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Inhibitory Regulation of Inhibin Gene Expression by Thyroid Hormone during Ovarian Development in Immature Rats

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To explore the role of the thyroid gland in ovarian development during the initiation process of puberty, we examined the effects of hypothyroidism on the secretion of ovarian hormones during equine chorionic gonadotropin (eCG)-induced follicle development in immature female rats. Immature rats at 22 days of age were thyroidectomized (Tx) to cause hypothyroidism and then given a single s.c. injection of 5 IU eCG at 26 days of age to induce normal first ovulation. The blood samples were collected at 0, 24, and 48 h after eCG treatment to measure inhibin and estradiol by radioimmunoassay. Serum inhibin and estradiol levels in eCG-primed Tx animals were significantly higher at 24 and 48 h after eCG treatment than those in controls (eCG treated non-Tx rats). The number of healthy follicles larger than 400 μm in diameter and ovarian weight were significantly increased in Tx rats at 48 h after eCG treatment, compared to those in controls. The number of oocytes which are ovulated by an injection of human chorionic gonadotropin (10 IU) was significantly increased on the day after eCG treatment, compared to that of eCG treated non-Tx rats. The increments in both hormones levels, the number of large antral follicles, and ovarian weight in eCG-primed Tx animals were suppressed up to control levels with daily administrations of 5.0 μg thyroxine (T_4) for 6 days during 22 to 27 days of age. The expression of mRNAs for inhibin α and β_A subunits increased in eCG-primed Tx rats at 48 hr after eCG treatment , and the increase in inhibin mRNAs was suppressed by T_4 treatment up to control levels. These results clearly demonstrate that thyroid hormone takes part in an inhibitory regulation of ovarian hormonal secretion and folliculogenesis in eCG-primed immature female rats. © 1998 Academic Press

It has already been well documented that the reproductive functions are influenced by the functions of the thyroid gland. Hypothyroidism induces menstrual disturbances, decreases secretion of gonadotropins, and results in impaired fertility and an increase in the frequency of miscarriage in adult women (1, 2). In adult female rats, hypothyroidism decreases gonadotropin secretion resulting in their irregular estrous cycles, and then the maturation of ovarian follicles is suppressed (3-5). Direct relationship between the thyroid gland and reproductive organs has been discussed. Thyroid hormone exerts stimulatory effects on granulosa cell differentiation in porcine and rat ovaries (6-8). Specific high-affinity binding sites of T_3 receptor are demonstrated in porcine (7, 8) and human (9) granulosa cells. Mattheij *et al* (10) have recently shown that hypothyroidism in adult female rats induced irregular and prolonged estrous cycles and an increase in the proestrous LH surge, and, therefore, it was concluded that hypothyroidism may affect steroid metabolism and secretion. Contrary to the stimulatory effects of thyroid hormone on ovarian functions of adult rats, it has been reported that hypothyroidism in male rats during the neonatal period also resulted in a large increase in adult testis size (11,12) and sperm production (13-15). However, no study examining acute effects of hypothyroidism or thyroid hormone treatment on the secretion of ovarian hormones during early follicular maturation before puberty has been performed.

Inhibin is a glycoprotein hormone, consisting of an α chain and one of two highly homologous β chains designated β_A and β_B (16,17). The heterodimer peptide is one of the most important ovarian local regulators, regulating ovarian growth (18,19) and steroidogenesis (20,21), as well as functioning as a circulating hormone. Granulosa cells of healthy follicles start to secrete inhibin prior to secreting estrogen from preantral stages,

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and continue to secrete it throughout follicular maturation in rats. Both inhibin and estrogen are important ovarian hormones as indexes of follicular maturation. Changes in the plasma levels of inhibin during rat estrous cycles provide a precise indicator for follicular recruitment, selection and ovulation (22,23). In the present study, in order to clarify the physiological roles of the thyroid gland in the development of immature female reproductive functions, we examined how hypothyroidism influences the secretion of inhibin and estrogen during equine CG (eCG)-induced ovarian development in immature rats.

MATERIALS AND METHODS

Animals and drug treatment. Immature female rats of the Wistar strain were maintained under controlled-temperature, humidity and 12 hr light lighting schedule (lights on at 0700h), with free access to laboratory rodent chow and water. Thyroidectomy (Tx) with self-implanted parathyroidgland was performed under ether anesthesia at 22 days of age. To induce earlier puberty, immature rats were injected s.c. with 5 IU of eCG (Teikoku Hormone MFG Co., Tokyo, Japan) dissolved in 0.2 ml saline at 0800 h at 26 days of age. To examine effects of exogenous thyroid hormone, 5 μg thyroxine (Sigma Chemical Co., St. Louis, MO, USA) , which can maintain physiological serum levels of T3 and T4, was injected i.p. at 0900 h once daily for 6 days (days 22 - 27) immediately after Tx. At various times after eCG or vehicle treatment, blood was collected via the abdominal aorta under ether anesthesia. Blood was allowed to clot at 4°C. Serum samples were separated by centrifugation and stored frozen at −80°C until assay for inhibin, estradiol and progesterone.

Radioimmunoassay (RIA) of inhibin, estradiol, and progesterone. Serum concentrations of inhibin were measured by double-antibody RIA, using a rabbit antiserum against bovine inhibin (TDNH-1) and ^{125}I - labeled 32 kDa inhibin as described previously (23). The intra- and inter-assay coefficients of variation were 5.1% and 11.0%, respectively. Concentrations of estradiol and progesterone in the serum were determined by double-antibody RIA, using ^{125}I-labeled radioligands as described previously (24). Antisera against estradiol (GDN244; 25) and progesterone (GDN377; 26) were kindly provided by Dr. GD Niswender, Colorado State University, Fort Collins, CO, USA. The intra- and inter-assay coefficient of variation were 5.8% and 18.1% for estradiol and 9.8% and 10.4% for progesterone, respectively.

Ovarian histology. Ovaries removed various time after eCG treatment were weighed and soaked in methacarn fixative for 8 h. After fixation, ovaries were dehydrated, embedded in paraffin, sectioned serially at 10 μm, and placed on poly-L-lysine coated slides. Each section was stained with hematoxylin-eosin for counting of follicles and morphological observation. All healthy and atretic antral follicles larger than 200 μm in diameter were counted using an ocular micrometer. About 300 ovarian sections in an individual ovary were examined. Follicular diameter was determined in the ovarian section containing the largest cross-section of the oocyte. Follicles were classified into either medium follicles (200-400 μm), large follicles (>400 μm), developing healthy small follicles (>200 μm) or atretic follicles(>200 μm). Degenerative phenomena of the oocyte and granulosa cells, i.e. nuclear changes such as pyknosis, shrinking, and fragmentation and hypertrophy of cells, were used as criteria for follicular atresia. Detachment from basement membrane of granulosa cell layer was also used as a criterion for atresia.

RNA preparation and Northern analysis. Ovaries which were removed at 48 h after eCG treatment were immediately frozen in liquid nitrogen for northern analysis. Tissues were homogenized in 4 M guanidine isothiocyanate solution. Total RNA was extracted according to the method reported by Chomczynski & Sacchi (27). Denatured 20 μg RNA was separated by formaldehyde-agarose gel electrophoresis and transferred to a nylon membrane (Zeta-Probe, BioRad). Before hybridization with the inhibin α- or β_A-subunit cDNA probe, the membrane was incubated at 42°C for 4 h in a solution containing 50% (vol/vol) formamide, 50 mM NaH_2PO_4 (pH 6.5) , 5 × SSC (1× SSC = 0.15 M NaCl, 15 mM sodium citrate, pH 7.0), 5 × Denhardt's solution, 0.1% sodium dodecyl sulfate (SDS) and 300 μg/ml denatured herring sperm DNA at 42°C for 4 h. The filter was then hybridized at 42°C for 16 h in the above buffer, which contained ^{32}P-labeled inhibin α- or β_A-subunit cDNA probe. After hybridization, the filter was washed with a solution with 5 × SSC and 0.1% SDS for 10 min each time. The filter was dried briefly and exposed to X-ray film (Reflection NEF, Dupont). Hybridization with a human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used to make internal mRNA control. The density of each G3PDH band was expressed as a ratio to that of the band on lane 1 in eCG group as control, and the densities of the corresponding bands after hybridization with the inhibin probe was divided by the ratio to obtain corrected values.

Statistical analysis. Data are presented as the mean ± S.E. of the number of animals. The significance of the difference was tested with an unpaired Student's *t*-test or Cochran-Cox test (two-tailed). Analysis of variance followed by Tukey's multiple range test was employed in experiments requiring multiple comparisons. Differences of $p < 0.05$ were considered as statistically significant.

RESULTS

Changes in the Serum Levels of Inhibin and Estradiol during eCG-Induced Follicular Development in Tx Rats

There was no significant difference in the levels of inhibin and estradiol between Intact and Tx groups just before eCG treatment at 26 days of age. (Fig.1). In eCG-primed Tx (Tx+eCG) rats, serum inhibin and estradiol levels 24 and 48 h after eCG treatment were significantly higher than those in eCG-primed (eCG) rats. The serum levels of inhibin in Tx+eCG group reached the maximal level within 24 h and the level was maintained until 48 h. The level of estradiol in Tx+eCG group was markedly increased from 24 to 48 h. The serum levels of both hormones at 48 h in Tx+eCG group were 3.0 fold higher than those in eCG group. An increase in ovarian weight was also noted at the same time (eCG: 32 ± 1.3 mg *vs.* Tx+eCG: 37 ± 2.1 mg, $p<0.05$). However, the levels of progesterone at 24 h after eCG treatment showed no difference between eCG group and Tx+eCG group (data not shown). The values of those hormones were almost the same between eCG group and sham operated eCG group, indicating that the effect was not due to anesthesia and/or surgery (data not shown).

Effects of Thyroxine Replacement on Secretion of Inhibin and Estradiol and Ovarian Weight in Tx Rats

In order to determine whether the elevations in the serum levels of inhibin and estradiol after eCG treat-

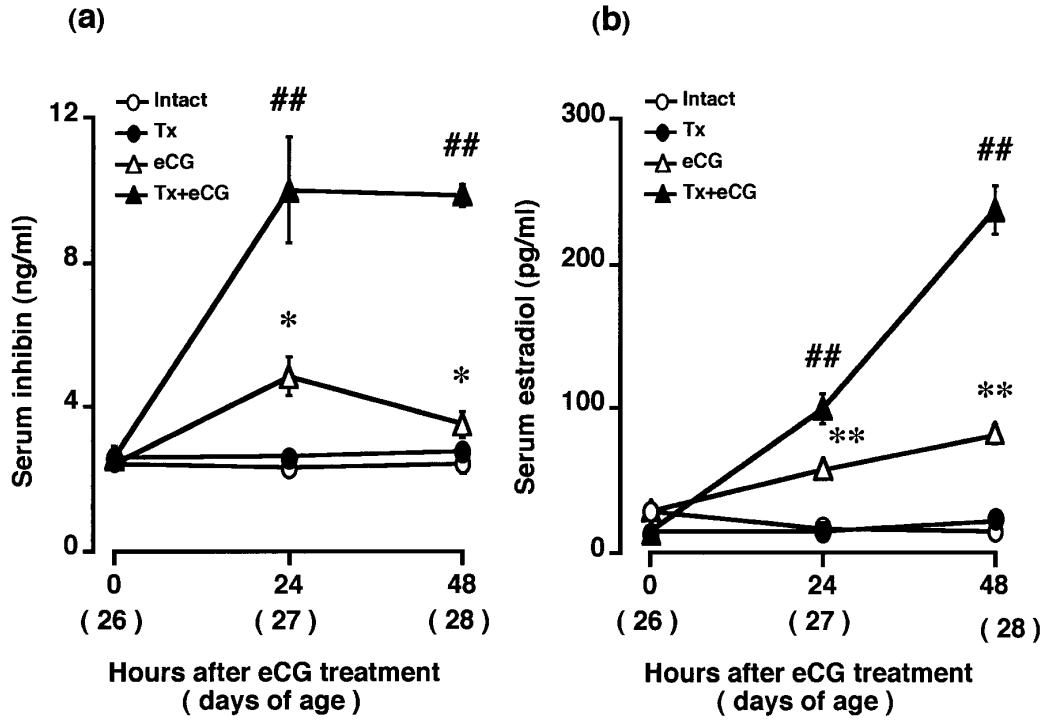

FIG. 1. Effects of thyroidectomy (Tx) on the serum levels of inhibin (a) and estradiol (b) in eCG-primed immature female rats. Tx was performed at 22 days of age. Animals were given a single s.c. injection of eCG (5 IU) at 26 days of age. Blood was collected via the abdominal aorta under ether anesthesia at 27 and 28 days of age (24h and 48 h after eCG treatment). Each point represents the mean ± S.E. of 5 or 6 animals. *p<0.05, **p<0.01; Significantly different from intact. ##p<0.01; Significantly different from eCG.

ment in Tx+eCG group were restored with administration of exogenous thyroid hormone, five μg thyroxine (T_4) were injected from day 22 to 27, and blood samples were collected on day 28 (Fig. 2). Effects of T_4 on the serum levels of ovarian hormones were examined at 48 h after eCG treatment. The increases in serum levels of each ovarian hormone and ovarian weight in Tx+eCG group were withdrawn by T_4 treatment to almost the same levels of eCG group (Ovarian weight: Tx+eCG+T_4: 31 ± 0.7 mg *vs.* Tx+eCG: 37 ± 2.1 mg, p<0.05, eCG: 32 ± 1.3 mg).

Influence of Thyroidectomy on Ovarian Follicular Development

Serial sections of ovaries which were removed at 0 and 48 h after eCG treatment were stained with hema-

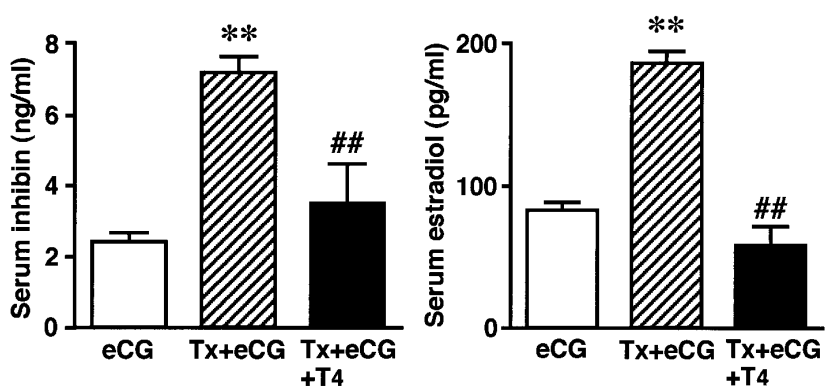

FIG. 2. Effects of thyroxine (T_4) replacement on the serum levels of inhibin and estradiol in thyroidectomized immature female rats. Thyroidectomy (Tx) and eCG treatment were performed as described in Fig 1. Animals were injected i.p. with T_4 (5 μg/rat) once a day for 6 days (total 6 injections) after Tx (Tx+eCG+T_4 group). Blood was collected under ether anesthesia at 48h after eCG treatment. Each value shows the mean ± S.E. of 5-6 rats. **p<0.01; Significantly different from eCG. ##p<0.01; Significantly different from Tx+eCG.

TABLE 1

Effects of Thyroidectomy on Follicular Size and the Number of Follicles in eCG-Primed Rat Ovary

Groups	Size of follicles (μm) 200–400	>400	Total >200	Atretic follicles >200
0 h				
Intact	55.0 ± 2.88	13.4 ± 1.72	68.4 ± 2.29	51.8 ± 4.84
Tx	44.6 ± 3.62	9.0 ± 0.89	53.6 ± 9.06	36.0 ± 3.90*
48 h				
eCG	41.4 ± 3.12	11.8 ± 1.53	53.2 ± 3.79	61.6 ± 8.80
Tx+eCG	44.2 ± 6.37	24.4 ± 1.47**	68.6 ± 5.30	43.8 ± 3.22
Tx+eCG+T4	40.3 ± 7.31	10.7 ± 2.19***	51.0 ± 8.02	56.3 ± 5.49

Note. Thyroidectomy (Tx), eCG and T_4 treatment were performed as described in the Figs. 1 and 2. Ovaries were removed before eCG treatment (0 h) or 48 h after eCG treatment. All follicles which were larger than 200 μm in diameter were counted. Each value shows the mean ± S.E. of 3-5 rats.

* $p<0.05$.

** $p<0.01$; Significantly different from intact or eCG.

*** $p<0.01$; Significantly different from Tx+eCG.

toxylin-eosin. The total number of healthy follicles larger than 200μm in diameter before eCG treatment was not significantly different between intact and Tx groups. However, the total number of atretic follicles which were larger than 200 μm in diameter was reduced by Tx (Intact *vs.* Tx, 0 h) (Table 1). At 48 h after eCG treatment, 2 fold of the number of ovarian follicles larger than 400 μm in diameter was observed in Tx+eCG group, compared to that of eCG group. Total number of follicles larger than 200 μm in diameter and atretic follicles in Tx+eCG group tended to increase or decrease when compared to eCG group, respectively. Further, changes in the number of those follicles in Tx+eCG group were restored to the level in eCG group with T_4 treatment (Tx+eCG+ T_4).

The hCG-Induced Ovulation in the Process of Follicular Development in eCG-Primed Tx and Non-Tx Rats

To determine whether thyroidectomy causes the changes in the number of mature follicles which can be ovulated on preovulatory day, 10 IU of hCG was injected to animals 33h (1700h on day 27) after eCG treatment and the number of oocytes ovulated in oviduct was counted (Table 2). Ovulation was confirmed all animals by hCG treatment in Tx and non-Tx (Control) groups and the number of oocytes was markedly increased about 1.7 fold in Tx group, compared to control group.

Northern Analysis of mRNAs for Inhibin α- and β_A-Subunits

Total RNA from rat ovaries at 48 h after eCG treatment was examined by northern analysis for detecting mRNA for inhibin. Hybridization with each cDNA probe of inhibin α- and β_A-subunits resulted in a single band of specific hybridization at 1.5 and 6.8 kilobase (kb) , respectively (Fig. 3a). The same blotted membrane were then hybridized with the human G3PDH probe and corrected values were calculated for the relative levels of inhibin gene expression. The values of inhibin α- and β_A-subunit mRNA signals were increased 1.5- and 2.3 fold by thyroidectomy, respectively. The increase in the hybridization signals in Tx+eCG group were suppressed to the almost same values of eCG group by T_4 administration (Fig 3b).

DISCUSSION

The present study is the first report which mentions the fact that thyroidectomy in immature female rats enhances the secretion of ovarian hormones (inhibin

TABLE 2

The Number of Oocytes on Enforced Ovulation with hCG Treatment at the Preovulatory Stages in Thyroidectomized Immature Rats

Groups	Ovulating rats/ rats examined	No. of oocytes in ovulating rats	Ovarian weight (mg)
eCG	5/5	9.4 ± 1.40	37.5 ± 1.46
Tx + eCG	5/5	16.4 ± 0.81***	40.4 ± 1.35

Note. Thyroidectomy (Tx) and eCG treatment were performed as described in Fig. 1. Animals were given a single i.p. injection of 10 IU of hCG at 1700 h on day 27 and sacrificed at 0800 h on day 28. The extent of ovulation was determined by counting the oocytes in the oviduct. Each value shows the mean ± S.E. of 5 rats.

*** $p<0.001$; Significantly different from eCG.

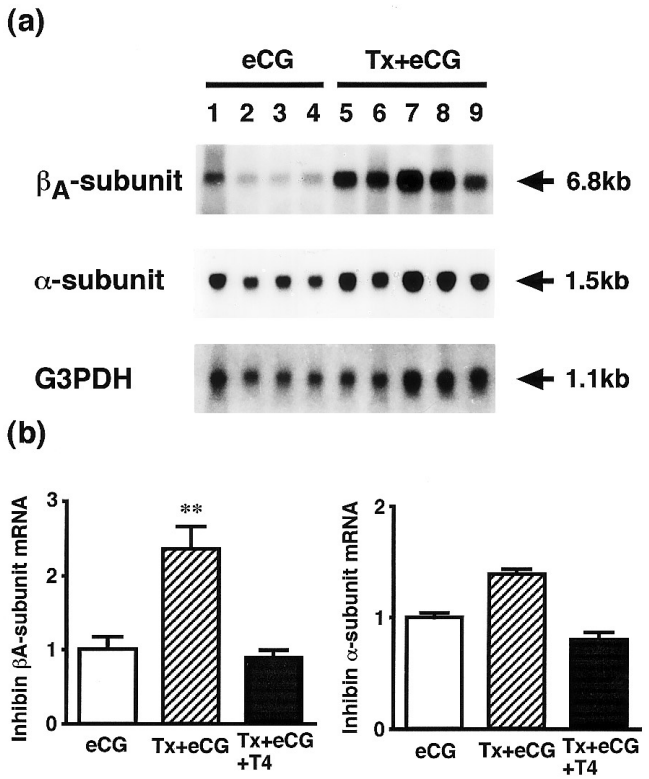

FIG. 3. Effects of thyroidectomy (Tx) and thyroxine (T_4) replacement on the expression of mRNA for ovarian inhibin α-and β_A-subunits in eCG-primed immature female rats. Tx, eCG and T_4 treatments were performed as described in Figs. 1 and 2. At 48 h after eCG treatment, ovarian total RNA was extracted and 20 μg RNA was subjected to northern analysis. (a) Representative Northern blot analysis of ovarian inhibin subunits mRNA in eCG (lane 1-4) and Tx+eCG (lane 5-9) rats. For standardization, blots were stripped and reprobed with a human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) cDNA probe. Each lane shows the result from individual animal. (b) Densitometric analyses of mRNA for inhibin α-and β_A-subunits detected by northern blot analysis. All results which were obtained by three experiments of northern blotting were analyzed. The mean value of mRNA in eCG group is represented as 1.0. Each column represents the mean ± S.E. of 9-10 rats. **$p<0.01$; Significantly different from eCG. ##$p<0.01$; Significantly different from Tx+eCG.

and estradiol) during ovarian development induced by eCG. The serum levels of the ovarian hormones were significantly increased in Tx+eCG group compared to those in eCG group. Further, high levels of both ovarian hormones in Tx animals were recovered to control levels by T_4 treatment. The increase in the plasma levels of the ovarian hormones in Tx animals was induced only after eCG administration. These results indicate that a certain important interaction exists between the actions of T_4 and gonadotropin in influencing the fate of developing follicles. To elucidate the mechanism for the enhancement of inhibin production in Tx rats after eCG administration, morphological changes and northern analyses of mRNAs for inhibin were examined. The number of healthy antral follicles in intact group and that in Tx group before eCG treatment were almost the same, although the number of atretic follicles was reduced by Tx. However, at 48 h after eCG administration, the number of healthy antral follicles larger than 400μm in diameter, which can be ovulated by the physiological ovulatory dose of hCG, was significantly increased over the normal number of oocytes in Tx+eCG group. The increase in the number of large antral follicles seems to be associated with the elevation in serum inhibin and estradiol levels observed in Tx animals 48 h after eCG treatment. As one of the possibilities, it is thought that the reduction of atretic follicles in Tx group before eCG injection might contribute to the increment of healthy follicles after eCG treatment in Tx animals. The expression of mRNAs for inhibin subunits in the ovary was also increased by thyroidectomy and suppressed by administration of thyroid hormone. The increase in serum inhibin levels in Tx rats is probably caused by stimulation of inhibin gene expression in ovarian follicles as well as by an increase in the number of follicles. There is a possibility that the local high concentration of ovarian inhibin in Tx animals might trigger ovarian folliculogenesis, because Woodruff *et al.* (19) have shown that recombinant human inhibin directly increases the follicular diameter in immature rat ovaries. Basal and FSH-induced aromatase activities were inhibited by thyroid hormone in rat Sertoli cells (28) and in porcine granulosa cells (29). It is strongly suggested that the decrease in the levels of serum thyroid hormone caused by thyroidectomy probably leads to an elevation of ovarian aromatase activities, and then enhances estradiol production as shown here. Further, our results from T_4 replacement suggest that endogenous thyroid hormone directly affects the ovarian hormones secretion through nuclear thyroid hormone receptors in granulosa and theca cells since T_3 receptors exist in these cells (7-9). However, we cannot exclude the local effects of other endocrine or autocrine factors, such as vasoactive intestinal peptide (VIP) (30, 31) and thyroid stimulating hormone (TSH) (32), elevated by thyroidectomy.

Although thyroidectomy induces an elevation of inhibin and estradiol secretion, this does not necessarily imply that the deprivation of thyroid hormone enhances normal follicular development and the secretion of both hormones. Supporting the present study, Copmann & Adams (33) have reported that hypothyroidism in immature rats changed ovarian sensitivity to gonadotropin and increased FSH binding, leading to polycystic ovaries *in vivo*. If the ovaries in Tx animals have such an abnormal responsibility to FSH in the present study, the stimulation of both hormones secretion by thyroidectomy may be due to the enhancement of ovarian sensitivity to gonadotropin. Chronic effects of 6-

propyl-2-thiouracil (PTU) on ovarian development have been recently reported (34), and the authors concluded that an inadequate thyroid hormone supply finally resulted in the disturbance of folliculogenesis after daily administration of PTU from birth to day 40 postpartum, because hypothyroidism suppressed the differentiation, not the proliferation of granulosa cells in prepubertal rats. In PTU-treated male rats, transient prepubertal hypothyroidism causes an increase in testis size, sperm production at the adult phase(11-15). The increased testis size was also caused by a prolongation of the period of Sertoli cell proliferation, and the prolongation was accompanied by a delay in the differentiation of the cells (14,15). It was shown that there was a critical period when hypothyroidism was effective for an increase in testis size (35), suggesting that the action of thyroid hormone on the testicular function suppressively regulates the process of testicular development. Therefore, a stagnation of granulosa cell differentiation induced by Tx might also be related with the increase in the levels of inhibin mRNAs and inhibin secretion. As for the long term effect of hypothyroidism, we recently observed that thyroidectomy induced a decrease in pre-ovulatory gonadotropin surge on the afternoon of the first proestrus (day 28) and then suppressed the first ovulation (data not shown).

Maruo *et al* (8) showed that thyroid hormone directly stimulated the differentiation and functions of granulosa cells in mature porcine ovary. Similarly, there is a report indicating that thyroid hormone stimulates rat testicular maturation and production of inhibin *in vitro* (36). Although our data in the present study seem to be opposed to those earlier studies, and the reason cannot be explained at present, the difference between the present data and the earlier published results might be due to the difference in experimental conditions such as the degree of maturation of ovarian cells and the dose of T_4 used, as well as the difference between *in vitro* and *in vivo* studies.

In conclusion, the present study indicates that gonadotropin treatment of hypothyroid rats induces greater responses in terms of the number of antral follicles, the production of inhibin subunits and their mRNAs and the ovarian weight compared to control animals, and that the augmentation of inhibin production in thyroidectomized animals is mediated partially through the stimulation of inhibin subunit gene expression in ovarian cells, which is suppressed by T_4. These data, therefore, suggest that thyroid hormone has inhibitory action on gonadotropin-stimulated ovarian development in immature female rats.

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