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Changes in ovarian steroidogenesis in insulin-resistant, type 2 diabetic Goto–Kakizaki rats after thyroidectomy and gonadotropin treatment

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Abstract

The present study used thyroidectomized insulin-resistant, type 2 diabetic Goto–Kakizaki (GK) rats to assess whether insulin resistance and hypothyroidism modulate ovarian physiology. Animals were treated with daily injections of 5 IU equine chorionic gonadotropin for 5 days starting 1 week after thyroidectomy. Control groups included rats of GK and control (Wistar) strains treated only with equine chorionic gonadotropin or thyroidectomy, or with no treatment (intact). In Wistar rats, equine chorionic gonadotropin injections tended to increase the serum concentrations of luteinizing hormone (LH) and testosterone more in the thyroidectomy group than in intact rats. Similar changes in LH and testosterone were observed in the thyroidectomy+equine chorionic gonadotropin and equine chorionic gonadotropin groups of GK rats, but the LH and testosterone levels in the thyroidectomy+equine chorionic gonadotropin group were significantly higher in GK rats. Expression of ovarian LH receptor messenger RNA (mRNA) was enhanced by thyroidectomy. The LH receptor mRNA levels were significantly higher in the thyroidectomy+equine chorionic gonadotropin group of GK rats than in the corresponding group of control rats. These results indicate that hypothyroidism in animals with insulin resistance and type 2 diabetes promotes LH and testosterone secretions, and suggests that the enhanced-testosterone levels is partially mediated by the enhancement of LH receptor expression and an increase in the serum level of LH.

Keywords: Insulin resistance; Ovarian hormone; The luteinizing hormone receptor

1. Introduction

In humans, hypothyroidism is clinically associated with menstrual disorders, menstrual irregularity, sterility, decreased ability to become pregnant, and increased frequency of spontaneous abortions (Longcope, 1991; Stradtman, 1993). In adult rats, hypothyroidism influences normal follicular maturation (Bruni et al., 1975; Jiang et al., 2000) and gonadotropin secretion (Bruni et al., 1975), resulting in irregular estrous cycles. Thyroidectomy before puberty increases the number of antral follicles in rats (Tamura et al., 1998b), but blocks equine chorionic gonadotropin-induced first ovulation, mainly due to a reduction in the preovulatory luteinizing hormone (LH) surge (Tamura et al., 1998a). Our recent study showed that administration of propyl-2-thiouracil (PTU, an antithyroid agent) causes irregular estrus cycles and significant changes in reproductive hormone levels in rats (Hatsuta et al., 2004a). Such reproductive changes caused by hypothyroidism are counteracted by the administration of thyroid hormone (Hatsuta et al., 2004a).

Polycystic ovary syndrome (PCOS), which is characterized by oligo-anovulation, an elevated LH/FSH ratio, and hyperandrogenism, is often seen in women with hypothyroidism (Slowey, 2001). Ovarian cysts associated with hypothyroidism in infancy are probably caused by the effect of thyrotropin-releasing hormone (TRH) on the gonadotropin-releasing hormone (GnRH) receptor of the gonadotropes

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(Stradtman, 1993). Recent reports have suggested that ovarian cysts and other abnormalities of ovarian follicles may also be associated with insulin resistance (Belaisch et al., 2001; Pugeat et al., 2000). Insulin-sensitizing drugs, including troglitazone, improve the ovulatory dysfunction, hirsutism, hyperandrogenemia, and insulin resistance of polycystic ovary syndrome in humans (Azziz et al., 2001; Ehrmann et al., 1997). Although insulin resistance and hypothyroidism are associated with disorders of ovarian follicular development in humans, their involvement in reproductive failure is not fully understood in an animal model.

Goto–Kakizaki (GK) rats are a highly inbred strain of Wistar rats that exhibit mild hyperglycemia, glucose intolerance, impaired glucose-induced insulin secretion (Portha et al., 1991), and modest insulin resistance (Bisbis et al., 1993), and spontaneously develop type 2 diabetes (Goto et al., 1976). Adult GK rats are used as a model of non-insulin-dependent diabetes mellitus (NIDDM) and provide an opportunity to investigate the pathogenesis of insulin resistance in the reproductive system. GK rats have decreased fertility, although no reason has yet been ascertained (personal communication from Charles River Japan, Inc.), and there have thus far been no studies addressing ovarian function in GK rats. To determine whether hypothyroidism produces ovarian hormonal changes in GK rats, we have, therefore, investigated the effects of equine chorionic gonadotropin on the secretion of gonadotropin and ovarian hormones in thyroidectomized GK rats.

2. Materials and methods

2.1. Animals and experimental schedule

Six-week-old female Wistar and GK rats (Charles River Japan, Inc., Kanagawa, Japan) were maintained in an air-conditioned room (temperature 23 ± 1 °C and humidity $55\% \pm 5\%$) under controlled lighting (12-h light/day schedule), with free access to food and water. All procedures in the animal care and surgery protocol were approved by the Institutional Animal Care Committees at Tokyo University of Pharmacy and Life Science, in compliance with institutional guidelines for experimental animal care. Hypothyroidism was induced by thyroidectomy when the rats were 6 weeks old, using a procedure previously described in detail (Hatsuta et al., 2004b). Some thyroidectomized Wistar and GK rats (the thyroidectomy + equine chorionic gonadotropin group) received 5 IU equine chorionic gonadotropin (Teikoku Hormone MFG Co., Tokyo, Japan) injected subcutaneously (s.c.) at 1000 h around 1 week (6–8 days) after thyroidectomy, during diestrus; these injections continued once daily for 5 days. Three other groups of animals were also used: the equine chorionic gonadotropin group received equine chorionic gonadotropin but not thyroidectomy, the thyroidectomy group received thyroidectomy but not equine chorionic gonadotropin, and the intact group received neither. Blood was collected 24 h after the final injection of equine chorionic gonadotropin (the equine chorionic gonadotropin and thyroidectomy + equine chorionic gonadotropin groups) or 13 days after thyroidectomy (the thyroidectomy group) and allowed to clot at 4 °C. Serum was separated by centrifugation and stored frozen at −30 °C until assayed for hormones. The ovaries were homogenized with RNA extraction buffer and frozen in liquid nitrogen until semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) analysis.

2.2. Radioimmunoassay of gonadotropins and ovarian steroids

Serum concentrations of FSH and LH were measured with a National Institute of Diabetes and Digestive and Kidney Diseases radioimmunoassay (RIA) kit (provided by Dr. A.F. Parlow, Director, Pituitary Program and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA, USA), using antisera against FSH (S-11) and LH (S-10) (Hatsuta et al., 2004a). The results were calculated by comparison to a standard curve created with FSH-RP-2 or LH-RP-3. Serum steroid levels were also measured by RIA. Antisera against testosterone (GDN #250) and 17β-estradiol (GDN #244) were provided by Prof. G.D. Niswender (Colorado State University, Fort Collins, CO, USA) (Hatsuta et al., 2004a). Serum progesterone (P4) concentrations were measured by RIA using ^{3}H-labeled radioligands, as described previously (Tamura et al., 1991). The intra- and inter-assay coefficients of variation were less than 10% for all RIA assay data.

2.3. Analysis of LH receptor mRNA levels

Poly $(A)^{+}$ RNA was isolated with a QuickPreps micro mRNA purification kit (Amersham Biosciences, Buckinghamshire, England). Poly $(A)^{+}$ RNA (0.5 μg) was used for RT-PCR. The primers for the LH receptor were sense: 5′- CGAGTCCCAGCTCTG-3′ and antisense: 5′-ATAGTCTGG CGAGGCCGGCTCGAGGGCCAG-3′. The predicted length of the LH receptor mRNA fragment is 165 bp. The PCR products were subjected to electrophoresis in a 1.8% agarose gel and visualized under UV light with ethidium bromide. For Southern hybridization, the products were transferred to a nylon membrane and hybridized in a high sodium dodecyl sulfate buffer containing a digoxigenin-labeled cDNA probe (25 ng/ml) (Tamura et al., 2001). Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was used as an internal control. The bands on the Kodak scientific imaging film (X-OMAT XB-1, Eastman Kodak Co., Rochester, NY) were analyzed using NIH Image, and each value was normalized against that of the G3PDH band in the corresponding lane, as described previously (Tamura et al., 2003).

2.4. Data analysis and statistics

Data are represented as means ± S.E.M. The significance of the differences was determined using t tests and analysis of variance. The RT-PCR results that are shown represent single experiments. A value of $P<0.05$ was considered significant.

3. Results

3.1. Effects of thyroidectomy and/or equine chorionic gonadotropin treatment on the body weight and reproductive tracts of Wistar and GK rats

Both Wistar and GK rats that received thyroidectomy had significantly lower body weight than non-thyroidectomized rats in the Intact and equine chorionic gonadotropin groups (Table 1), indicating that the operations were successful. Equine chorionic gonadotropin treatment increased the weight of the ovaries, but there was no difference between the equine chorionic gonadotropin and thyroidectomy+equine chorionic gonadotropin groups. No statistical difference was found in the weight of the uterus among any of the groups, although the weights of the body, ovary, and uterus in GK rats tended to be lower than those in Wistar rats.

3.2. Effects of thyroidectomy and/or equine chorionic gonadotropin treatment on the serum levels of LH and FSH in Wistar and GK rats

This study evaluated the effects of 5 days of equine chorionic gonadotropin treatment on the serum levels of LH and FSH in Wistar and GK rats with hypothyroidism (Fig. 1). Treatment with equine chorionic gonadotropin increased serum LH levels in Wistar and GK rats. In GK rats, the thyroidectomy+equine chorionic gonadotropin group had significantly higher serum levels of LH than the equine chorionic gonadotropin group. Similar changes were observed in Wistar rats, but the difference was smaller and not significant. Further, the serum levels of LH in the thyroidectomy+equine chorionic gonadotropin group of GK rats were significantly higher than in the same group

Table 1
The weight of body, ovary and uterus in Wistar and GK rats received thyroidectomy and/or equine chorionic gonadotropin treatment

Groups		Weight		
		Body (g)	Ovary (mg)	Uterus
Water	Intact	180.3 ± 3.0	74.2 ± 4.0	354.5 ± 41.3
	eCG	187.5 ± 1.0	87.8 ± 4.5*	360.1 ± 22.3
	Tx	167.2 ± 2.8*	66.4 ± 4.4	326.3 ± 16.8
	Tx+eCG	164.3 ± 2.4*	82.5 ± 5.2**	358.6 ± 22.3
GK	Intact	175.6 ± 3.6	65.7 ± 2.2	285.9 ± 18.7
	eCG	171.1 ± 3.9	76.0 ± 2.4*	290.2 ± 13.5
	Tx	164.8 ± 1.6*	58.4 ± 2.0	265.4 ± 13.2
	Tx+eCG	160.9 ± 3.5*	74.5 ± 2.5**	281.8 ± 16.2

Thyroidectomy was performed on 6-week-old female rats. In the thyroidectomy+equine chorionic gonadotropin groups (Tx+eCG), the rats were given an s.c injection of equine chorionic gonadotropin (5 IU) for 5 days starting approximately 1 week after thyroidectomy. Animals in the equine chorionic gonadotropin (eCG) or thyroidectomy (Tx) groups received only equine chorionic gonadotropin treatment or thyroidectomy, respectively. Animals were sacrificed 24 h after the final injection of equine chorionic gonadotropin (the eCG and Tx+eCG groups) or 13 days after thyroidectomy (the Tx group). Each value shows the mean ± S.E.M. of 8 to 12 rats. Intact: untreated 8-week-old animals.

* $P<0.05$ vs. Intact in each animal species.

** $P<0.05$ vs. Tx in each animal species.

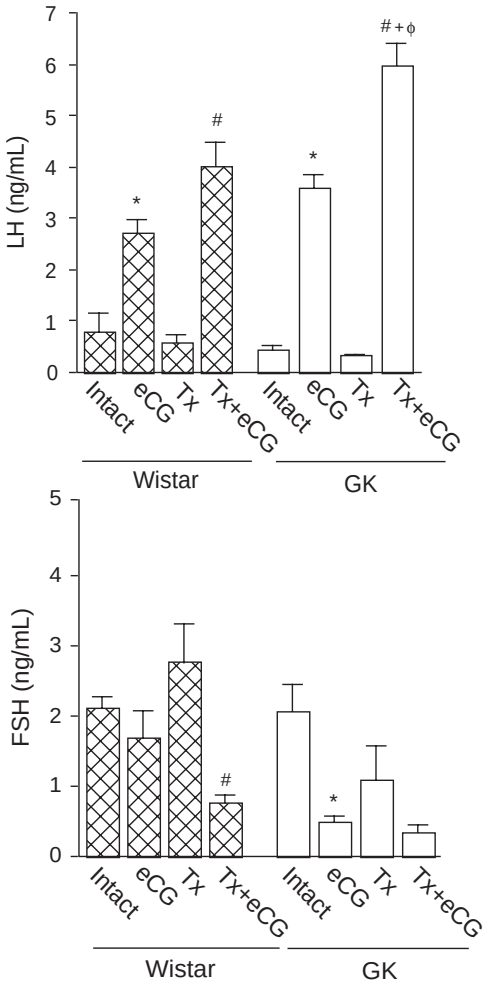

Fig. 1. Effects of thyroidectomy and/or equine chorionic gonadotropin treatment on the serum levels of LH and FSH in Wistar and GK rats. Thyroidectomy was performed at 6 weeks of age. Rats were given an s.c. injection of equine chorionic gonadotropin (5 IU) for 5 days starting 1 week after thyroidectomy (the thyroidectomy+equine chorionic gonadotropin groups). Animals in the equine chorionic gonadotropin group did not receive thyroidectomy and animals in the thyroidectomy group did not receive equine chorionic gonadotropin treatment. Blood samples were obtained 24 h after the last injection of equine chorionic gonadotropin (the equine chorionic gonadotropin and thyroidectomy+equine chorionic gonadotropin groups) or 13 days after thyroidectomy (the thyroidectomy group). Each value shows the mean ± S.E.M. of 8 to 12 rats. Intact: intact animals at 8 weeks old. Tx+eCG: the thyroidectomy+equine chorionic gonadotropin group. $^{*}P<0.05$, vs. Intact in the same strain of rats; $^{\#}P<0.05$, vs. Tx in the same strain; $^{+}P<0.05$, vs. eCG in GK; $^{\phi}P<0.05$, vs. Tx+eCG in Wistar.

of Wistar rats. In contrast, treatment with equine chorionic gonadotropin tended to decrease serum FSH levels in Wistar and GK rats. In all three treated groups, FSH levels tended to be lower in GK rats than in Wistar rats, although there were no differences in FSH levels between Wistar and GK rats in the intact groups.

3.3. Effects of thyroidectomy and/or equine chorionic gonadotropin treatment on the serum levels of ovarian steroids and inhibin in Wistar and GK rats

Fig. 2 shows the serum levels of testosterone and P4 in Wistar and GK rats 1 week after thyroidectomy. Serum testosterone levels increased following equine chorionic gonadotropin treatment in Wistar and GK rats, and were higher still with combined thyroidectomy and equine chorionic gonadotropin treatment, although thyroidectomy alone did not increase testosterone levels. Testosterone levels in the equine chorionic gonadotropin and thyroidectomy + equine chorionic gonadotropin groups in GK rats were higher than the levels in the respective groups in Wistar rats. In both strains of rat, P4 levels in the thyroidectomy + equine chorionic gonadotropin group were significantly increased compared with the levels in either the equine chorionic gonadotropin or thyroidectomy group. The serum levels of 17β-estradiol tended to rise after equine chorionic gonadotropin treatment in both strains (Fig. 3), and the levels of 17β-estradiol in the thyroidectomy + equine chorionic gonadotropin group were significantly higher than those in the equine chorionic gonadotropin group in Wistar rats. However, this difference was not significant in GK rats, and there were no significant differences between Wistar and GK rats in 17β-estradiol levels. The serum levels of inhibin were enhanced by thyroidectomy, but, similar to the 17β-estradiol findings, no difference was found in the levels between the Wistar and GK rats.

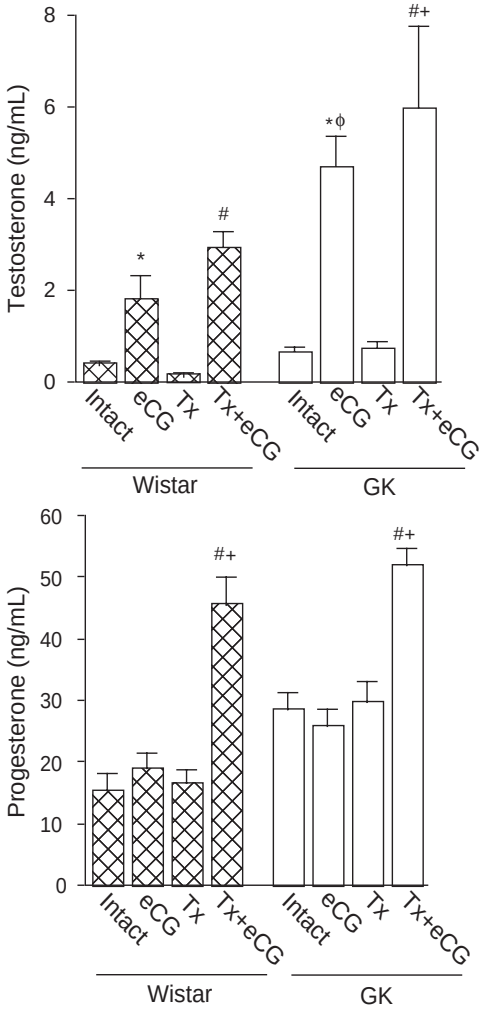

Fig. 2. Effects of thyroidectomy and/or equine chorionic gonadotropin treatment on the serum levels of testosterone and progesterone in Wistar and GK rats. Animal treatment and blood collection were performed as in Fig. 1. Each value shows the mean ± S.E.M. of 8 to 12 rats. Tx + eCG: the thyroidectomy + equine chorionic gonadotropin group. $^{*}P<0.05$, vs. Intact in the same strain; $^{\#}P<0.05$, vs. Tx in the same strain; $^{+}P<0.05$, vs. eCG in the same strain; $^{\phi}P<0.05$, vs. eCG in Wistar.

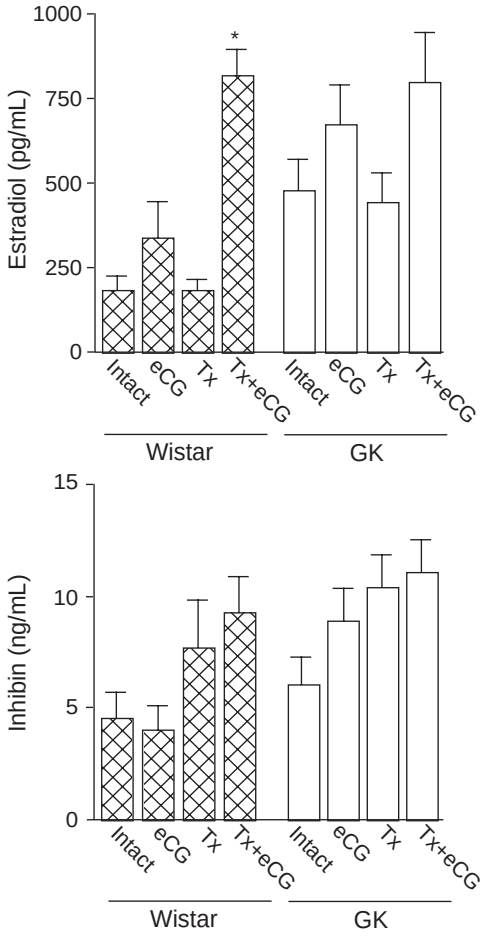

Fig. 3. Effects of thyroidectomy and/or equine chorionic gonadotropin treatment on the serum levels of estradiol and inhibin in Wistar and GK rats. Animal treatment and blood collection were performed as in Fig. 1. Each value shows the mean ± S.E.M. of 8 to 12 rats. Tx + eCG: the thyroidectomy + equine chorionic gonadotropin group. $^{*}P<0.05$, vs. Intact, eCG, or Tx in Wistar.

3.4. Influences of thyroidectomy and equine chorionic gonadotropin treatment on ovarian LH receptor mRNA levels in Wistar and GK rats

To compare the levels of the LH receptor mRNA in the equine chorionic gonadotropin and thyroidectomy+equine chorionic gonadotropin groups, we used RT-PCR to examine ovarian poly $(A)^+$ RNA on the day after the final equine chorionic gonadotropin treatment (Fig. 4). The blots in Fig. 4A and B show representative data for LH receptor mRNA expression; densitometric analysis of these data is shown in Fig. 4C. The levels of the LH receptor mRNA were increased by thyroidectomy in both animals. Furthermore, the LH receptor mRNA levels in the thyroidectomy+equine chorionic gonadotropin group was higher in GK rats than in Wistar rats.

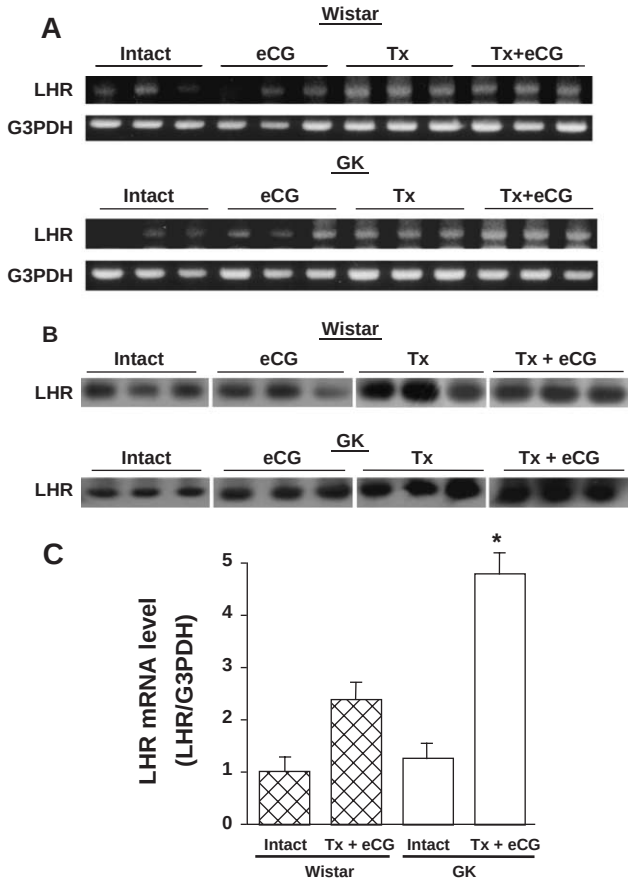

Fig. 4. Effects of thyroidectomy and/or equine chorionic gonadotropin treatment on the expression of ovarian LH receptor (LHR) mRNA in Wistar and GK rats. Thyroidectomy was performed on 6-week-old rats. Rats were given an s.c. injection of equine chorionic gonadotropin (5 IU) at 1000 h for 5 days after thyroidectomy. Ovaries were removed 24 h after the final injection of equine chorionic gonadotropin and ovarian poly $(A)^+$ RNA was extracted. Each lane shows the data obtained from 0.5 μg poly $(A)^+$ RNA from an individual animal. (A) Detection of LHR mRNA by RT-PCR. (B) Representative blot for LHR mRNA expression detected by Southern hybridization using a digoxigenin-labeled LHR probe. (C) Relative differences in mRNA abundance for LHR. Quantification of LHR mRNA levels detected by two independent experiments ($n=6$), including the data in B, was conducted using NIH Image. Expression of LHR mRNA was normalized for G3PDH expression. Tx+eCG: the thyroidectomy+equine chorionic gonadotropin group. *$P<0.05$, vs. Tx+eCG in Wistar.

4. Discussion

We designed this study to examine the differences in the secretory functions of the reproductive system between normal (Wistar) rats and GK rats, a diabetic strain with insulin resistance, by investigating the effects of thyroidectomy and equine chorionic gonadotropin on animals of both strains. Hypothyroidism has been reported to increase the formation of ovarian follicles including polycystic follicles (Copmann and Adams, 1981), the number of LH receptors and ovarian weight (Fitko et al., 1984) in rats. The body weight of thyroidectomized animals was significantly lower than that of non-thyroidectomized animals in both rat strains, regardless of equine chorionic gonadotropin treatment, because thyroid hormone is profoundly involved in body growth. Due to the induction of follicular development by equine chorionic gonadotropin, the ovarian weights increased in the equine chorionic gonadotropin and thyroidectomy+equine chorionic gonadotropin groups compared with the intact and thyroidectomy groups, respectively. Although there was no significant difference between the equine chorionic gonadotropin and thyroidectomy+equine chorionic gonadotropin groups in body, ovary, or uterus weights, these weights tended to be lower in GK rats compared to Wistar rats. In this study, GK rats had significantly greater elevations than Wistar rats in the serum levels of LH and testosterone after thyroidectomy and equine chorionic gonadotropin treatment. The levels of LH, testosterone, estradiol, and inhibin were enhanced more by equine chorionic gonadotropin in thyroidectomized animals than in non-thyroidectomized animals of both strains. The decreases in FSH levels in the thyroidectomy+equine chorionic gonadotropin groups of both species were possibly due to the enhanced inhibin levels. LH and testosterone levels after thyroidectomy and equine chorionic gonadotropin injection were significantly higher in GK rats than in Wistar rats. Further, the expression of ovarian LH receptor mRNA was stimulated by thyroidectomy in GK and Wistar rats, remarkably so in the GK rats. However, no differences were detected between Wistar and GK rats or between the thyroidectomy and thyroidectomy+equine chorionic gonadotropin groups in the mRNA expression of key enzymes for steroidogenesis (3β-hydroxysteroid dehydrogenase, steroidogenic acute regulatory protein [StAR], and cholesterol side chain cleavage enzyme [p450scc]; data not shown). LH activates the intracellular signals for steroidogenesis in the growing follicle or corpus luteum through the LH receptor (Ji et al., 1997; McFarland et al., 1989). The increases in the number of LH receptors or the enhancement of the responsiveness to LH in the ovary augment the production of ovarian steroids. The prominent

elevation in the serum levels of testosterone in GK rats may be due to the increase in ovarian LH receptor expression as well as the enhancement of pituitary LH secretion. Additional studies will be needed to determine why the difference is seen only in the levels of testosterone and not in the levels of other ovarian steroids. Future studies should also investigate the molecular mechanisms leading to the increase in LH receptor expression and the elevation of serum LH and testosterone levels in GK rats receiving thyroidectomy. The factors that cause changes in ovarian function might be insulin resistance, the diabetes itself, or a hereditary factor in GK rats. Insulin-sensitizing agents may be useful to determine the involvement of insulin resistance on these fluctuations.

Insulin resistance was recently suggested to be a trigger for polycystic ovary syndrome (Dunaif, 1997). It is well known that the patients with polycystic ovary syndrome show excess LH secretion and ovarian LH receptor expression (Jakimiuk et al., 2001; Patel et al., 2004). These clinical characteristics are similar to the changes in GK rats observed in this study. Fitko et al. (1984) suggested that treatment of hypothyroid rats with equine chorionic gonadotropin and human chorionic gonadotropin (hCG) for several days may provide a useful procedure for the induction of polycystic ovary syndrome. There are several rat models for polycystic ovary syndrome (Szukiewicz and Uilenbroek, 1998); however, a fully convincing animal model for the study of polycystic ovary syndrome has not been established. Although it is necessary to further investigate the endocrine changes in detail, including morphological changes in the ovary, the present GK model might be appropriate as a model of human polycystic ovary syndrome. There are some reports that abnormalities in LH secretion are observed in women who have insulin resistance (Butzow et al., 2000; Anttila et al., 1993), but the mechanism has not yet been clarified. Possibly, the compensatory increases in serum insulin levels induced by insulin resistance are involved in abnormal LH secretion in women (Slowey, 2001). Insulin promotes LH release in rat pituitary cells (Weiss et al., 2003) and stimulates testosterone production in ovarian thecal cells (Zhang et al., 2000). However, there was no significant difference in the serum levels of insulin between Wistar and GK rats after thyroidectomy and equine chorionic gonadotropin treatment in the present study (data not shown), although GK rats exhibit impaired glucose-induced insulin secretion (Portha et al., 1991). These results indicate that the increases in LH and testosterone observed here were not due to the elevation of serum insulin levels.

In conclusion, the present data indicate that acquisition of hypothyroidism in animals with insulin resistance and type 2 diabetes may promote LH secretion and ovarian testosterone production. The enhancement of ovarian LH receptor expression in addition to the increase in the serum level of LH probably results in the elevation of testosterone levels in thyroidectomized GK rats. Our results seem to suggest that hypothyroidism and insulin resistance cooperatively increase the production of ovarian testosterone.

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