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Actions of a Gonadotropin-Releasing Hormone Antagonist on Gonadotropin II and Androgenic Steroid Hormone Secretion in Precocious Male Rainbow Trout

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We used a mammalian GnRH antagonist, [Ac-3,4-dehydro-Pro[1], D-p-F-Phe[2], D-Trp[3,6]]-GnRH, to examine the details of the salmon type gonadotropin-releasing hormone (sGnRH) and GnRH agonist analog (Des-Gly[10][d-Ala[6]]-ethylamide GnRH; GnRHa) functions in the control of maturational gonadotropin (GTH II) secretion, in precocious male rainbow trout, in both *in vivo* and *in vitro* experiments. In the *in vivo* study, plasma GTH II levels increased by sGnRH or GnRHa treatment, but the response was more rapid and stronger in the GnRHa treatment group. The increase in GTH II was significantly suppressed by the GnRH antagonist, while the antagonist had no effect on basal GTH II levels in both groups. The GnRH antagonist showed stronger suppression of GTH II levels in the sGnRH treatment fish than in the GnRHa treatment fish. In addition, plasma androgenic steroid hormones (testosterone and 11-ketotestosterone) increased by the sGnRH or GnRHa treatment. The GnRH antagonist significantly inhibited the increases in plasma androgenic steroid hormone levels stimulated by the sGnRH or GnRHa, while the antagonist had no effect on basal androgenic steroid hormone levels in both groups. In the *in vitro* study, treatment with sGnRH or GnRHa increased GTH II release from the cultured dispersed pituitary cells, but the response was stronger in the GnRHa treatment group. The increase in GTH II release by GnRH was suppressed by adding the GnRH antagonist, dose-dependently. On the other hand, basal release of GTH II did not decrease by the GnRH antagonist treatment in both groups. These results suggest that the GnRH antagonist, [Ac-3,4-dehydro-Pro[1], D-p-F-Phe[2], D-Trp[3,6]]-GnRH, used in this study is effective in blocking the action of GnRH-induced GTH II release from the pituitary gland both *in vivo* and *in vitro*.

Key words: Fish, Reproduction, GTH II, Androgen, GnRH peptides

Introduction

As in other vertebrates, gonadal maturation in teleosts is regulated by an endocrine system, consisting of the brain-pituitary-gonad axis. This gonadal maturation is regulated by gonadotropin-releasing hormone (GnRH) via the stimulation of gonadotropin (GTH) synthesis and release (Fink, 1988). In most teleost species examined, including salmonids, salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II) have been detected in the brain (Amano et al., 1991). With respect to GnRH, sGnRH but not cGnRH-II seems to be involved in GTH synthesis and release in salmonid fish, since only sGnRH has been detected in the pituitary immunocytochemically and radioimmunologically (Amano et al., 1991; Kah et al., 1993). Although both sGnRH and cGnRH-II molecules stimulate the release of GTH in the fish pituitary both *in vivo* and *in vitro* under exogenous administration (Peter et al., 1991), their physiological roles in the brain and pituitary are not fully understood.

The two classical approaches used to study the role of GnRH in regulation of pituitary function are immunoneutralization and inhibition by antagonist

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(Murthy et al., 1993). However, immunoneutralization has not been a practical approach in teleosts, due to the direct innervation of gonadotrophs by neurosecretory fibers (Kah et al., 1993). In salmonid fish, [D-Phe^{2,6}, Phe^{3}] a GnRH antagonist can block the increase in maturational gonadotropin (GTH II) release induced by treatment of mammalian GnRH (mGnRH) from cultured pituitaries of male brown trout (Crim et al., 1981). Flett et al. (1994) also reported that [D-pGlu^{1}, D-Phe^{2}, D-Trp^{3,6}] GnRH antagonist inhibited the release of GTH II response to GnRH agonist from perifused pituitary glands of testosterone-primed immature rainbow trout. In goldfish, mGnRH antagonist, [Ac-Δ^{3}-Pro^{1}, 4FD-Phe^{2}, D-Trp^{3,6}]-GnRH has also been shown to block the action of sGnRH- and cGnRH-II-induced GTH II release from pituitary fragments perifusion system (Murthy et al., 1993). These results suggest that the GnRH peptides stimulate GTH II release in teleosts. However, none of these studies provides information on whether the GnRH antagonist acts directly at the pituitary level, and the antagonists used in these studies did not completely block the actions of GnRH.

To address the role of the various GnRH forms on gonadotropic function, parallel studies of their pituitary levels and of their respective abilities to stimulate GTH II secretion have been used. Notably, finding a potent GnRH antagonist will be useful 1) as a probe in studies on regulation of GTH secretion by hypothalamic and gonadal factors in teleost, 2) in studies on GnRH receptors and associated intracellular second messenger systems, and possibly also in controlling the reproduction of fish in aquaculture. The objective of present study was to examine the details of the actions of sGnRH and Des-Gly^{10}[d-Ala^{6}]-GnRH ethylamide on the control of GTH II and androgenic steroid hormone secretion in precocious male rainbow trout using [Ac-3,4-dehydro-Pro^{1}, D-p-F-Phe^{2}, D-Trp^{3,6}] GnRH antagonist in *in vitro* and in vivo experiments.

Materials and Methods

Experimental Fish

One-year-old spermiating (precocious) male rainbow trout were selected from stock held in a recirculating freshwater tank under natural photoperiod at 14 ℃. Mean body weight (wt) was 92.3 ± 7.4 g and mean gonadosomatic index (GSI) was 11.5 ± 4.2 %.

Hormone

Salmon gonadotropin-releasing hormone (sGnRH), Des-Gly^{10}[d-Ala^{6}]-mammal GnRH ethylamide (GnRHa) and [Ac-3,4-dehydro-Pro^{1}, D-p-F-Phe^{2}, D-Trp^{3,6}]-mammal GnRH antagonist (GnRH anta) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Peptides were dissolved in a physiological saline (PS) consisting of 0.75 % NaCl, stored at -80 ℃. Appropriate concentrations of the peptide were prepared by diluting the stock solutions immediately before use in an experiment.

Radioimmunoassys (RIAs)

Salmon GTH II was iodinated according to the method of Kim (1997). The procedures were the same as that in the salmon GTH II RIA (Kim, 1997). Testosterone (T) and 11-ketotestosterone (11-KT) measurements were carried out with RIA developed by Lou et al. (1986).

In vivo experiment

We examined the effects of GnRH antagonist administration on sGnRH- and GnRHa-stimulated GTH II and androgenic steroid hormone levels in precocious males. Sixty-four fish were divided into two experimental groups (sGnRH and GnRHa administration groups). Each GnRH administration group of thirty-two fish was divided into 4 groups (8 fish each), respectively, such as physiological saline (PS), sGnRH (0.1 μg/g body wt) or GnRHa (0.1 μg/g body wt)+PS, GnRH antagonist (1 μg/g body wt)+PS and sGnRH (0.1 μg/g body wt) or GnRHa (0.1 μg/g body wt)+GnRH antagonist (1 μg/g body wt). GnRH peptides were injected intraperitoneally. Blood samples were taken at 0, 6 and 12 hr after injection by puncturing the caudal vasculature using heparinized syringes. After centrifugation (6000 rpm, 15 min.), the plasma was stored at -40 ℃ until assayed for GTH II, T and 11-KT levels.

In vitro experiment

In the *in vitro* study, we observed GTH II release from pituitary gland cells following administration of GnRH and/or related molecules. The pituitary glands were removed from precocious male fish, and the pituitary gland cells were prepared by

collagenase/DNAse enzyme treatment as previously described (Kim et al., 1999). The pituitary cells were preincubated for 3 days in RPMI-1640 medium [medium RPMI-1640 (Sigma) containing 25mM HEPES, 4mM $NaHCO_3$, 1 % Antibiotic-antimycotic agent (GIBCO Lab) and 10 % fetal bovine serum (GIBCO Lab), pH 7.5] at a density of 2.5×10^5 cells/ml/well in 0.1 % poly- L-lysine treated-well plates under a humidified atmosphere at 18 ℃. Prior to experiments, cells were washed with RPMI-1640 (medium RPMI-1640 containing 25mM HEPES, 4 mM $NaHCO_3$, 1 % Antibiotic-antimycotic agent and 0.1 % BSA, pH 7.5). Thereafter, GnRH peptides were added into a fresh RPMI-1640 medium (medium RPMI-1640 containing 25mM HEPES, 4mM $NaHCO_3$ and 1 % Antibiotic-antimycotic agent, pH 7.5) for 24 hr. Culture media were separated and stored at -40 ℃ until assayed for GTH II.

Statistical analysis

Data were analyzed for statistical significance ($P<0.05$) using one-way ANOVA and Dancan's new multiple range test. In Figs. 1 and 3, the difference of GTH II levels for sGnRH and GnRHa treatment during the same time period were tested for significance ($P<0.05$) using Student's t test.

Results

In vivo experiment

sGnRH- and GnRHa-treated precocious male showed significantly higher plasma GTH II levels at 6 and 12 hr post-injection compared to PS-injected controls (Figs. 1A, 1B). At 12 hr post-injection, however, GTH II levels in fish receiving sGnRH were significantly lower than the levels in fish receiving GnRHa. Coinjection of GnRH antagonist with sGnRH or GnRHa significantly suppressed the sGnRH- and GnRHa-stimulated GTH II increase in plasma GTH II levels at both 6 and 12 hr post-injection (Figs. 1A, 1B, respectively). However, the GnRH antagonist showed a stronger inhibition in sGnRH-stimulated GTH II levels than in GnRHa-stimulated GTH II levels at 6 and 12 hr post-injection, respectively. The GnRH antagonist alone treatment had no significant effects on basal plasma GTH II levels through the sampling times in both groups.

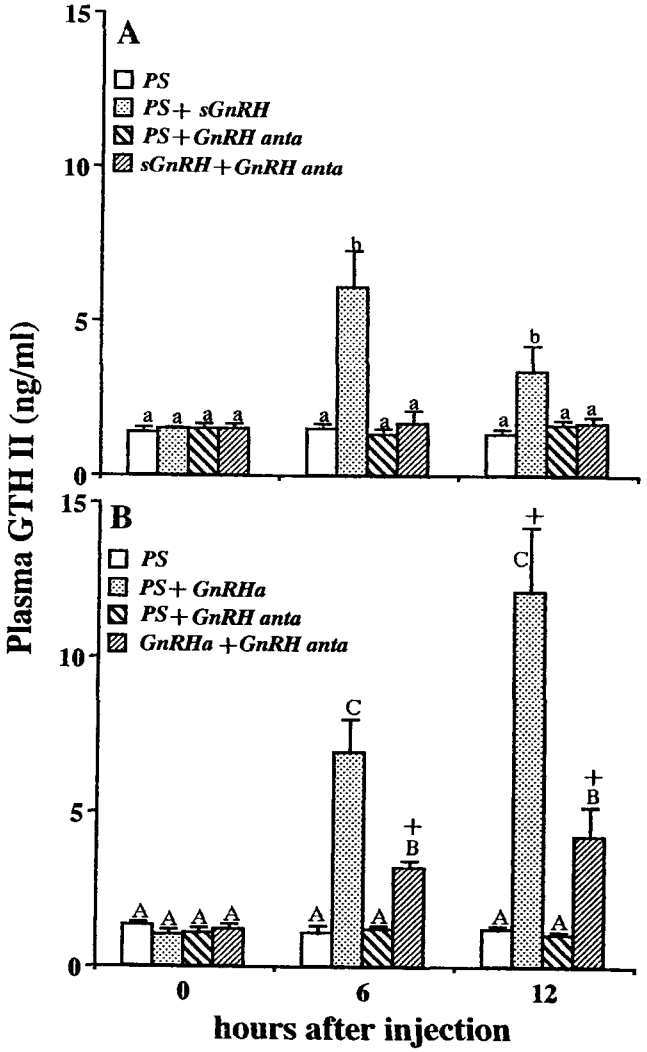

Fig. 1. Effects of GnRH antagonist on the plasma GTH II levels by sGnRH and GnRH agonist in precocious male fish. Blood samples were taken at 0, 6 and 12 hr post-injection. Results are expressed as columns to indicated by different letters at each sampling time.+indicated the levels of significant differences ($P<0.05$) from sGnRH- and GnRHa-stimulated GTH II at the same sampling time.
A : Effects of GnRH antagonist on the sGnRH-stimulated GTH II levels.
B : Effects of GnRH antagonist on the GnRHa-stimulated GTH II levels.

Plasma T and 11-KT levels remained steady in PS-injected control males for the duration of this experimental period in both groups (Figs. 2A, 2B). In contrast, following a single sGnRH or GnRHa injection, plasma T and 11-KT levels in males rapidly increased at 6 hr, and the levels of the

increased androgenic steroid hormone declined to the levels of PS-injected control males at 12 hr in both groups. The GnRH antagonist alone did not significantly alter the plasma androgenic steroid hormone levels through the sampling times in both groups. In a group of combination of GnRH antagonist and sGnRH or GnRHa, the GnRH antagonist completely inhibited the plasma T and 11-KT levels increased by sGnRH as well as the plasma 11-KT levels increased by GnRHa, but it was partially inhibit in the plasma T levels increased by GnRHa at 6 hr post-injection (Figs. 2 A, 2B, respectively).

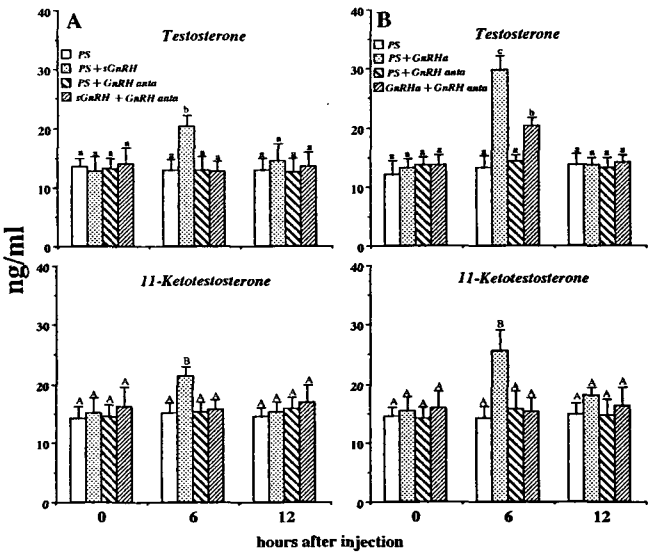

Fig. 2. Effects of GnRH antagonist on the plasma androgenic steroid hormone levels by sGnRH and GnRH agonist in precocious male fish. Blood samples were taken at 0, 6 and 12 hr post-injection. Presentation of statistical significance is as in Fig. 1.
A : Effects of GnRH antagonist on the sGnRH-stimulated androgenic steroid hormone levels.
B : Effects of GnRH antagonist on the GnRHa-stimulated androgenic steroid hormone levels.

In vitro experiment

Figure 3 shows the effects of GnRH antagonist on the GTH II release induced by sGnRH or GnRHa from cultured pituitary cells of precocious male. Basal release of GTH II was not altered by the GnRH antagonist (10^{-6} M) alone treatment. Treatment with sGnRH (10^{-8} M) or GnRHa (10^{-8} M) significantly increased GTH II release from

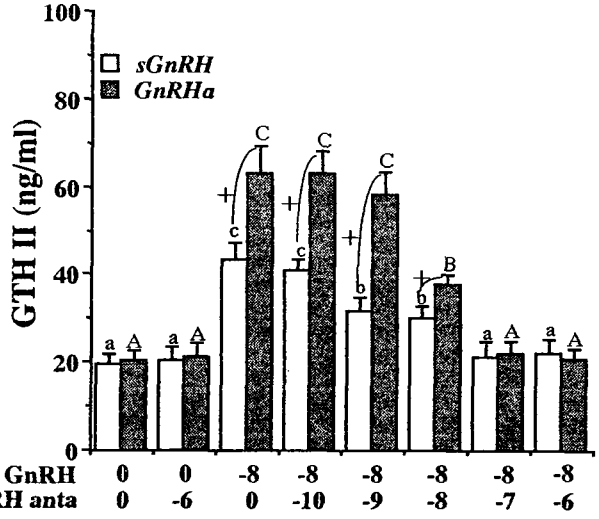

Fig. 3. Effects of GnRH antagonist on the GTH II release by sGnRH and GnRHa from cultured pituitary cells of precocious male fish. Data are expressed as the mean ± SEM (n=6). Presentation of statistical significance is as in Fig. 1.+indicated the levels of significant differences ($P<0.05$) between sGnRH and GnRHa treatments.

cultured pituitary cells, respectively. Compared to GnRHa, however, sGnRH level was significantly lower in potency to stimulate GTH II release. sGnRH (10^{-8} M)-stimulated GTH II release began to decrease by GnRH antagonist at a concentration of 10^{-9} M and was totally inhibited at 10^{-7} M. In addition, GnRHa (10^{-8} M)-stimulated GTH II release partially decreased by GnRH antagonist at a concentration of 10^{-8} M and was totally inhibited at 10^{-7} M.

Discussion

Our findings from the present studies indicate that GnRH antagonist, [Ac-3,4-dehydro-Pro[1], D-p-F-Phe[2], D-Trp[3,6]]-GnRH, is effective in blocking the actions of exogenous GnRH on GTH II release in precocious male rainbow trout. However, this antagonist alone had no significant effect on basal GTH II and basal androgenic steroid hormone levels. These results indicate that a physiological effect of endogenous GnRH in regulating GTH II release seems to be stronger in precocious male fish. This notion is supported by the fact that sGnRH-stimulated GTH II release decreased by GnRH

antagonist shown to be much more pronounced in sexually immature male fish than in maturing male rainbow trout (Kim et al., 1996).

With the discovery of additional forms of GnRH in teleosts, it has become of practical interest to assess the capacity of these GnRHs and their analogs to stimulate GTH II secretion from the fish pituitary gland. The sGnRH and cGnRH-II forms of GnRH have been found in a wide range of teleost species (Kah et al., 1993). In goldfish, sGnRH and cGnRH-II are equally effective in stimulating *in vitro* GTH II release (Chang et al., 1990) and they have similar pituitary levels (Yu et al., 1988). This indicates that these two forms possess the same potential role in regulating gonadotropic function. In contrast, in salmonids, while sGnRH and cGnRH-II are also both active on *in vitro* GTH II release (Kim, 1997), the absence of cGnRH-II in the pituitary suggests that this form is not directly involved in the control of gonadotropic function (Amano et al., 1991).

The teleost GnRH analog ([D-Arg^6, Trp^7, Leu^8, Pro^9-NEt]-GnRH) is a superactive agonist analog in goldfish, having greater biological activity than native GnRH peptides in terms of pituitary GTH II release *in vitro* and *in vivo* (Peter et al, 1991) and receptor-binding affinity in the pituitary (Habibi et al., 1987). Crim et al. (1988) also found that all the native forms of fish, bird, and mammalian GnRH stimulate GTH II release in salmonid fish, but the native peptides are relatively weak compared to certain of the GnRH analogs. Generally, native GnRH peptides are rapidly degraded by enzymes in the pituitary, kidney and liver of gilthead seabream (Zohar et al., 1990), which limits the effectiveness of native forms *in vivo*. We have found that native sGnRH on GTH II levels is relatively weak, compared to a mammalian GnRH analog, both *in vivo* and *in vitro*. Such discrepancies in the biological activities between of sGnRH and GnRHa on GTH II levels may be due to the different potential role in regulating gonadotropic function and to the different affinity for GnRH receptors and to the different resistance to enzymatic degradation.

Recently, Murthy et al. (1993) observed that [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}]-GnRH antagonist (probably the same structure of GnRH antagonist used in the present study) completely inhibited both sGnRH- and cGnRH II-induced GTH II release in a dose-dependent manner from dispersed pituitary cells of goldfish. It has been also reported that GnRH antagonists inhibit GnRH-stimulated GTH II release by competitively binding to GnRH receptors in goldfish pituitary cells (Murthy et al., 1994). In salmonid fish, Crim et al. (1981) have shown that [D-Phe^{2,6}, Phe^3]-GnRH antagonist can block the increase in GTH II release induced by treatment of mammalian type GnRH agonist, *in vivo* and *in vitro*. Flett et al. (1994) also reported that [D-pGlu^1, D-Phe^2, D-Trp^{3,6}] GnRH antagonist inhibited the release of GTH II response to GnRH agonist from perifused pituitary fragments of testosterone-primed immature rainbow trout. However, the antagonists used in salmonid fish did not completely block the actions of GnRH. Also, whether the antagonists directly acted on pituitary cells to inhibit the GTH II release is not clear. The antagonist used in the present study completely suppressed sGnRH- and GnRHa-stimulated GTH II release from the pituitary gland both *in vivo* and *in vitro*. Notably, this GnRH antagonist acted directly at the pituitary cell level to inhibit GnRH actions.

In this investigation, the plasma androgenic steroid hormone (T and 11-KT) levels were elevated by sGnRH or GnRHa treatment and were inhibited by the GnRH antagonist. Harmin and Crim (1993) have shown that a single injection of [D-Ala^6, Pro^9-NHEt]-LHRH increased the plasma levels of T and 11-KT within 12 hr in maturing male winter flounder, *Pseudopleuronectes americanus* and the steroid hormone levels remained elevated for long periods, lasting several days. Sun et al. (1992) also reported that a single injection of [D-Ala^6, des-Gly^{10}]-LHRH ethylamide at a dose of 30 μg/kg wt or 60 μg/kg wt can induce ovulation in ayu, *Plecoglossus altivelis*; levels of plasma T, estradiol-17β and 17α-hydroxyprogesterone increased significantly in both female and male ayu within 6~12 hours following injection. Therefore, the increase of androgen levels in precocious male rainbow trout are considered to be mediated by the elevation of GTH II concentration following treatment with GnRHa. A question arising here is whether the GnRH antagonist directly influences the gonad by binding GnRH receptors or not. Unfortunately, existence of GnRH receptors in the gonads of salmonid fish has not been reported yet. However, in cyprinid fish, specific GnRH binding was observed in testis and ovary of goldfish

(Habibi and Pati, 1993), and that GnRH directly acts on oocyte meiosis and/or gonadal steroidogenesis in goldfish (Habibi et al., 1988, 19 89) and carp (Pati and Habibi, 1992). Furthermore, testicular development was inhibited by the long-term treatment of GnRH antagonist in precocious male masu salmon (Amano; personal communication). Therefore, it may possible that the GnRH antagonist regulates in testis of rainbow trout.

In summary, the GnRH antagonist, [Ac-^{3,4}-dehydro-Pro^{1}, D-p-F-Phe^{2}, D-Trp^{3,6}]-mGnRH, is effective in modulating the action of GnRH-induced GTH II release from the pituitary gland as well as androgen secretion from testis in precocious rainbow trout. A direct regulatory action of a GnRH antagonist on the control of sex steroids secretion in the rainbow trout gonad still awaits further investigations.

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