRACT—Our previous study suggested that, in the pituitaries of pre-spawning chum salmon, salmon gonadotropin-releasing hormone (sGnRH) stimulates expression of genes for gonadotropin (GTH) IIβ but not for Iβ, since the levels of mRNAs encoding sGnRH and GTH II but not I were increased during the final stages of spawning migration. In the present study, a capsule of GnRH analog (GnRHa) was implanted into the dorsal muscle of maturing sockeye salmon to clarify function of GnRH on expression of GTH subunit genes in pre-spawning homing salmonids. The amounts of GTH subunit mRNAs in the individual pituitaries were analyzed by a quantitative dot blot analysis using single-stranded sense DNA as the standard. The levels of GTH α and IIβ mRNAs in the GnRHa-implanted fish were significantly higher than those in the control fish in both the males and females, whereas the levels of GTH Iβ mRNA did not show any significant differences in both sexes. These results indicate that GnRH elevates expression of GTH subunit genes which encode the components of GTH II, α and IIβ chains, in the pituitary of maturing sockeye salmon, and then accelerates final maturation.

INTRODUCTION

The presence of two gonadotropins (GTH) in teleosts, namely GTH I and II, was well established by Kawauchi *et al.* (1989). GTH I is considered as vitellogenic, and GTH II as maturational. They are composed of common α and unique β subunits. Complete amino acid sequences of GTH α (Itoh *et al.*, 1990) and β subunits (Itoh *et al.*, 1988) were first determined in chum salmon. Further, complete nucleotide sequences of cDNAs encoding GTH subunits were determined in several salmonids (Trinh *et al.*, 1986; Sekine *et al.*, 1989; Gen *et al.*, 1993; Kato *et al.*, 1993; Suzuki *et al.*, 1995). Because a certain sequence of the GTH subunit mRNA conserves high similarity (about 95%) with their homologues in salmonids, a cDNA probe designed for particular subunit mRNA in one species can be used as a hybridization probe in the pituitaries of various salmonid species (see Hiraoka *et al.*, 1993).

Secretion of GTH is mainly controlled by gonadotropin-releasing hormone (GnRH), which is involved in the control of reproduction in a wide range of vertebrate species including teleosts (see Sherwood *et al.*, 1993). In salmonids, salmon GnRH (sGnRH) functions as reproductive GnRH despite the presence of two GnRH variants, sGnRH and chicken GnRH-II (Amano *et al.*, 1997; Kobayashi *et al.*, 1997). sGnRH neurons in the ventral telencephalon (VT) and the preoptic area (POA) which send their axons to the pars distalis may control pituitary GTH cells to release GTH into circulation. Recent studies in masu salmon indicated that expression of sGnRH gene in these neurons seemed to correlate well with sexual maturation (Amano *et al.*, 1995a, b). Further, these VT and POA sGnRH neurons in chum salmon showed increase in the levels of sGnRH mRNA and also the number of hybridization positive cells during the final stages of spawning migration (Kudo *et al.*, 1996). Arising question here was whether GTH gene expression is controlled by sGnRH in pre-spawning salmon.

In our previous study, we first developed a quantitative dot blot analysis by which the levels of GTH subunit mRNAs in the salmonid pituitary were determined by use of single-stranded sense DNA (ssDNA) encoding the same mRNA sequence. Utilizing this method, we found that the levels of GTH α and IIβ mRNAs in freshwater chum salmon ready to spawn

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were higher than those in coastal seawater animals, however such difference was not seen in the level of GTH Iβ mRNA (Kitahashi *et al.*, 1997). Since, as mentioned above, the number of hybridization positive sGnRH neurons in the VT and POA increased in pre-spawning chum salmon, it is highly probable that sGnRH stimulates expression of genes encoding GTH α and IIβ but not Iβ, and promotes final sexual maturation in homing salmonids. In the present study, we examined whether this hypothesis is true by implantation of GnRH analog (GnRHa) into the dorsal muscle of maturing sockeye salmon, *Oncorhynchus nerka*. Because of difficulty in obtaining chum salmon at appropriate stages of sexual maturation, we used freshwater sockeye salmon which were maintained for artificial fertilization.

MATERIALS AND METHODS

Fish

Maturing sockeye salmon were caught in the Bibi river upstream to Lake Utonai, Hokkaido, Japan, in the end of June 1996, and were kept in a holding pond in National Salmon Resources Center (Chitose, Hokkaido) under natural photoperiod condition. Fish were tagged by an intraperitoneal pit tag to be discriminated individually. On September 6, experimental animals were randomly selected, anesthetized with 0.05% ethyl m-aminobenzoate methanesulfonate (MS222, Nakalai tesque, Kyoto, Japan) buffered with sodium bicarbonate, and were measured of body weight and fork length. Body weights were about 1.88 kg in the males and 1.18 kg in the females, while fork length were about 55.0 cm in the males and 49.5 cm in the females. Blood samples (1 ml) were collected from the caudal vein for radioimmunoassay of plasma steroid hormones the result of which will be published elsewhere with the observation on improvement of gonadal fecundity (Fukaya *et al.*, in preparation). Afterward, fish received in the dorsal muscle a 2 mm implant capsule containing 75 or 150 μg of GnRHa, [Des-Gly^{10}, D-Ala^{6}, Pro^{9}]-GnRH ethyl amide in an ethylene vinyl acetate copolymer matrix (Zohar *et al.*, 1990; Zohar, 1996). The dose of implanted GnRHa was determined by consulting the previous papers (Zohar *et al.*, 1990). Sham operated animals served as controls. Control and GnRHa-implanted fish were sampled three weeks after the implantation. Those fish sampled on the day of implantation were assigned as initial controls. When fish were sampled, gonads were taken out and measured of their weights to calculate gonadosomatic index (GSI). The GSI and the number of animals in each group are shown in Table 1.

Table 1. Changes in gonadosomatic index in the initial, control and GnRHa-implanted sockeye salmon

	Male	n	Female	n
Initial	1.94 ± 0.11	3	7.81 ± 0.55	6
Control	1.22 ± 0.24*	5	13.39 ± 0.28**	4
75 μg GnRH	not tested		12.40 ± 0.46**	5
150 μg GnRH	0.85 ± 0.13**	5	11.94 ± 0.83**	3

Mean ± SEM.
Differences to initial: *, $P < 0.05$; **, $P < 0.01$; by the *t*-test.

Tissue preparation

The pituitaries were taken out upon decapitation, frozen in liquid nitrogen and stored at –80°C. Total RNA was extracted from single pituitaries by the acid guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987). The pituitary weight was about 20 mg and the amount of total RNA extracted from a single pituitary was 50-100 μg.

Assay method

ssDNAs which have the same sequences of mRNAs encoding GTH α, Iβ and IIβ were synthesized by use of a polymerase chain reaction (PCR) method with a single common sense primer. Template DNA for PCR was prepared by the restriction enzyme digestion at one site of plasmid DNA containing cDNA insert for the salmon GTH subunit (Sekine *et al.*, 1989). The GTH α cDNA used in the present study is a cDNA encoding α2 subunit, which is commonly obtained from GTH I and II according to Itoh's designation (Itoh *et al.*, 1990). Synthetic 20mer oligonucleotide, 5'-GTTACTTCTGCTCTAAAAGC-3', was used as the PCR primer. The PCR mixture contained 1.6 pmol template DNA described above, PCR buffer (50 mM KCl, 1.5 mM $MgCl_2$ and 10 mM Tris-HCl, pH 8.3), 200 μM dNTPs, 1.0 μM primer, 1 unit *Taq* DNA polymerase (TaKaRa) and sterile distilled water to 50 μl, overlaid with 30 μl light mineral oil. The profile of amplification was: 30 cycles of incubation at 93°C for 1.5 min, at 53°C for 1.5 min and 72°C for 2 min after denaturation at 95°C for 4 min. Amplified DNA was subjected to low melting temperature agarose gel electrophoresis to separate the band that contained ssDNA. Afterward, ssDNA was purified by phenol extraction. We obtained ssDNAs for GTH α, Iβ and IIβ whose lengths were about 780, 790 and 840 bases, respectively.

Labeled cDNA probes which were specific to particular GTH subunit mRNAs were prepared by a primer extension method using Megaprime DNA labeling system (Amersham) and [α-^{32}P]dCTP (Amersham) with synthetic oligonucleotide primers. They are about 200 bases long and specific to corresponding standard DNAs. The sequences used as cDNA probes were carefully selected to avoid cross-hybridization.

Total RNA extracted from the pituitaries was blotted to Hybond™-N^{+} membranes (Amersham) using a MilliBlot™-D (Millipore) in duplicate. As the standard to determine the amount of mRNA, serially diluted ssDNA (1-333 amol) were blotted to each membrane in triplicate.

Hybridization with the labeled probe was performed in a solution containing 5 × SSPE (0.05 M phosphate buffer, pH approx. 7.4, containing 0.76 M NaCl and 5 mM EDTA), 5 × Denhardt's solution (0.1% solution of bovine serum albumin, Ficoll and polyvinylpyrrolidone), 100 μg/ml denatured calf thymus DNA (Sigma) and 1.5-2 × 10^{6} cpm of labeled cDNA probe/ml hybridization buffer at 65°C for 20 hr. The membranes were then washed with 2 × SSPE/0.1% SDS at room temperature (RT) for 15 min, twice with 2 × SSPE/0.1% SDS at 65°C for 30 min, and twice with 0.1 × SSPE/0.1% SDS at 65°C for 30 min. The membranes were then exposed to a Fuji imaging plate (Fuji Photo Film Co., Ltd.) for 24-48 hr. Radioactivity expressed as the intensity of photostimulated luminescence (PSL) was analyzed by a Bioimaging analyzer (Fuji Photo Film Co., Ltd.), and the intensity of signals were estimated by subtraction of background.

Statistical analysis

For statistical analysis, Student's or Welch's *t*-test was applied after Scheffé's F test for variance. Correlations between the plasma steroid levels and GTH subunit mRNA levels were analyzed using Pearson's correlation coefficient method.

RESULTS

Changes in gonadosomatic index (GSI)

The stock of sockeye salmon used in the present study usually spawn in the middle to end of October. Hence, the initial controls sampled in the beginning of September were still in immature state. The GSI values in Table 1 indicate that, in both the male and female control groups, gonadal matura-

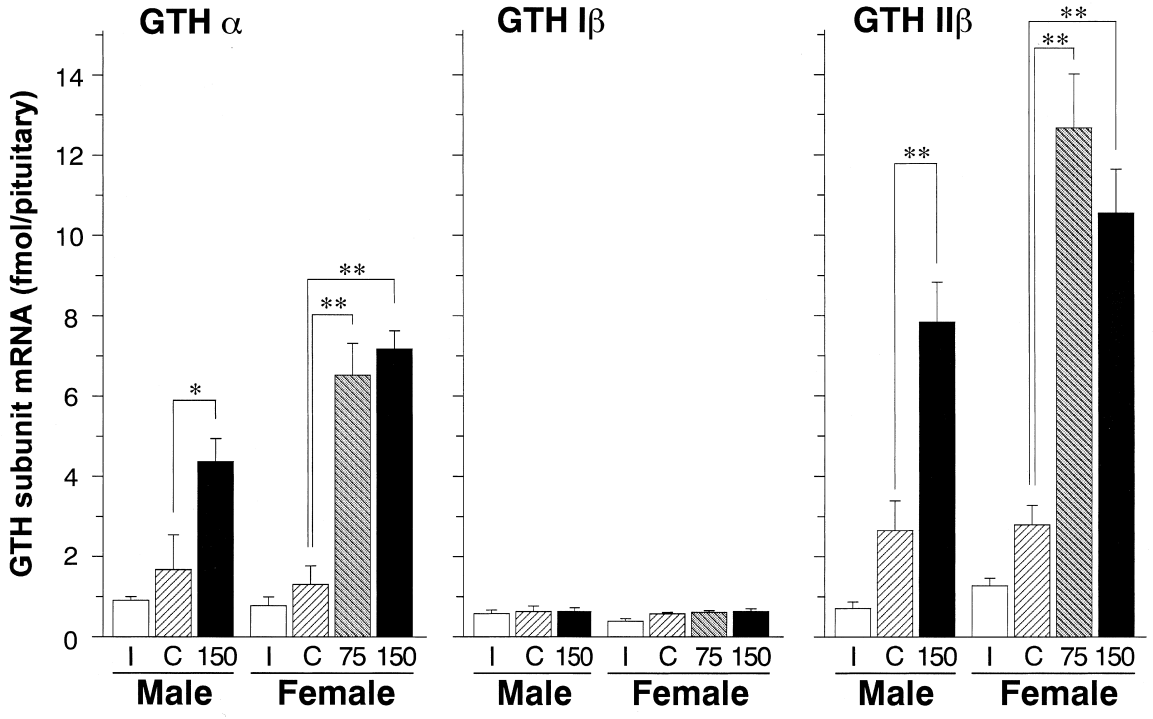

Fig. 1. Changes in the levels of GTH subunit mRNAs in terms of whole pituitary in GnRHa-implanted maturing sockeye salmon. GTH α, Iβ and IIβ subunit mRNA levels in each group are shown. I, initial control; C, control; 75, 75 μg of GnRHa-implanted; 150, 150 μg of GnRHa-implanted. Mean ± SEM (n = 3-6). *, $P < 0.05$; **, $P < 0.01$; by the *t*-test.

tion proceeded during the three-week experimental period. However, most of the control females were still preovulatory state, although all the control males excrete a little milt by pressing the abdomen. Implantation of GnRHa accelerated this naturally occurring gonadal maturation. All females which received GnRHa actually ovulated by three week GnRHa treatment (data not shown). The amount of excreted milt was increased in GnRHa treaded males.

The levels of GTH subunit mRNAs

Males: Probably due to progress of sexual maturation, the pituitary levels of GTH α and IIβ mRNAs but not Iβ mRNA were elevated 2-3 fold in the control males compared to those in the initial control (Fig. 1). The treatment with GnRHa significantly increased these α and IIβ mRNA levels 2-fold or more, whereas it had little effect on the level of GTH Iβ mRNA (Fig. 1). The pattern of changes in the levels of GTH subunit mRNAs which are expressed in terms of the unit weight of pituitary (mg pituitary), (data not shown) is nearly the same with that in Fig. 1 expressed in terms of whole pituitary.

Females: The changes in the levels of GTH subunit mRNAs in the female pituitaries showed similar pattern to those in the male pituitaries (Fig. 1). The levels of GTH α and IIβ mRNAs were drastically elevated in the GnRHa-implanted females compared to those in the controls. The more than 3-fold increases in the levels of α and IIβ mRNAs in the GnRHa-implanted females were more prominent than the 2-fold increases in the males. There was little difference in the levels of GTH subunit mRNAs between animals received 75 μg of GnRHa and those received 150 μg of GnRHa.

DISCUSSION

In the present study, we examined effects of GnRHa on expression of GTH subunit genes in the pituitary gland of maturing sockeye salmon. The effects of GnRHa on expression of GTH α and IIβ genes were markedly different from that of GTH Iβ gene. The levels of GTH α and IIβ mRNAs in the GnRHa-implanted fish were significantly higher than those in the control fish, whereas no significant difference was seen in the level of GTH Iβ mRNA between the controls and GnRHa-implanted fish. These findings indicate that, in maturing salmonids, GnRH stimulates expression of GTH α and IIβ subunit genes in pituitary GTH II-cells.

The previous immunohistochemical and *in situ* hybridization study clearly demonstrated that GTH I and II are synthesized in separate pituitary GTH cells in salmonids (Naito *et al.*, 1991). GTH I gene expression was elevated in association with initiation of vitellogenesis, whereas GTH II gene expression was concomitantly elevated with final gonadal maturation in the pituitary of rainbow trout. Taking the previous studies (Kudo *et al.*, 1996; Kitahashi *et al.*, 1997) mentioned in Introduction into consideration, we consider that sGnRH selectively stimulates GTH II cells, but not GTH I cells, to elevate expression of GTH α and IIβ genes in pre-spawning salmonids.

The levels of GTH α and IIβ mRNAs in the control fish were slightly higher than those in the initial controls. The magnitudes of increases and the amounts of mRNAs were comparable to our previous results in pre-spawning chum salmon in which the levels of GTH α and IIβ mRNAs were also elevated. It is therefore possible that endogenous sGnRH synthesis and release is stimulated during this period to accelerate final maturation through GTH II secretion.

Differences of GTH α and IIβ mRNA levels between the control and sGnRHa-implanted fish were prominent in the females when compared to those in the males. Difference in maturity between both sexes may account for this phenomenon, since the previous paper from our research group reported sexual differences in homing profiles and shortening of homing duration by GnRHa implantation in lacustrine sockeye salmon (Sato *et al*., 1997). GTH II synthesis in the control males in the present study may be already somewhat stimulated.

It is well known in salmonids that plasma levels of steroid hormones, in particular 17α, 20β-dihydroxy-4-pregnen-3-one (DHP), are elevated in association with the final gamete maturation (Nagahama and Adachi, 1985). Hence we compared the present results of GTH subunit mRNA levels with plasma levels of steroid hormones, such as DHP, testosterone (T), and 11-ketotestosterone (11KT) in the males and DHP, T and estradiol-17β (E_2) in the females in the same animals (Fukaya *et al*., in preparation). The results show that GnRHa induced a significant elevation of plasma DHP levels in both sexes, however, did not induce any noticeable changes in plasma T and 11KT levels in the males, and T and E_2 levels in the females. The increase in the levels of GTH α and IIβ mRNAs in the GnRHa-implanted fish correlated well with the increase in the plasma DHP level, particularly in the males.

In conclusion, implantation of GnRHa stimulates GTH II synthesis at the transcriptional level, and accelerates final maturation in maturing sockeye salmon. The acceleration of final maturation may be induced through elevation of plasma DHP level, which is stimulated by increase in plasma GTH II level.

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