Blockage of gonadotropin-induced first ovulation caused by thyroidectomy and its possible mechanisms in rats

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The direct or indirect relationship between the thyroid gland and reproductive organs has been documented on the basis of in vivo and in vitro studies (25, 34). Hypothyroidism is closely associated with the changes in folliculogenesis and the formation of corpus luteum in rats, and thyroid dysfunction causes disorders in ovarian functions in women. When mature female rats are thyroidectomized, estrous cycles become irregular and their ovaries become atrophic (25). It has been shown that the response of ovaries to human chorionic gonadotropin (hCG) was enhanced in hypothyroid rats, and ovaries turned into large cystic forms (5, 13). A single injection of equine chorionic gonadotropin (eCG) in prepubertal rats induces the first ovulation between 9 and 12 h after autonomous ovulatory luteinizing hormone (LH) surge, which occurs ~57 h after eCG treatment (10). In our previous study, thyroidectomy (Tx) in eCG-primed immature rats markedly stimulated the secretion of ovarian hormones at least until 48 h after eCG treatment (36). In eCG-primed thyroidectomized rats (Tx rats), the number of mature follicles, which were ovulated by an injection of hCG at a dose comparable to the physiological LH dose at the ovulatory LH surge, was significantly increased by ~75% at 33 h after eCG treatment compared with control (eCG-treated non-Tx rat). In the present study, we examined the effects of hypothyroidism on the eCG-induced first ovulation in immature rats. In addition, serum concentrations of gonadotropin, prolactin, and inhibin were measured to determine whether the effects were accompanied by changes in the levels of pituitary or ovarian hormones. We show here, together with the possible mechanisms, that prepubertal hypothyroidism results in the blockage of the first ovulation induced by a single injection of eCG.

MATERIALS AND METHOD

Animals. Immature female rats of the Wistar strain were maintained under controlled temperature (23 ± 1°C) and humidity (55 ± 5%) and a 12:12-h light-dark lighting schedule (lights on at 0700), with free access to laboratory rodent chow and water. Tx was performed under ether anesthesia at 22 days of age.

Drug treatment and experimental schedule. To induce earlier puberty, immature rats were injected subcutaneously with 5 IU eCG (Teikoku Hormone, Tokyo, Japan) dissolved in 0.2 ml saline at 0800 at 26 days of age. To examine ovarian responsiveness to gonadotropin for the first ovulation, 10 IU hCG (Teikoku Hormone) were injected intraperitoneally at 1700 on day 28. To examine pituitary responsiveness to luteinizing hormone-releasing hormone (LHRH), 1 µg of LHRH (National Institute of Diabetes and Digestive and Kidney Diseases, National Hormone and Pituitary Program) was injected intravenously at 1600 on day 28. Blood samples were collected via the abdominal aorta under ether anesthesia at 0800 on day 28 for prolactin and at 1700 on day 28 for gonadotropins and inhibin. Blood was allowed to clot at 4°C. Serum samples were separated by centrifugation and stored frozen at ~80°C until assay for each hormone. The occurrence of ovulation on day 29 was determined by examining whether oocytes were present in the ampulla of oviducts, using a dissecting microscope.

RIA of LH, follicle-stimulating hormone, inhibin, and prolactin. Concentrations of gonadotropins, inhibin (35, 41), and prolactin (37) were determined by RIA as previously reported. The intra- and interassay coefficients of variation were 8.6 and 9.8% for LH, 5.7% and 20.4% for follicle-stimulating hormone (FSH), 5.1% and 11.0% for inhibin, and 9.8 and 20.6% for prolactin, respectively.

Immunohistochemistry of inhibin. To evaluate the immunohistochemical localization of inhibin, paraffin-embedded ovarian sections were stained using antisera against inhibin α-subunit, as previously reported (31). Briefly, sections in
which endogenous peroxidase activity was quenched by incubation with 3% H2O2 in methanol and were incubated with Block Ace (Dainippon Pharmaceutical, Osaka, Japan) to prevent nonspecific reactions. Sections were incubated with a polyclonal antibody against inhibin α-subunit at 37°C overnight. Polyclonal rabbit antibody to inhibin was developed against Tyr56-labeled inhibin α-chain (1–30) conjugated to rabbit serum albumin. Subsequently, sections were incubated with 0.5% biotinylated goat anti-rabbit secondary antibody (ABC-serum albumin. Subsequently, sections were incubated with a poly- Ace (Dainippon Pharmaceutical, Osaka, Japan) to prevent nonspecific reactions. Sections were incubated with an unpaired Student’s t-test or Cochran-Cox test (2-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 statistically significant.

RESULTS

Effects of Tx on eCG-induced autonomous first ovulation and influence of exogenous ovulatory hormones on first ovulation. To examine the first ovulation in the Tx group, the occurrence of ovulation was checked when the rats were 29 days old (72 h after eCG treatment; Table 1). The number of ovulating animals in the Tx-eCG group was decreased (40%), although the number of oocytes in ovulating rats was the same as that in the intact-eCG group. We then examined the effects of hCG or LHRH on the suppression in the rates of ovulating animals in the Tx groups. Injection of hCG and LHRH was performed at 1700 on day 28, that is, around the time at which endogenous ovulatory gonadotropin surge occurs (41), and at 1600 on the same day, which is considered to be just after “the critical period” in first proestrus (16). Treatment with hCG or LHRH restored the rates of ovulating animals in the Tx group up to \( > 80\%\), although the ovulation was not completely recovered. There was no statistical difference in the number of ovulations per ovulating rat among all groups. Ovarian weight in the Tx-hCG group was significantly larger than that in the intact group or intact-hCG group.

Effect of Tx on eCG-induced follicular development. To determine whether Tx influences the localization of inhibin as an indicator of follicular development, ovarian sections were stained with an antibody against inhibin α-chain (Fig. 1). Positive staining of inhibin α-subunit was seen in granulosa cells of developing follicles at all stages, including primary to tertiary follicles at 0 h in both groups. At 48 h after eCG treatment, immunostaining was also detected in healthy and atretic follicles of various sizes. Some other cell types (theca interna and interstitial cells) also showed a modest staining. Antral follicles larger than 400 μm in diameter were increased in the Tx group, and granulosa cells of the follicles also had a positive staining. Tx significantly decreased the body weight on day 28, which is 6 days after operation compared with intact rats (Table 2). It is well known that attainment of a critical body weight is necessary for normal puberty to occur (3, 8). To determine whether the body weight in Tx animals influences the occurrence of ovulation in the present model, we checked both body weight and ovarian weight in ovulating rats and nonovulating rats. The weight of the animals that did not ovulate was almost the same as the weight of those that ovulated (58 ± 1.7 vs. 56 ± 1.6 g, 72 h after eCG), indicating that a decrease in the body weight in Tx rats played no role in the blockage of ovulation. An increase in ovarian weight was observed in the Tx-eCG group, whereas a decrease in uterine weight was seen in this group on the same day compared with that in the intact-eCG group.

Effects of Tx on the serum levels of gonadotropins, inhibin, and prolactin 2 days after eCG treatment. To confirm the effects of Tx on the secretion of gonadotropin and the effects of LHRH on its levels, serum concentrations of LH and FSH were measured at 1700 on day 28 (Fig. 2). Serum levels of LH and FSH in the Tx-eCG group were significantly lower than those of control (intact-eCG group) at 1700 on day 28. Treatment with LHRH on Tx animals enhanced the serum concentrations of LH, but not FSH, up to control levels. At the same time, the serum levels of inhibin in the Tx-eCG and Tx-eCG + LHRH groups were higher than those in the intact-eCG group (Table 3). We then examined whether the suppression in preovulatory LH surge in the Tx-eCG group is associated with the serum levels of prolactin. When serum levels of prolactin were measured on the morning of first proestrus (day 28) before gonadotropin surge (Fig. 3), the serum levels of prolactin in the Tx-eCG group were 2.2-fold higher than those in the intact-eCG group.

Table 1. Effects of thyroidectomy and hCG and LHRH treatment on first ovulation in eCG-primed immature rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Ovulating Rats/Rats Examined</th>
<th>Rate of Ovulation, %</th>
<th>No. of Oocytes in Ovulating Rats</th>
<th>Ovarian Weight, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact-eCG</td>
<td>10/10</td>
<td>100</td>
<td>10.1 ± 0.48</td>
<td>36.0 ± 2.46</td>
</tr>
<tr>
<td>eCG + hCG</td>
<td>10/10</td>
<td>100</td>
<td>9.4 ± 0.43</td>
<td>33.1 ± 3.93</td>
</tr>
<tr>
<td>Tx-eCG</td>
<td>4/10</td>
<td>40</td>
<td>11.5 ± 0.29</td>
<td>39.2 ± 2.56</td>
</tr>
<tr>
<td>eCG + LHRH</td>
<td>14/17</td>
<td>82</td>
<td>14.3 ± 1.97</td>
<td>49.0 ± 1.86*†</td>
</tr>
<tr>
<td>eCG + hCG + LHRH</td>
<td>4/5</td>
<td>80</td>
<td>11.3 ± 1.38</td>
<td>41.0 ± 2.06</td>
</tr>
</tbody>
</table>

Each value shows the mean ± SE of 5–17 rats. Tx, animals received thyroidectomy. Rats were given a subcutaneous injection of equine chorionic gonadotropin (eCG; 5 IU) at 26 days of age after they were thyroidectomized on day 22. Luteinizing hormone-releasing hormone (LHRH; 1 µg iv) and human chorionic gonadotropin (hCG; 10 IU ip) were injected 56 and 57 h after eCG treatment (1600 and 1700 on day 28), respectively. Animals were killed at 0800 on day 29. The number of ovulations was determined by counting oocytes in the oviduct. * \( P < 0.01\), significantly different from intact-eCG group. † \( P < 0.01\), significantly different from eCG + hCG of intact group.
DISCUSSION

Irregular menstrual cycles and amenorrhea are often induced in hypothyroid female patients (25, 34). The relationship between ovulation disorders, such as amenorrhea, menorrhagia, and luteal phase insufficiency, and thyroid functions has been suggested in humans and experimental animals. We recently showed that hypothyroidism induced an elevation in serum inhibin and estradiol, together with an acceleration in the growth of mature follicles, which are capable of ovulating on day 28 by exogenous hCG injected on day 27 (33 h after eCG treatment) in eCG-primed immature rats (36). Hypothyroidism stimulated the expression of mRNA for inhibin, and the stimulation was suppressed by thyroxine treatment up to control levels, suggesting that thyroid hormone has a direct inhibitory action on ovarian inhibin and estradiol secretion in gonadotropin-

Table 2. Effects of thyroidectomy on the weights of body, ovary, and uterus in eCG-primed immature rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Weight</th>
<th>Ovary, mg</th>
<th>Uterus, mg</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Body, g</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intact</td>
<td>70.2 ± 1.20</td>
<td>22.5 ± 0.88</td>
<td>59.4 ± 7.50</td>
</tr>
<tr>
<td>No additions</td>
<td>68.0 ± 0.80</td>
<td>30.9 ± 0.82*</td>
<td>102.1 ± 5.47*</td>
</tr>
<tr>
<td>eCG</td>
<td>56.5 ± 1.26*</td>
<td>19.2 ± 0.49</td>
<td>51.8 ± 4.89</td>
</tr>
<tr>
<td>Tx</td>
<td>58.6 ± 1.75‡</td>
<td>36.1 ± 1.87†</td>
<td>143.5 ± 6.82‡</td>
</tr>
</tbody>
</table>

Each value shows the mean ± SE of 5–11 rats. Thyroidectomy (Tx) and eCG treatment were performed as described in Table 1. Tissues were removed 48 h after eCG treatment (day 28). *P < 0.01, significantly different from intact-no eCG group. †P < 0.01 and ‡P < 0.05, significantly different from intact-eCG group.
primed prepubertal rats. However, in the previous study, it was not clarified whether the occurrence of the spontaneous first ovulation at 3 days after eCG treatment was affected by Tx or not. We then determined here the role of thyroid hormone in the induction of the first ovulation and hormonal changes in the process of ovulation. Our data demonstrated that Tx reduced the number of ovulating rats, and that the Tx-induced blockage of ovulation was mainly due to the suppression of preovulatory LH surge, because additional treatment with hCG or LHRH significantly increased the rates of ovulation.

Hypothyroidism has been shown to be associated with secondary hyperprolactinemia (38), which significantly increases the frequency of anovulation in patients. We observed the increased levels of prolactin on the day of first proestrus (day 28) in Tx animals. Similar to our results, there is evidence indicating that plasma prolactin levels are high in Tx rats with high levels of plasma estrogen (32, 43). An increase in prolactin levels causes the inhibition of normal LH levels in humans (9) and reduces the pituitary response to LHRH in rats (26, 33). The inhibition of LHRH secretion mediated by the elevation of prolactin levels may result in an impairment of preovulatory LH secretion on day 28 in the present model. Administration of LHRH on day 28 reversed the levels of serum LH up to control levels, although the levels of serum FSH could not be recovered. The inhibin levels in the Tx-eCG and Tx-eCG + LHRH groups on day 28 were significantly higher than control levels. Therefore, the suppression of preovulatory FSH secretion and the levels of FSH after LHRH treatment in Tx rats may be caused by high levels of peripheral inhibin, because the extent of FSH secretion strongly depends on the levels of circulating inhibin. Our results implicate that the suppression of LH surge in the Tx-eCG group is probably generated by the inhibition of LHRH secretion at the hypothalamus and/or the decrease in pituitary responsiveness to LHRH for LH release. Hypothyroidism resulted in an increase in the concentration of vasoactive intestinal peptide (VIP) in the pituitary (24). Because thyrotropin-releasing hormone (TRH; see Ref. 19) and VIP (1, 30) are thought to be regulators for prolactin secretion, the increased levels of VIP in the pituitary in addition to the increase in TRH in the hypothalamus might result in an elevation of prolactin. It has also been shown that corticotropin-releasing hormone (CRH), in which levels were increased by thiouracil treatment (39) and prolactin (42), may be an inhibitory factor for LHRH release at rat hypothalamus (6, 23). Figure 4 represents a possible hormonal mechanism for the blockage of ovulation in the eCG-primed immature Tx animals. A decrease in circulating thyroid hormones causes the elevation of pituitary prolactin levels, which is in part mediated by increases in the levels of TRH, thyroid-stimulating hormone, CRH and VIP. The increase in prolactin levels may inhibit LHRH secretion and/or LHRH action on LH secretion from the pituitary. Furthermore, Tx directly enhances the secretion of ovarian estrogen and inhibin as previously reported (36). Estrogen suppresses LHRH levels at the hypo-

### Table 3. Effects of thyroidectomy and LHRH treatment on serum levels of inhibin in eCG-primed immature rats

<table>
<thead>
<tr>
<th>Groups</th>
<th>Inhibin, ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact-eCG</td>
<td>1.9 ± 0.17</td>
</tr>
<tr>
<td>Tx-eCG</td>
<td>2.5 ± 0.18*</td>
</tr>
<tr>
<td>Tx-eCG + LHRH</td>
<td>2.5 ± 0.14*</td>
</tr>
</tbody>
</table>

Each value shows the mean ± SE of 5–17 rats. Thyroidectomy (Tx) and eCG treatment were performed as described in Table 1. LHRH (1 µg iv) injection was performed 56 h after eCG treatment (1600 on day 28), and blood was collected 1 h after LHRH treatment. *P < 0.05, significantly different from intact-eCG group.
hormone by its negative feedback, and inhibit blocks FSH release from the pituitary.

Interestingly, in the previous study, all Tx animals were ovulated by hCG treatment at 33 h after eCG treatment, and the number of oocytes became 1.75-fold as great compared with control (36). In the present study, at 48 h after eCG treatment, we also observed that Tx induced an increase in the number of large healthy follicles and ovarian weight in Tx rats. However, the number of oocytes that were ovulated by hCG injection at 57 h after eCG treatment did not increase significantly. We could not clarify the reason why the effect of Tx on the number of oocytes in ovulating rats time dependently changes after eCG treatment. It is known that transient hypothyroidism in immature male rats causes an increase in testis size (12) and sperm production (11), and excessive 3,5,3'-triiodothyronine (T₃) administration promotes Sertoli cell differentiation and reduces the period of the cell proliferation (40). Our observations may indicate that Tx in immature rats temporarily and abnormally accelerates gonadotropin-induced follicular development and lifespan, that is, the number of Graafian follicles with normal maturity has already started to decrease around the time of preovulatory LH surge (57 h after eCG treatment) in Tx rats. The elevated levels in prolactin during the preovulatory period may also be associated with the reduction of oocytes. It was shown that ovulation in perfused ovaries was inhibited by prolactin treatment in rabbits (18) and that prolactin inhibited the luteinization of granulosa cells in rats (2) and reduced the steroidogenesis of the cells in humans (14) in vitro. Prolactin might have directly suppressed the reduction of oocytes. It was shown that during the preovulatory period may also be associated with Tx-induced hyperprolactinemia, which inhibits the sensitivity of the ovary to hCG and LH. In addition to the changes in prolactin levels, it is well established that Tx results in a decrease in the synthesis and secretion of growth hormone (GH; see Refs. 27, 29) and the gene expression of GH-releasing factor receptor (28) in rat pituitary. GH exerts numerous effects on ovarian differentiation through binding to specific GH receptors in rat ovary (7). For example, GH stimulates FSH-induced LH receptor synthesis (20, 21) and the activity of tissue plasminogen activator (4) in granulosa cells, suggesting a role of GH in ovulation events as a paracrine mediator. The ovary may fail to ovulate without the normal serum GH environment. Therefore, the partial recovery of ovulation induced by hCG and LHRH in Tx rats may be brought about by inevitable changes in serum GH levels caused by Tx. Tx decreased the normal increase in uterine weight after eCG treatment, although an elevation of estradiol occurred. It is known that T₃ potentiates estradiol-induced increases in the uterine weight (17, 22) via a mechanism involving cross talk with T₃ receptor in the uterus (15), suggesting a “permissive” role of T₃ for estrogen action. This result may imply that T₃ is essential for the normal uterine development induced by eCG in the presence of estradiol, because we can assume that the levels of estradiol in both eCG and Tx-eCG groups were high enough to induce the maximal response to uterine weight.

In conclusion, the present study suggests that hypothyroidism inhibits the first ovulation in eCG-primed immature female rats and that the blockage of ovulation is mainly mediated through the inhibition of preovulatory LH surge from the pituitary.

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