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Effect of Testosterone on the mRNA Levels of Gonadotropin Subunits in the Immature Rainbow Trout Pituitary

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In order to clarify the role of gonadal sex steroids in the synthesis of gonadotropin (GTH) subunits in immature rainbow trout, we examined *in vitro* and *in vivo* effects of testosterone (T) on the pituitary mRNA levels of GTH I β, GTH II β and α subunits by Northern blot analysis and on the pituitary content levels of GTH I β and GTH II β by radioimmunoassay (RIA). The mRNA levels of the α subunit in T-treated fish were not changed more dramatically than those in control fish both *in vivo* and *in vitro*. Interestingly, the mRNA levels of GTH I β in T-treated fish were shown to be slightly lower than those in the control fish under these experimental conditions, but no differences were observed in pituitary GTH I β contents. In contrast, the mRNA levels and pituitary contents of GTH II β subunit were strongly increased by T both *in vivo* and *in vitro*. These results demonstrate that the expressions of GTH I β and II β subunit genes in immatue rainbow trout pituitary are subjected to differential regulation by T.

Key words: GTH subunit mRNAs, testosterone, rainbow trout

Introduction

Gonadotropins (GTHs; GTH I and GTH II) are produced by two distinct types of gonadotropes in rainbow trout (Nozaki et al., 1990), and changes in staining intensities, reflecting immunoreactivity or mRNA, show a pattern that correlates with the reproductive cycle (Naito et al., 1991). It consists of two nonidentical α and β subunits that are linked noncovalently (Suzuki et al., 1988). The primary structures of the α and β subunits of several species of fish have been determined (Trinh et al., 1986; Suzuki et al., 1988). Sequence analysis revealed that the homology of the α subunit (approximatly 70%) is much higher than that of the β subunit (approximatly 40%) between fish and mammalian GTHs (Trinh et al., 1986). These data indicated that the α subunit is highly conserved, whereas the β subunit is diversified during the evolution of vertebrate GTH.

In teleost fish, the expression of GTH I and GTH II is tightly regulated and subject to the control of many factors including the gonadal steroids and hypothalamic releasing factors (Xiong et al., 1994a). In particular, sexual maturation in rainbow trout involves increases in the circulating levels of the GTHs (Prat et al., 1996), and of the gonadal steroids (Low et al., 1986). Because these hormones change with the season in a similar manner, the potential for interactions among these hormones is great. In salmonid fish, on the other hand, both positive and negative feedback effects of the gonadal steroids have been observed. Studies in salmonid fish demonstrated negative feedback effects of gonadal steroids primarily in mature fish (Billard, 1978), whereas positive feedback effects were observed in immature fish (Crim et al., 1981; Crim and Evans, 1983; Kim, 1997). The physiological relationship between positive and negative feedback mechanisms in the control of GTHs is, however, not very clear.

Recently, the synthesis of the GTHs has been examined by molecular techniques to explore the

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differential regulation of expression of the α-subunit, GTH I β and GTH II β genes by gonadal steroid (Dickey and Swanson, 1995; Sohn et al., 1998). However, since these studies were conducted *in vivo*, it has been difficult to identify the primary site of the steroid action and its mode of action. Therefore, the purpose of this study was to investigate the *in vitro* and *in vivo* effect of testosterone (T) on GTH subunits synthesis by using Northern blot analysis coupled with laser densitometry in immature rainbow trout pituitary. In addition, pituitary GTH subunits accumulation by T was also investigated using radioimmunoassay.

Materials and Methods

Fish

One-year-old immature rainbow trout of mixed sex were selected from stock held in a recirculating freshwater tank under natural photoperiod at 14℃. Mean body weight was 95.3 ± 5.4 g and mean gonadosomatic index (GSI) were 0.14 ± 0.03% (female) and 0.03 ± 00.009% (male). Fish were fed once daily with commercial trout pellets at a rate of 1 to 2% body weight.

Time-related effect of testosterone (T) *in vivo*

We examined the effects of T on the mRNA levels and pituitary contents of GTH subunits in immature rainbow trout *in vivo*. Fish were divided into two groups (60 fish each) such as control and T-injected group. Immature rainbow trout of mixed sexes were injected intraperitoneally (i.p.) with 100 μg/fish concentration of T (Wako Pure Chemicals Co. Japan). T was initially dissolved in ethanol and then diluted appropriately in oleum-palmeum oil to give the desired doses. Control fish received only 250 $\mu\ell$ of the oleum-palmeum oil. At various time intervals after injection (3 hr, 3 day, 1 week, 2 week), the fish were anesthetized by immersion in 2-phenoxyethanol (200 ppm). Blood samples were taken at each sampling time after injection by puncturing the caudal vasculature using heparinized syringes. After centrifugation (6000 rpm, 15 min.), the plasmas were stored at -40℃ until assayed for T levels. The pituitary glands were removed and frozen immediately by immersion in liquid nitrogen to extract the total RNA and GTHs from pituitary glands as described below.

Time-related effect of testosterone (T) *in vitro*

Pituitary contents and mRNA levels of GTH subunits were determined in response to treatment with T by means of a pituitary organ culture system in sexually immature rainbow trout as described previously (Kim, 1997). The fish were rapidly anesthetized in 2-phenoxyethanol (200 ppm). Pituitary glands were removed and incubated in a 48-well plate containing RPMI 1640 medium [medium RPMI-1640 (Sigma Chemical Co.) containing 25 mM HEPES, 4 mM $NaHCO_3$, 1% Antibiotic-antimycotic agent (GIBCO Lab.) pH 7.5]. The pituitary glands were incubated at 15℃ under an atmosphere of 95% O_2/5% CO_2, resulting in a pH of 7.5~7.7. After 18 hr preincubation, the medium containing T (100 ng/mℓ) was changed at 24 hr intervals during the experiment period. At various time intervals after treatment (1 day, 4 day, 7 day), the pituitary glands were frozen immediately by immersion in liquid nitrogen to extract the total RNA and GTHs from pituitary glands as described below.

Radioimmunoassays (RIAs)

Extraction of GTH from the pituitary gland was done according to Amano et al. (1992). Chum salmon GTH I β and II β and antisera against GTH I β and II β were kindly provided by Dr. H. Kawauchi of Kitasato University. GTH I β and II β were iodinated according to the methods of Kim (1997). The procedure for each RIA was the same as that in the salmon GTH RIA (Kim, 1997). Displacement curves for pituitary samples were parallel to the standard curves in both GTH I β and II β RIA (Amano et al., 1992). The antiserum against GTH I β cross-reacted with GTH I, GTH II and GTH II β at 1.7%, 4.0% and 4.4%, respectively, at 50% binding. The antiserum against GTH II β was found to cross-react with GTH I, GTH II and GTH I β at 0.22%, 3.7% and 1.0%, respectively, at 50% binding.

Testosterone levels were determined by RIA as described by Lou et al. (1986). Details on the RIA for T have been described previously (Lou et al., 1986).

RNA preparation and Northern blot analysis

Frozen pituitary glands were pooled into two tubes, and stored at -80℃ until required for use. RNA extraction and Northern blot analysis were performed as described previously by Kim et al. (1999). Total RNA was extracted from the pooled pituitaries with RNA extraction solution, Isogen (Nippon Gene). The total RNAs (each 5 μg for α, Iβ and IIβ) were denatured at 65℃ for 15 min in 50% formamide and subjected to electrophoresis on a 0.9% agarose gel in 0.2% MOPS, pH 7.0, containing 2.2 M formamide, 0.05 M sodium acetate, and 5 mM EDTA, then transferred to Hybond N^+ Nylon membrane (Amersham Corp.). The membranes were air-dried and baked at 80℃ for 15 min prior to being hybridized with randomly labeled identical [^{32}P] dCTP cDNA probes encoding masu salmon pituitary GTH α, Iβ and IIβ. The masu salmon cDNA probes for α, Iβ and IIβ were provided by Dr. K. Gen (National Research Institute of Aquaculture, Japan). The membranes were washed at room temperature for 15 min in 2X SSC and at 65℃ for 15 min in 1X SSC containing 0.5% SDS. The hybridized membranes were scanned by a Fujix BAS 1000 Mac Bio-Imaging Analyzer (Fuji Film, Tokyo, Japan) to count the hybridization signals. The quantified α, Iβ, IIβ mRNA levels were represented with respect to total RNA.

Statistics

Data were analyzed for significance ($P<0.05$) using one-way ANOVA and Duncan's new multiple range test.

Results

Time-related effect of testosterone (T) *in vivo*

After a single injection with T (100 μg/fish), plasma T levels in immature rainbow trout were found to be 169.4 ± 29.3 ng/mℓ (3 hr after injection), 115.7 ± 18.3 ng/mℓ (3 day after injection), 72.6 ± 9.5 ng/mℓ (1 week after injection) and 36.9 ± 5.5 ng/mℓ (2 week after injection). However, plasma levels of T in the control group did not exceed 0.5 ng/mℓ during the experiment.

The effects of T on pituitary α, GTH Iβ and GTH IIβ mRNA levels in sexually immature rainbow trout are presented in Fig. 1. Pituitary α-

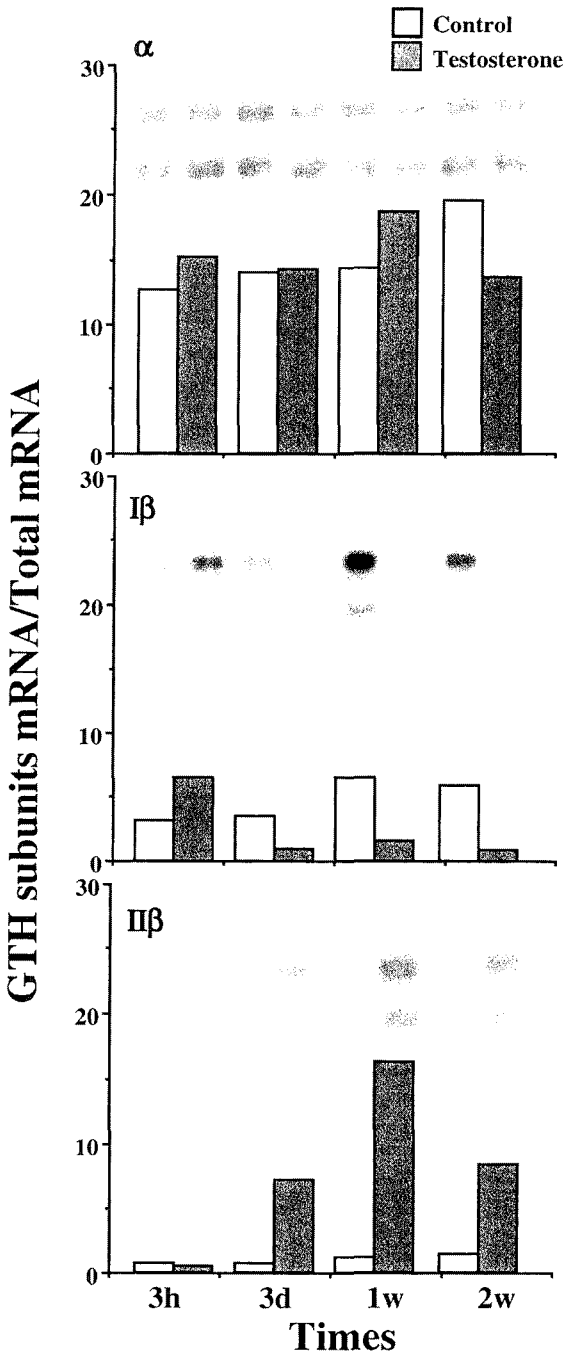

Fig. 1. Time-related effect of testosterone (T; 100 μg /fish) on pituitary mRNA levels of GTH subunits in immature rainbow trout *in vivo*. Pituitary total RNA of control and T-injected group was extracted at 3 hr, 3 day, 1 week and 2 week after injection, respectively. The mRNA levels were measured by Northern blot analysis and quantified using a computerized densitometer, Fujix BAS 1000 Bio-Imaging Analyzer (Fuji Film). The quantified mRNA levels were standardized by pituitary total RNA.

subunit mRNA levels in control and T-injected fish did not change during the experiment until 2 week after injection. Although the GTH I β mRNA levels induced by T injection were higher than those of control fish at 3 hr post-injection, they were lower than those of control fish for up to 2 week post-injection. GTH II β mRNA levels in control and T-injected fish did not change at 3 hr post-injection, but they were more sharply increased in T-injection fish than in control fish from 3 day after injection.

The effects of T on pituitary GTH I β and GTH II β contents in sexually immature rainbow trout are shown in Fig. 2. No significant change in pituitary GTH I β contents were observed in control nor T-injection fish throughout the sampling times. Pituitary GTH II β contents elevated by T-injection were significantly maintained for up to 2 week after injection. However, pituitary GTH II β contents in control fish did not significantly change throughout the sampling times.

Time-related effect of testosterone (T) *in vitro*

The effects of T on pituitary α, GTH I β and GTH II β mRNA levels from cultured pituitary organs in sexually immature rainbow trout are presented in Fig. 3. The mRNA levels of the pituitary α-subunit in T treatment were slightly higher than those in the control group at 1 day, but no marked differences were observed at 4 and 7 day after treatment. The mRNA levels of the pituitary GTH I β subunit showed no differences between T-treated and control group for up to 4 day. At 7 day, however, the mRNA levels of the pituitary GTH I β subunit were markedly lower in T-treated than in the control group. The pituitary GTH II β subunit mRNA levels showed no differences between T-treated and the control group at 1 day after treatment. The GTH II β mRNA levels in T treatment were extremly higher than those of the control group at 4 day, but no marked differences were observed at 7 day after treatment.

The effects of T on pituitary GTH I β and GTH II β contents from cultured pituitary organ in sexually immature rainbow trout are shown in Fig. 4. No significant change in pituitary GTH I β contents were observed in T-treated or in the control group throughout the sampling times. However, pituitary GTH II β contents increased by T treatment were more significantly maintained than those of the control group for up to 7 day after treatment.

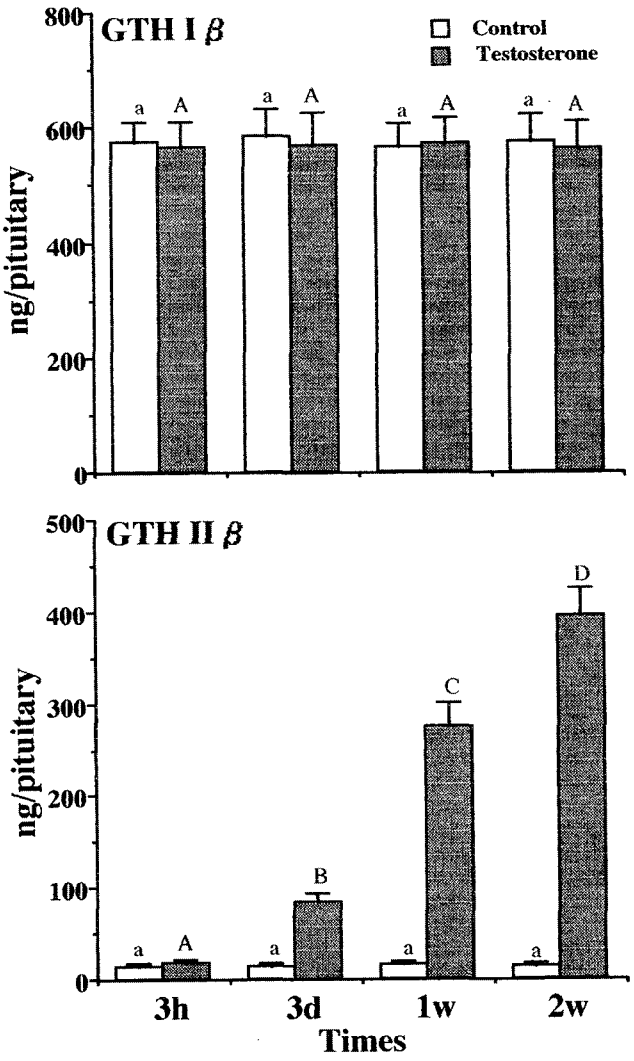

Fig. 2. Time-related effect of testosterone (T; 100 μg/fish) on pituitary GTHs contents in immature rainbow trout *in vivo*. Pituitary GTHs contents of control and T-injected group were extracted at 3 hr, 3 day, 1 week and 2 week after injection, respectively. Pituitary GTHs contents were measured by RIA, respectively. A significant difference ($p<0.05$) was observed between columns indicated by different letters.

Discussion

Gonadal steroid feedback regulation on synthesis

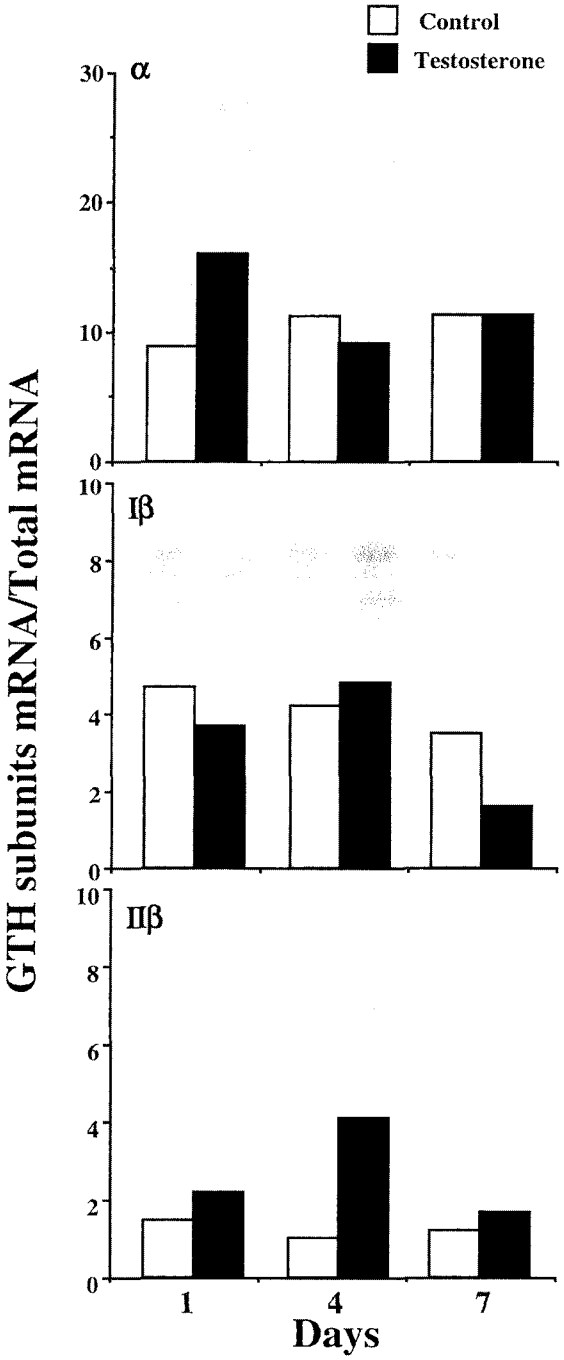

Fig. 3. Time-related effect of testosterone (T; 100 ng/mℓ) on pituitary mRNA levels of GTH subunits in immature rainbow trout *in vitro*. Pituitary total RNA of control and T-treated group was extracted at 1 day, 4 day and 7 day after treatment, respectively. The mRNA levels were measured by Northern blot analysis and quantified using a computerized densitometer, Fujix BAS 1000 Bio-Imaging Analyzer (Fuji Film). The quantified mRNA levels were standardized by pituitary total RNA.

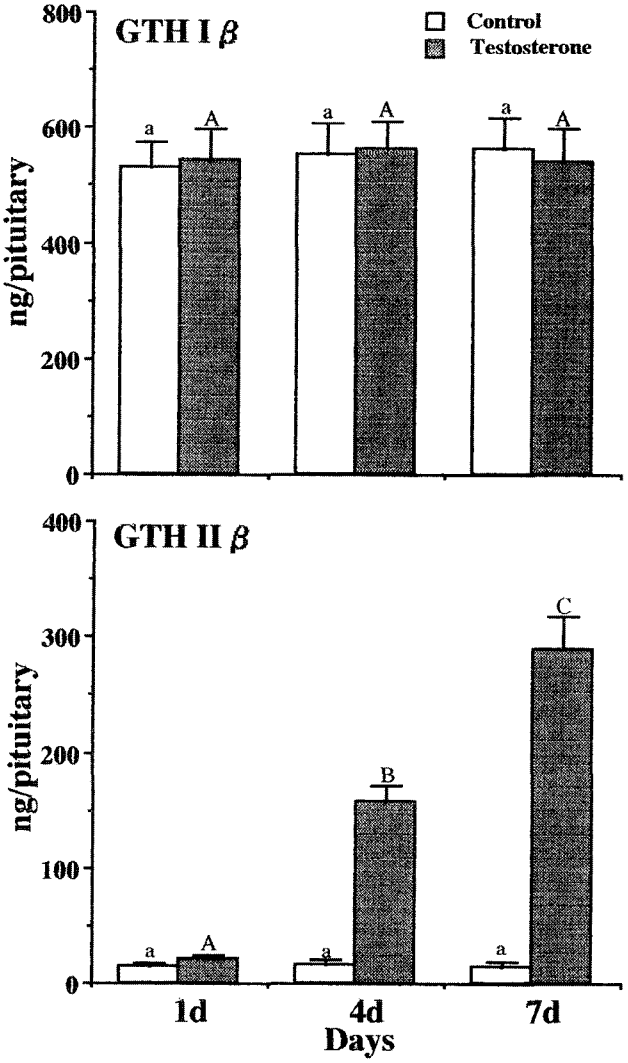

Fig. 4. Time-related effect of testosterone (T; 100 ng/mℓ) on pituitary GTHs contents in immature rainbow trout *in vitro*. Pituitary GTHs contents of control and T-injected group were extracted at 1 day, 4 day and 7 day after treatment, respectively. Pituitary GTHs contents were measured by RIA, respectively. A significant difference ($p<0.05$) was observed between columns indicated by different letters.

and secretion of GTH is an important component of the neuroendocrine control of reproduction in teleosts as in other vertebrates (Callard et al., 1990). In fish, although the effects of gonadotropin-releasing hormone (GnRH), neurotransmitters (e.g., dopamine), and gonadal steroids on GTHs, particularly GTH II, have been well studied (Saligaut et al., 1999), very limited information is available about GTH I involvement in the regulation of

gonadal maturation. With regard to the mechanism of inhibitory effects of the sex steroids on GTH I β mRNA expression, it is not known whether sex steroids act directly on the pituitary GTH I cells or indirectly via the hypothalamus. It has been reported that in immature coho salmon no significant effects of estradiol-17β (E_2) were observed on GTH I β mRNA levels by using RNase protection assays (Dickey and Swanson, 1995). However, Sohn et al. (1998) observed marked inhibitory effects of sex steroids on GTH I β mRNA levels in early recrudescent goldfish but not in mature fish by using Northern blot analysis coupled with laser densitometry. In the present study, the mRNA levels of GTH I β subunit were decreased by long-term effects of T both *in vivo* and *in vitro* by using Northern blot analysis coupled with laser densitometry. The different results obtained for coho salmon and rainbow trout investigations may be due to differences in the assay methods of hybridized signals. Therefore, the study presented here indicates that inhibitory effects of GTH I β mRNA expression by T may be mediated via the pituitary GTH I cells directly.

Pituitary GTH I β contents did not show a clear correlation to the pituitary mRNA levels of GTH I β after T treatment in both *in vivo* and *in vitro*. Nor did our previous study on time-course of T treatment show any remarkable changes in pituitary GTH I contents (Kim, 1997). According to Amano et al. (1994), the oral administration of 17α-methyltestosterone in immature masu salmon had no effect on the contents of GTH I β in the pituitary, and the administrations of T, E_2 and 11-ketotestosterone in juvenile coho salmon also had no influence on GTH I contents (Swanson and Dickhoff, 1988). In both juvenile and previtellogenic white sturgeon (*Acipenser transmontanus*), however, *in vivo* long-term T treatment stimulates the accumulation of pituitary GTH I but does not affect basal or gonadotropin-releasing hormone analog (GnRHa)-induced GTH I (Raymond et al., 1997). The different results obtained for white sturgeon and rainbow trout investigations may be due to differences of sensitivity of the pituitary GTH I cells to T depending on the maturity of the fish. Therefore, these results suggest that the transcript levels and accumulation of GTH I β by sex steroids treatment are differentially regulated in immature rainbow trout.

In the present study, the levels of GTH II β mRNA by T treatment resulted in a dramatic increase in immature rainbow trout both *in vivo* and *in vitro*. Gene expression and accumulation of the GTH II β are known to be stimulated by T treatment in immature fish (Trinh et al., 1986; Dickey and Swanson, 1995; Huggard et al., 1996; Huang et al., 1997; Sohn et al., 1998). Studies in immature rainbow trout demonstrated an increase in GTH II β mRNA levels in response to steroid treatment *in vivo* (Trinh et al., 1986) and *in vitro* (Xiong et al., 1994b). Pituitary cells from sexually mature (prespawning) trout, which actively synthesize GTH II β mRNA, responded positively to steroid treatment. However, steroid treatment of pituitary cells from spawning fish had no effect on GTH II β mRNA levels. In goldfish, the levels of GTH II β mRNA by a chronic treatment with T or E_2 only resulted in a slight increase in early recrudescent fish and almost no increase in mature fish (Huggard et al., 1996; Sohn et al., 1998). These results are not surprising due to the presence of the steroid response element in the promoter region for the GTH II β subunit gene (Xiong et al., 1994b).

In the present study, pituitary GTH II β contents did show a clear correlation to the pituitary mRNA levels of GTH II β after T treatment in both *in vivo* and *in vitro*. Crim et al. (1981), Amano et al. (1994) and Kim (1997) demonstrated an accumulation of GTH II in the pituitary of immature salmonid fish after treatment with aromatizable androgens and estrogens. Gielen and Goos (1983) provided evidence that the positive action of steroid hormones on the gonadotropes might be a direct one, not necessarily mediated by the hypothalamus. In all these studies the development of GTH II cells and the accumulation of GTH II were not accompanied by an enhanced release of GTH II. Crim and Evans (1983) treated immature trout with T over a longer period of time and apparently with high doses. They noticed increased plasma GTH II levels and precocious maturity. These results suggested that long-term treatment with gonadal steroids not only has a positive influence on GTH II synthesis but also exerts a positive feedback on the GnRH producing system. Indeed, Breton and Sambroni (1996) and Amano et al. (1995) found an increase

in the amount of salmon type GnRH (sGnRH) and the expression of sGnRH mRNA present in the preoptic area after sex steroid administration to rainbow trout and masu salmon, respectively. Thus, these results suggest that the transcript levels and accumulation of GTH IIβ by sex steroids treatment are synchronouslly regulated in immature rainbow trout.

In the present study, the levels of α-subunit mRNA were not changed dramatically by T in both *in vivo* and *in vitro* experiments of immature rainbow trout pituitary. This result corresponds well to those in immature rainbow trout by E_2 treatment (Xiong et al., 1994b). Also, α-subunit mRNA levels were not changed significantly by sex steroids in both early recrudescent and sexually mature goldfish (Sohn et al., 1998). In the European eel (*Anguilla anguilla*), however, implantation of T and E_2 for four weeks results in a 5-fold increase in α-subunit mRNA (Querat et al., 1991). Furthermore, Nagae et al. (1996) also reported that the increase in mRNA levels of α-subunit in Japanese eel, *Anguilla japonica* is probably due to positive feedback of T and E_2 produced by ovarian follicles in response to the GTH contained in chum salmon pituitary homogenate. The relationship between α-subunit mRNA and sex steroids remains unknown.

In summary, treatment with T in both *in vivo* and *in vitro* experiments of immature rainbow trout pituitary exerts an inhibitory effect on GTH Iβ gene expression but not on pituitary GTH Iβ contents. These results suggest that the transcript levels and protein levels of GTH Iβ by sex steroids treatment are differentially regulated in immature rainbow trout pituitary. However, treatment with T exerts a stimulatory effect on GTH IIβ gene expression and pituitary GTH IIβ contents in both *in vivo* and *in vitro* experiments of immature rainbow trout pituitary. These results indicate that the transcript levels and protein levels of GTH IIβ by sex steroids treatment are synchronouslly regulated in immature rainbow trout. Therefore, the present findings demonstrate that testosterone, at physiological levels, directly regulates GTH subunits gene expression in the rainbow trout pituitary. The observed changes in GTH subunits mRNA production may be an important factor in steroidogenic regulation of rainbow trout reproduction through mechanisms directed at the level of the pituitary.

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