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Effects of Gonadotropin-Releasing Hormone on *in vitro* Gonadotropin Release in Testosterone-Treated Immature Rainbow Trout

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Abstract: The control mechanism of gonadotropin-releasing hormone (GnRH) on gonadotropin (GTH) release was studied using cultured pituitary cell or cultured whole pituitary obtained from Testosterone (T) treated and control immature rainbow trout. The release of FSH was not changed by salmon type GnRH (sGnRH), chiken-II type (cGnRH-II), GnRH analogue ([des-Gly^{10}D-Ala^{6}] GnRH ethylamide) and GnRH antagonist ([Ac-3, 4-dehydro-Pro^{1}, D-p–F-Phe^{2}, D-Trp^{3,6}] GnRH) in cultured pituitary cells of T-treated and control fish. Indeed, FSH release was not also altered by sGnRH in cultured whole pituitary. All tested drugs had no effect on the release of LH in both culture systems of control fish. The levels of LH, in contrast, such as the pituitary content, basal release and responsiveness to GnRH were increased by T administration in both culture systems. In addition, the release of LH in response to sGnRH or cGnRH-II induced in a dose-dependent manner from cultured pituitary cells of T-treated fish, but which is not significantly different between in both GnRH at the concentration examined. Indeed, LH release was also increased by sGnRH in cultured whole pituitary of T-treated fish. GnRH antagonist suppressed the release of LH by sGnRH (10^{-8} M) and GnRH analogue (10^{-8} M) stimulation in a dose-dependent manner from cultured pituitary cells of T-treated fish, and which were totally inhibited by 10^{-7} M GnRH antagonist. These results indicate that the sensitivity of pituitary cells to GnRH is elevated probably through the T treatment, and that GnRH is involved in the regulation of LH release. GnRH-stimulated LH release is inhibited by GnRH antagonist in a dose-dependent manner. The effects of gonadal steroids on FSH levels are less clear.

Key words: rainbow trout, pituitary cell culture, GTH release, GnRH peptides

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INTRODUCTION

Gonadal maturation in teleost is regulated by hypothalamus-pituitary axis via modulation of a complex interrelationships between the environmental and physiological factor (Saligaut et al., 1999). Among the latter, it is also included negative and positive feedback of gonadal steroids, which are produced from gonad by stimulation of gonadotropin (GTH) appearing in the pituitary. Negative feedback of gonadal steroids on pituitary GTH secretion has been established in adult rainbow trout by using gonadectomy (Billard et al., 1977) ; gonadectomy-induced elevations of plasma LH are abolished by testosterone (T) or estoradiol-17β (E2) treatment. Despite the evidence implicating the inhibitory actions of steroids on GTH secretion in adults fish, it is has also been observed that gonadal steroids stimulate pituitary GTH accumulation in immature rainbow trout (Crim and Evans, 1983). It has been established in sexually regressed goldfish, *Carassius auratus* (Trudeau et al., 1993) and sexually immature rainbow trout, *Oncorhynchus mykiss* (Crim et al., 1981a) that both T and E2 can enhance LH release in response to gonadotropin–releaging hormone (GnRH). These results indicate that gonadal steroids is a main factor which regulate the function of the hypothalamus-pituitary axis.

Physiological levels of GTH are modulated by complex interrelationships between the gonadal steroids and the hypothalamic GnRH, and the steroids and GnRH individually regulate GTH secretion (Saligaut et al., 1999). Gonadal steroids may act directly at the pituitary level or indirectly via GnRH producing system in the hypothalamus, and they can have positive or negative feedback actions, depending on physiological state (for review see Chang et al., 2009). However, salmonid fish have two types of

GnRH, salmon type GnRH (sGnRH) and chiken-II type GnRH (cGnRH-II) in the brain, but only sGnRH is detectable in the pituitary of rainbow trout (Okuzawa et al., 1990) and masu salmon, *Oncorhynchus masou* (Amano et al., 1991). Recently, Kawauchi and co-workers have also characterized that the two pituitary GTHs, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), from salmonids (Suzuki et al., 1988; Swanson et al., 1991). The participation of GnRH in the regulation of LH release in well understood (Saligaut et al., 1999; Popesku et al., 2008). However, it is little known whether the synthesis of FSH is regulated by steroid hormone or which is released by GnRH peptides in the teleosts.

We examined the possible interaction between steroid hormone and GnRH peptides on the regulation of FSH and LH using the cultured pituitary cells or whole pituitary gland of T-treated immature rainbow trout. Indeed, we also tested the effects of GnRH antagonist as a useful tool in understanding the function and mode of GnRH peptides on the regulation of GTHs release.

MATERIALS AND METHODS

Fish

Immature rainbow trout (*Oncorhynchus mykiss*), weighting about 100 g, were obtained from a commercial source. Before experiment, fish were recirculating freshwater tank at 14°C for more than week.

Experimental design

Four separate experiments were conducted. In experiment 1, changes in GTHs content and sGnRH-stimulated GTHs release were examined in the time course of testosterone (T) treatment to know adequate period for T treatment. Fish were treated with pellets containing T as described later. Before and after 1, 2, 3, 4, and 8 weeks of T treatment, 30 to 40 fish at a time were sacrificed. Dispersed pituitary cells were cultured to analyze GTHs release profiles in response to sGnRH.

In experiment 2, we examined the effects of sGnRH and cGnRH-II on GTHs release *in vitro* from the pituitaries in T-treated and control (T-untreated) fish, since the salmonid fish have two types of GnRH, sGnRH and cGnRH-II in the brain. Fish were fed with pellets containing T or without T for 30 days, and pituitaries were used for cell culture.

In experiment 3, we also examined whether GnRH antagonist acts directly at the pituitary cells levels, and it also inhibits GnRH actions on GTHs release. Thus, we tested the effects of GnRH antagonist ([Ac-3,4-dehydro-Pro^{1}, D-p-F-Phe^{2},D-Trp^{3,6}] GnRH) on GnRH analogue (Des-Gly^{10}[d-Ala^{6}]GnRH ethylamide) and sGnRH-stimulated GTHs release from cultured pituitary cells of T-treated and control fish. Fish were fed with pellets containing T or without T for 30 days, and pituitaries were used for cell culture.

In experiment 4, we examined the possibility that GnRH effects on GTHs release from pituitary organs are mediated by indirect actions on the remaining nerve terminals or by the interaction of cells into pituitary organ. Therefore, we tested the effect of sGnRH on GTHs release in pituitary organ culture using T-treated and control fish. Fish were fed with pellets containing T or without T for 30 days, and pituitaries were used for organ culture.

In vivo Treatment of Testosterone (T)

T containing pellets were prepared by spraying T ethanol solution, (25 mg/100 mL) on 1kg commercial pellets for rainbow trout (Oriental Co., Tokyo), followed by evaporation at room temperature overnight. Final content of T was 25 μg/g dry pellets. Fish were fed with these pellets twice daily at a ration of 1.5% of body weight per day. The control group (T-untreated) of immature trout was fed T-free pellets sprayed with only ethanol.

Dispersion of pituitary cells for cell culture

The fish were rapidly anesthetized in 2-phenoxyehanol (0.5 mL/L). Pituitary glands were removed from T-treated and control fish, respectively, and placed in ice-cold Hank's balanced salt solution (HBSS, Gibco Laboratories, New York), which was buffered with 25 mM HEPES and 4 mM $NaHCO_3$ containing 1%(v/v) antibiotic-antimycotic agent (Gibco Laboratories), pH 7.5. Excised pituitaries were washed three times with HBSS, and diced into fragments by tissue slicer (Narishige Scientific Instrument Lab. Tokyo). The tissue were transferred to a siliconized culture flask (Wheaton Instruments, New jersey) filled with 10 mL HBSS containing 20 mg collagenase (Sigma, St. Louis) and were then incubated in the dispersion solution at 15°C for 75 min. During this period, dissociation rate of cell dispersion was mechanically aided by aspirating the fragments in a siliconized pasteur pipette. After that, 500 μL of 0.04% DNAse I (w/v; Boehringer Maunheim, GmbH, Germany) solution was added and incubated for further 15 min. Dispersed cells were filtered through 50 μm nylon mesh, and harvested by centrifugation at 150×g for 5 min. Harvested cells were then resuspended in RPMI-1640 (Sigma, St. Louis) containing 25 Mm HEPES, 4 Mm $NaHCO_3$, 10% fetal bovine serum (Gibco Laboratories) and 1% antibiotic-antimycotic agent, and had a final pH of 7.5. The cell population was counted directly on a hemato-cytometer under the light microscope, the cell viability rate was determined by mixing 40 μL of cell suspension with 10 μL of 0.1% trypan blue (Sigma) dissolved in 0.01 M phosphate buffer (pH 7.5). The cell yield is 0.3-0.35×10^{6} cells per pituitary with a cell viability percentage of 91±1%.

Cell culture

The cell suspension was adjusted to be 2.5×10^5 cells/mL and 500 μL was plated into each wells of 48-well plate (Sumitomo Co. Tokyo). The wells were prepared before plating with 500 μL/well of 0.1% poly-L-lysine for 30 min, rinsed 3 times with distilled water and then dried in a clean bench before use. The cell were cultured at 18°C (Weil et al., 1986) under a humid atmosphere. After 3 days of culture (preincubation), test incubations were carried out. Before exposing to salmon and chicken-II type GnRH (Peninsula Labratories, Belmont, CA), GnRH analogue (GnRHa; Sigma), GnRH antagonist (Sigma) according to experiment, cells were washed twice with 500 μL serum-free culture medium containing 0.1% BSA (Sigma). Thereafter, fresh medium containing each GnRH was added and incubation was conducted for 24 hr. Culture media were separated and stored at −40°C until assayed for GTHs. Dispersed pituitary cells in the each wells were sonicated in 500 μL RIA assay buffer, and the suspentions were centrifuged at 1500X *g* and the supernatant was used for the assay.

Organ culture

After washing with HBSS, removed pituitaries were preincubated at 18°C under an atmosphere of 95% O_2/5% CO_2 for up to 3 days. Serum-free RPMI 1640 medium was changed every 24 hr. After 3 days of preincubation, fresh medium containing sGnRH was added and incubation was conducted for 24 hr. For determination of GTHs release, the incubation media were stored at −40°C. Pituitary glands in the each wells were sonicated in 500 μL RIA assay buffer, and the suspentions were centrifuged at 1500×*g* and the supernatant was used for the assay.

Radioimmunoassays (RIAs)

Culture media and the remained pituitary cells were measured by specific RIAs for salmon FSH and LH. Stable FSH and antisera against FSHβ were kindly provided by Prof. H. Kawauchi of Kitasato University. FSH and LH were iodinated according to the method of Kobayashi *et al.* (1987). The procedure of each RIA was the same as that in the LH RIA (Kim and Aida, 2000a). Displacement curves for culture media and the extract of dispersed pituitary cells were parallel to the standard curves in each FSH and LH RIA system (Fig. 1). The cross-reactions of FSH in the LH RIA and LH in the FSH RIA were approximately 2.1% and 9.2%, respectively.

Statistic

The Student's *t*-test, Cochran-Cox text, and the new multiple range test of Duncan were used for statistical analysis.

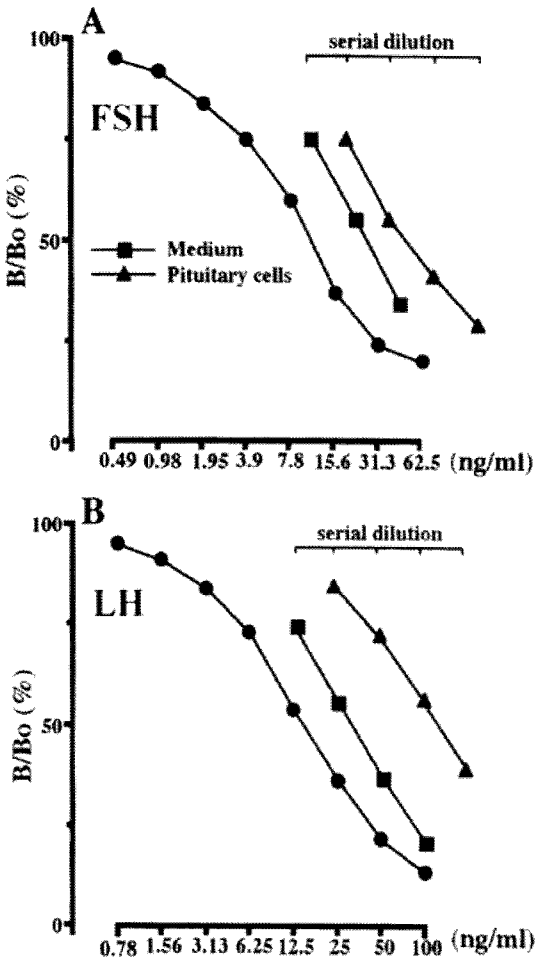

Fig. 1. Competitive binding curves for stable FSH standards (A) and serial dilutions of culture media and dispersed pituitary cells of T-treated immature trout, and LH standard (B) and serial dilutions of culture medium and dispersed pituitary cells of T-treated immature trout. Each point represents the average of duplicate determinations.

RESULTS

Experiments using dispersed pituitary cells (experiment 1, 2 and 3)

Figure 2 shows the changes in GTHs content in the pituitary cells of T-treated fish in EXP. 1. Pituitary LH content was significantly increased after 1 week and it increased with the lapse of time until 6 weeks. On the other hand, pituitary FSH content and plsma LH levels showed no significant changes by T treatment. Figure 3 shows the changes in FSH and LH release properties in response to various doses of sGnRH from dispersed pituitary cells of T-treated fish with time course. Although pituitary LH content reached plateau after 6 weeks, LH release in responses to sGnRH become maximum after 4 weeks. Basal LH release also increased, and peaked after 6 weeks. On the other hand, FSH of basal release and response to sGnRH showed no significant changes.

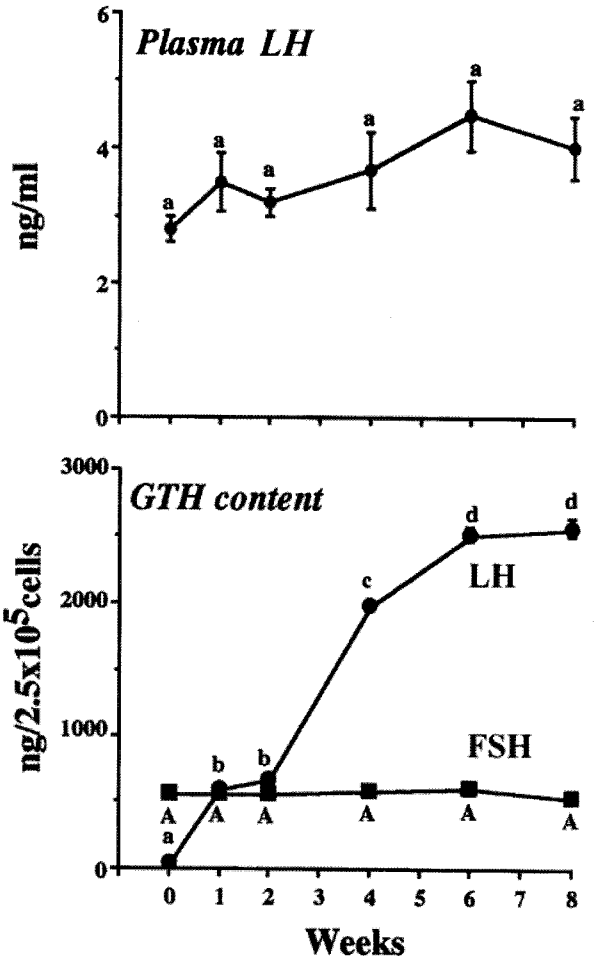

Fig. 2. FSH and LH content from dispersed pituitary cells with T treatment time course *in vivo*. Data are expressed as the Means±SEM (n=6). A significant difference was observed between columns indicated by different letters.

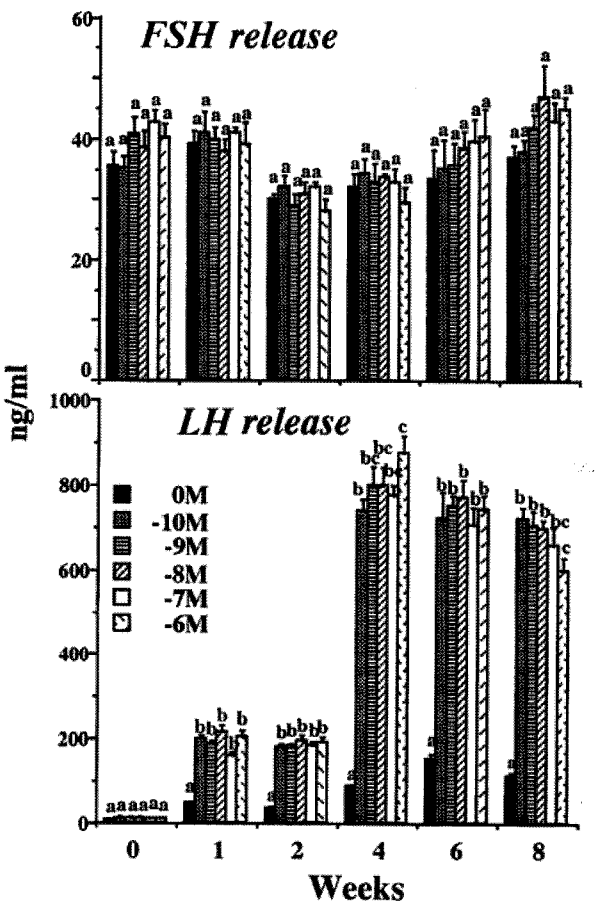

Fig. 3. Effects of FSH and LH release to sGnRH in medium from dispersed pituitary cells with T treatment time course *in vivo*. Data are expressed as the Means±SEM (n=6). Presentation of statistical is as in Fig. 2.

In Exp. 2, the effects of *in vivo* T treatment on FSH and LH release from dispersed pituitary cells in response to sGnRH and cGnRH-II are shown in Fig. 4. Both sGnRH and cGnRH-II induced significantly LH release from dispersed pituitary cells (Fig. 4A), and LH content of gonadotrophs present in dispersed pituitary cells following a 24 hr incubation period with GnRHs decrease with GnRHs dose-dependent manner (Fig. 4B) in T-treated fish. The patterns of LH release were not significantly different between sGnRH and cGnRH-II at the concentration examined. On the other hand, LH release was not stimulated in control fish. FSH release from dispersed pituitary cells of T-treated and control fish was not stimulated by sGnRH or cGnRH-II. LH content in control fish, and FSH content from dispersed pituitary cells of T–treated and control fish was not alter by sGnRH and cGnRH-II, respectively.

In Exp. 3, the effects of GnRH antagonist on the sGnRH (10^{-8} M)- and GnRH analogue (10^{-8} M)-induced GTHs release from dispersed pituitary cells of T-treated and control fish are shown in Fig. 5. No responses to GnRH antagonist were observed on the FSH release both in the T-treated and control fish, and the LH release in the control fish. On the other hand, GnRH antagonist caused dose-dependent decreases in the LH release induced by 10^{-8} M of sGnRH and GnRHa in the T-treated fish. Indeed, in the presence of 10^{-6} M GnRH antagonist the basal LH release was not significantly altered in T-treated fish.

Experiment using pituitary organ culture (experiment 4)

Figure 6A shows the FSH and LH release patterns in response to sGnRH from pituitaries organ-cultured. Significant increase in FSH release in response to various dose of sGnRH was not observed in both groups. On the other hand, LH release from the pituitaries of T-treated fish significantly increased in response to 10^{-9}~10^{-6} M sGnRH. Indeed, LH content from the pituitaries of T-treated fish decrease with sGnRH concentration (Fig. 6B). LH release was not stimulated by sGnRH in the pituitaries of control fish. LH pituitaries contents in control fish, and FSH

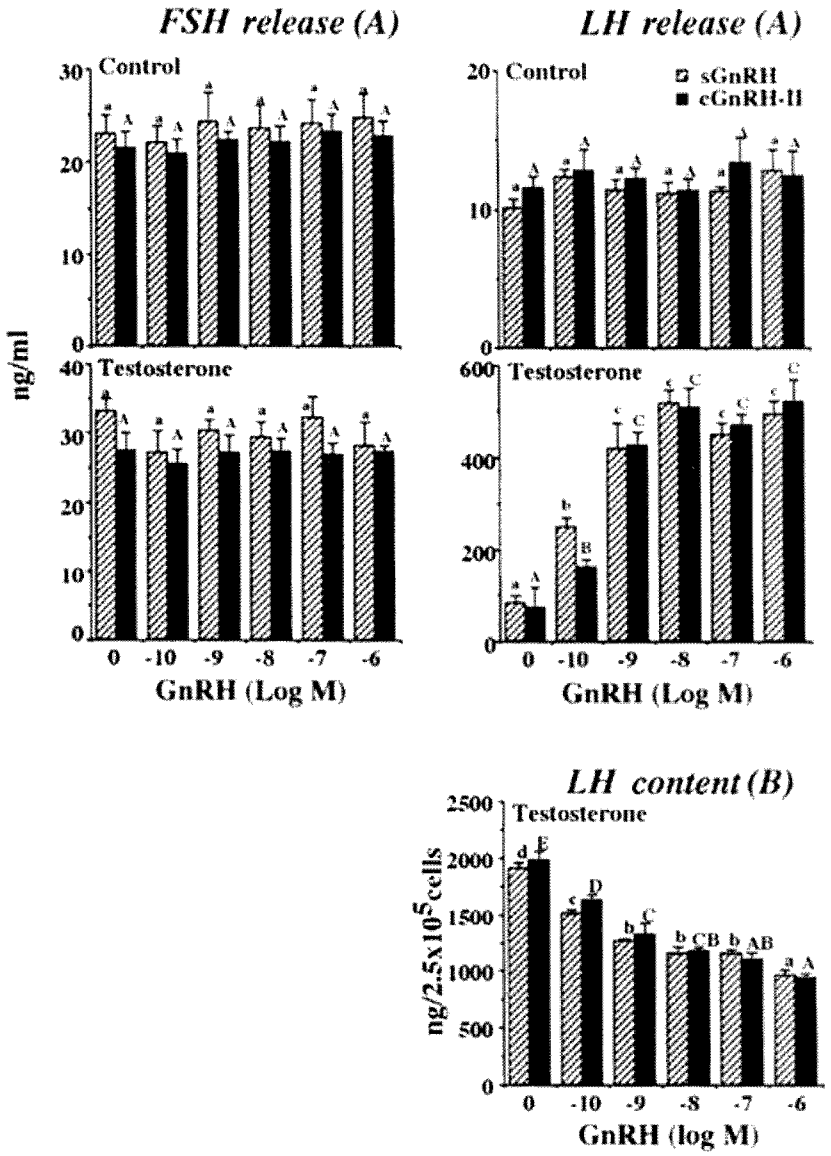

Fig. 4. Effects of FSH and LH release to sGnRH and cGnRH-II from dispersed pituitary cells of control and T-treated fish (A). Analyses of LH content in the remaining after the removal of the incubation media following 24h incubation period with GnRH on dispersed pituitary cells of T-treated fish alone (B). Data are expressed as the Means±SEM (n=4). Presentation of statistical is as in Fig. 2.

pituitaries contents in T-treated as well as control fish was not changed by sGnRH, respectively.

DISCUSSION

We demonstrated in the present study that the pituitary content of LH of sexually immature fish is very low and it is not augmented by treatment with sGnRH or cGnRH-II, but *in vivo* T treatment increase the pituitary content of LH, basal release of LH and pituitary sensitivity to GnRHs. In the levels of FSH, however, pituitary content, basal release and responsiveness to GnRHs were not significantly influenced by T treatment when compared with control fish.

According to Amano et al. (1994), the oral administration of 17α-methyltestosterone in immature masu salmon, *Oncorhynchus masou*, had no effect on the content of FSHβ in the pituitary, and the administration of E_2, T and 11-KT in juvenile coho salmon, *Oncorhynchus kisutch*, also gave no influences on FSH content (Swanson and Dickhoff, 1988). However, Pavlick and Moberg (1997) reported that longterm T treatement stimulates the accumulation of pituitary FSH in both juvenile and previtellogenic white strogeon, *Acipenser transmontanus*, but dose not affect basal or GnRH analogue (GnRHa)-induced FSH secretion. Furthermore, increase of FSH release by GnRHa from organ-cultured pituitaries of juvenile coho salmon had been demonstrated (Swanson et al., 1989). In contrast, the present study shows that stimulation of FSH release was not observed both in organ-cultured pituitaries and dispersed

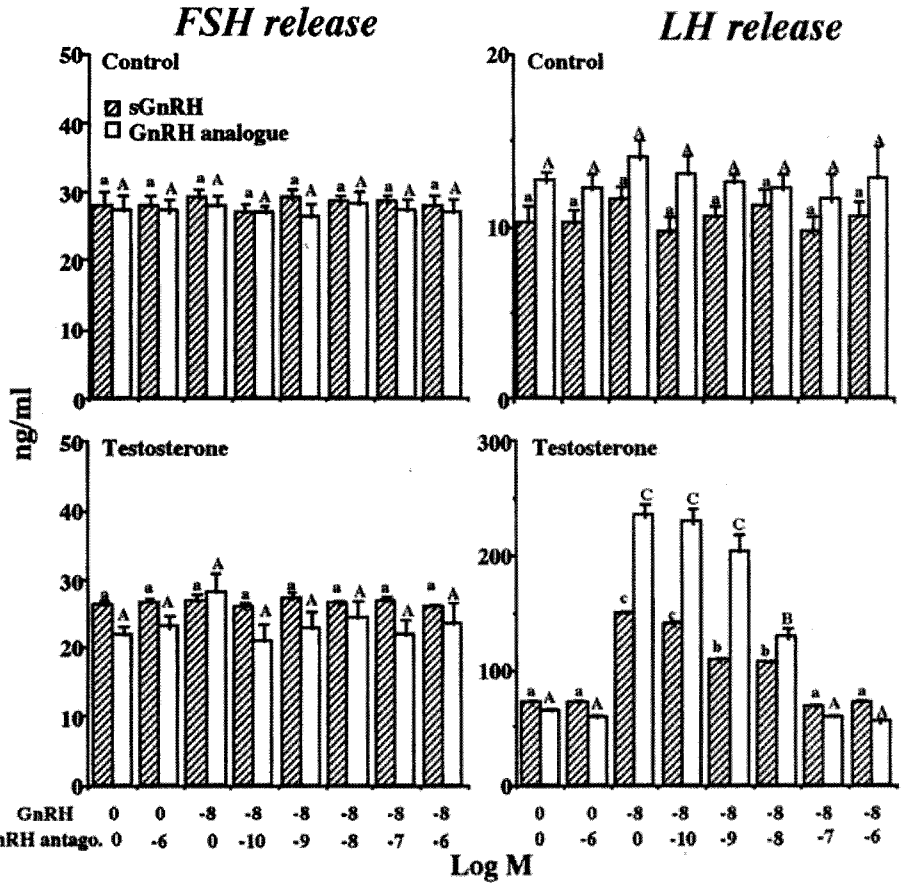

Fig. 5. Effects of a dose-dependent of GnRH antagonist on sGnRH (10^{-8} M)- and GnRH analogue (10^{-8} M)-induced GTHs release from dispersed pituitary cells of control and T-treated fish. Data are expressed as the Means±SEM (n=6). Presentation of statistical is as in Fig. 2.

pituitary cells. This discrepant result may be related to the *in vitro* pituitary organ culture method used and/or the differences of bioactivity between native GnRH peptides and GnRH analogue. In this investigation, we used native forms of GnRH to understand the physiological response of FSH cells, and FSH release was not influenced by them at the concentration of 10^{-10} M to 10^{-6} M. Therefore, we may conclude that FSH release at physiological conditions is not regulated by native GnRH.

The present study shows that stimulation of experiment of pituitary LH content and responsiveness to GnRHs according to the time course of T treatment significantly increased at 4 weeks. This suggests that the effects of T treatment in immature rainbow trout seem to involve up-regulation of the release of LH response to GnRH peptides as well as increases the responsiveness of pituitary LH cells to GnRH peptides. Notably, *in vitro* basal release of LH from dispersed pituitary cells of T-treated rainbow trout was increased and LH content present in dispersed pituitary cells and pituitaries following a 24 hr incubation period with GnRH decrease with GnRHs concentration in T-treated fish. On the other hand, no significant changes were observed in control fish. Furthermore, the possibility of responsiveness of GnRH on GTHs release from pituitary organ may be mediated by indirect actions on the remaining nerve terminals or by the interaction of cells into pituitary organ. Therefore, we tested the effect of sGnRH on GTHs release from pituitary organ culture using T-treated and control fish. These results are supported by the fact that sex steroids appear to have a predominantly positive-feedback effect in sexually immature salmonid fish. For example, pituitary content of GTH (presumably LH) increases following treatment of juvenile male and female rainbow trout with testosterone (Crim *et al.*, 1981a; Crim and Evans, 1983), and it also enhances responsiveness of pituitary to native GnRH and of fragmented pituitary to GnRH analogue in perifused culture *in vitro*, respectively (Crim and Evans, 1980; Flett *et al.*, 1994). In addition, gonadal steroids increased the number of immunoreactive gonadotrophs as well as the pituitary GTH content of immature trout *in vitro* (Fahraus-van Ree *et al.*, 1983), and sex steroids increase the pituitary sensitivity to sGnRH and cGnRH-II in goldfish (Trudeau *et al.*, 1993). However, the letter authors suggested that LH release to responsiveness of sGnRH and cGnRH-II from dispersed pituitary cells are different in goldfish (Trudeau *et al.*, 1993).

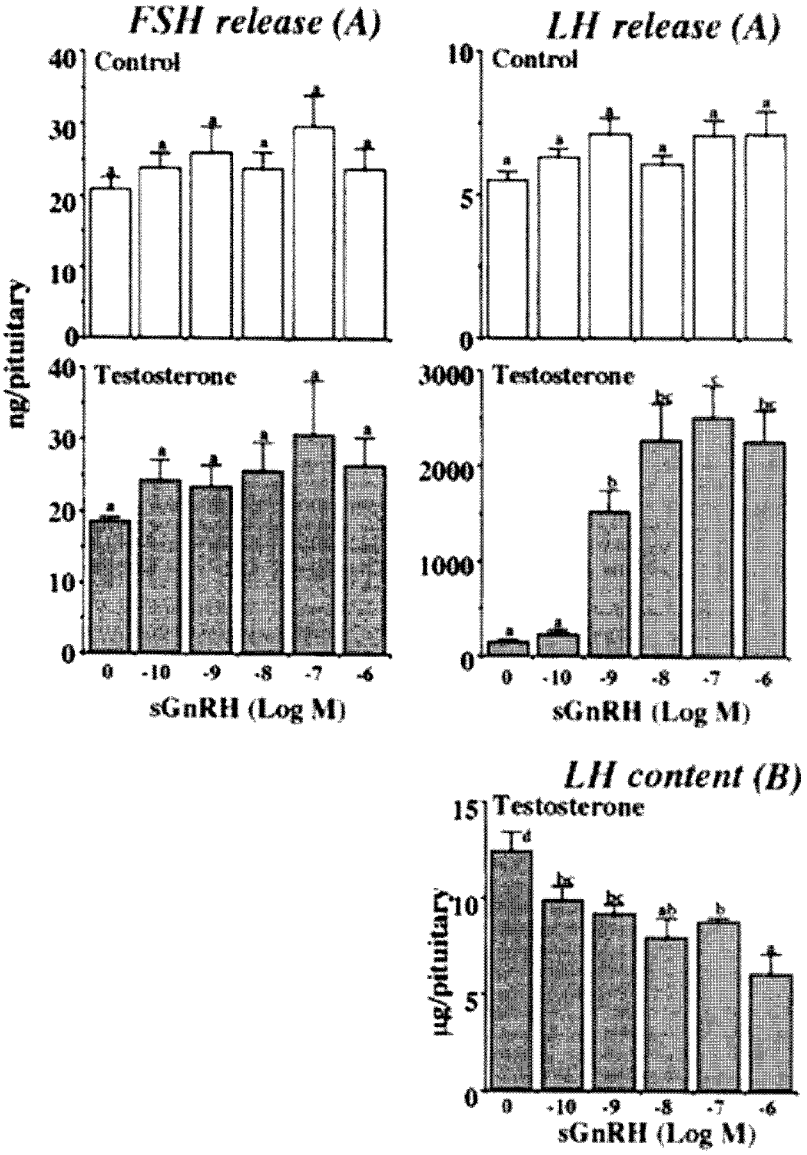

Fig. 6. Effects of FSH and LH response to sGnRH from whole pituitary of control and T-treated fish (A). Analyses of LH content in the remaining after the removal of the incubation media following 24h incubation period with sGnRH on whole pituitary of T-treated fish alone (B). Data are epressed as the Means±SEM (n=8). Presentation of statistical is as in Fig. 2.

In the goldfish brain and pituitary gland, there are two endogenous GnRH molecules, sGnRH and cGnRH-II, which have been shown to stimulate LH release *in vivo* and *in vitro* (Peter et al., 1987). Although both sGnRH and cGnRH-II are found in the brain of rainbow trout (Okuzawa *et al.*, 1990) and masu salmon (Amano *et al.*, 1991), only sGnRH is present in the pituitary, implying the involvement of only sGnRH in regulation of LH release in these salmonid fish. However, Chang and co-workers showed that the two native GnRHs in goldfish activate somewhat different second messenger components in stimulating LH release (Chang *et al.*, 1991, 2009). They proposed a novel hypothesis that two closely related peptides can compete for the same receptors on gonadotrophs and stimulate hormone release via activation of different postreceptor messenger systems (Chang *et al.*, 2009).

In the present study, the responsiveness to sGnRH and cGnRH-II-induced LH from dispersed pituitary cells of T-treated fish were equivalent over a dose range of 10^{-9} M to 10^{-6} M. This is because that two peptides are structurally similar, so it may be bind to the same receptors, and can show similar effect on the biological activity of LH secretion. In contrasts to the situation with goldfish (Habibi, 1991), a down-regulation of LH release by high GnRH dosages has not been studied in detail in salmon, and the possibility can not be excluded that our experimental conditions contributed to the decrease in LH release at concentrations of 10^{-8} or 10^{-7}~10^{-6} M GnRHs. However, the fact that the apparent partial inhibition of LH release was more obvious in T-treatment fish may be indicative of a

mechanism restricting an overstimulation of the pituitary at GnRH peptides.

In the present study, [Ac-3,4-dehydro-Pro^1, D-p-F-Phe^2, D-Trp^{3,6}] mGnRH antagonist completely inhibit sGnRH and GnRH analogue actions on LH release from dispersed pituitary cells of T-treated immature rainbow trout, without any effects on basal release of LH. Notably, [Ac-3,4-dehydro-Pro^1, D-p-F-Phe^2, D-Trp^{3,6}] mGnRH antagonist acted directly on dispersed pituitary cells to inhibit LH release. However, FSH release in response to mGnRH antagonist was not also influenced in dispersed pituitary cells of T-treated fish. Previously, we reported that [Ac-3,4-dehydro-Pro^1, D-p-F-Phe^2, D-Trp^{3,6}] mGnRH antagonist was effective in modulating the action of GnRH-induced LH release from the pituitary gland as well as androgen secretion from testis in precocious rainbow trout (Kim et al., 2000b), and Crim et al., (1981b) also reported that [D-Phe^{2,6,} Phe^3] GnRH antagonist can block the increase in the LH release induced by treatment of GnRH analogue in trout pituitary culture. In goldfish, furthermore, Murthy et al., (1994) observed that [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}] mGnRH antagonist (probably same structure of GnRH antagonist used in present study) inhibited both sGnRH- and cGnRH-II-induced LH in a dose-dependent manner from dispersed pituitary cells of goldfish. Therefore, these results indicate [Ac-Δ^3-Pro^1, 4FD-Phe^2, D-Trp^{3,6}] mGnRH antagonist bind to GnRH receptors in pituitary gland, and competitively inhibit the stimulatory actions GnRH, resulting in a loss of LH realese.

In summary, the present studies demonstrate that in sexually immature rainbow trout, *in vivo* treatment with T potentiates native GnRHs-induced LH secretion *in vitro*. Therefore, T has a direct effect on the pituitary and/or indirect effect via GnRH to increase GnRH responsiveness. Notably, GnRH antagonist exerts its actions directly at the pituitary cells levels, and which also inhibits GnRH actions on LH release. However, FSH release *in vitro* in T-treated and control fish was not affected by native GnRHs. So it is though that FSH release may be regulated by other endogenous neuropeptides and neurotransmitter factor, because its immunoreactive fibers also directly innervate the pituitary.

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