Endocrine, *vol. 27, no. 3, 301–305, August 2005* 0969–711X/05/27:301–305/$30.00

Effect of Troglitazone on the Excess Testosterone and LH Secretion in Thyroidectomized, Insulin-Resistant, Type 2 Diabetic Goto–Kakizaki Rats

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Our previous study suggested that hypothyroidism in Goto–Kakizaki (GK) rats with insulin resistance and type 2 diabtetes elevates their serum testosterone and luteinizing hormone (LH) levels and ovarian LH receptor messenger RNA (mRNA) expression. The present study assessed the effects of troglitazone (Tro), an insulin-sensitizing agent, on these hypothyroidism-induced hormonal changes in GK rats. GK and normal (Wistar strain) female rats were thyroidectomized (Tx) and then injected with 5 IU of equine chorionic gonadotropin (eCG) for 5 d starting 1 wk after thyroidectomy (the control groups). In the test groups, Tx GK and Wistar rats were injected with both eCG and Tro (100 mg kg^{-1}) po for 5 d. Tro treatment had no effect on the elevated LH serum levels in eCG-treated Tx GK rats but suppressed their enhanced serum testosterone levels as well as significantly decreasing their LH receptor mRNA expression. Tro lowered testosterone and LH receptor mRNA levels in cultured theca cells. These results indicate that Tro lowers the elevated testosterone secretion and ovarian LH receptor mRNA expression that is induced in GK rats by Tx and gonadotropin treatment, which suggests that insulin resistance may be involved in enhancing testosterone production and LH receptor expression in the ovary.

Key Words: Troglitazone; testosterone; luteinizing hormone receptor; hypothyroidism; insulin resistance; Goto–Kakizaki (GK) rat.

Introduction

The thyroid is involved in normal ovarian physiology because hypothyroidism disturbs folliculogenesis *(6)* and results in irregular estrus cycles in rats *(14,20)*. Moreover, administration of the antithyroid agent propyl-2-thiouracil (PTU) causes irregular estrus cycles in rats and significantly changes their secretion of reproductive hormones *(14)*. Such hypothyroidism-induced reproductive changes can be counteracted by administering thyroid hormone. In humans, hypothyroidism is clinically associated with menstrual disorders, menstrual irregularity, sterility, decreased ability to become pregnant, and increased frequency of spontaneous abortions *(18,26)*.

It has been reported that polycystic follicles increase in number in the ovaries of rats with hypothyroidism that are treated with equine chorionic gonadotropin (eCG) *(4)*. Our recent study showed that continuous eCG administration after rats were thyroidectomized (Tx) increased their serum levels of testosterone and luteinizing hormone (LH) and enhanced their expression of the LH receptor (LHR) in the ovary *(27)*. Moreover, we observed that these changes are more prominent in Goto–Kakizaki (GK) rats, an animal model of non-insulin-dependent diabetes mellitus (NIDDM), than in Wistar (normal) rats. It is now recognized that insulin resistance leads to dysfunction of the reproductive organs and abnormal secretion of ovarian hormones *(8,29)*. For example, insulin resistance is associated with polycystic ovary syndrome (PCOS), a major disorder in premenopausal women that is characterized by anovulatory infertility and hyperandrogenism *(23,29)*. When the insulin resistance of these women is improved by treatment with the insulin-sensitizing agent troglitazone (Tro), the ovulatory disorder, hirsutism, and hyperandrogenism of this syndrome also improves *(2,9)*, although Tro has been withdrawn in clinical use because of its hepatotoxic side effects.

On the basis of these observations, we examined the effect of Tro on the eCG-stimulated ovarian physiology in Tx GK rats to determine whether insulin resistance contributes to the abnormal functions (testosterone secretion and LHR expression) of the ovary in animals with insulin resistance and type 2 diabetes.

Results

Effect of Insulin-Sensitizing Agents on the Elevated Testosterone and LH Serum Levels in eCG-Treated Tx GK Rats

The effect of Tro or Pio on the enhanced testosterone and LH levels that arise in GK rats after thyroidectomy and

Received March 31, 2005; Revised June 28, 2005; Accepted June 29, 2005.
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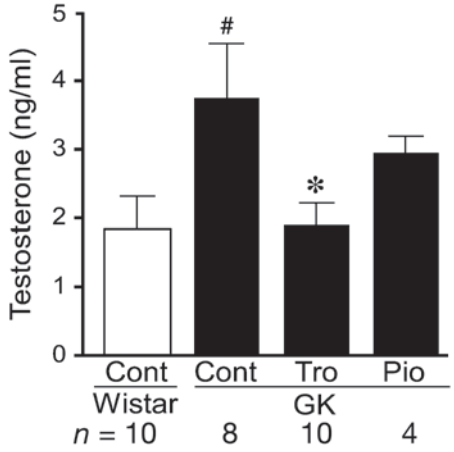

Fig. 1. Effect of Tro and Pio on serum testosterone levels in eCG-treated Tx GK rats. Thyroidectomy was performed at 6 wk of age and about 1 wk later, the Tx rats were given sc injections of eCG (5 IU) and the insulin-sensitizing drug Tro (100 mg kg^{-1}) or Pio (10 mg kg^{-1}) for 5 d at 10:00 h. The Wistar and GK control group animals received a thyroidectomy and were only treated with eCG (Cont). A blood sample was obtained 24 h after the last eCG injection. Each value is the mean ± SE of 4–10 rats. $^{\#}p < 0.05$ vs Wistar control; $^{*}p < 0.05$ vs untreated GK control.

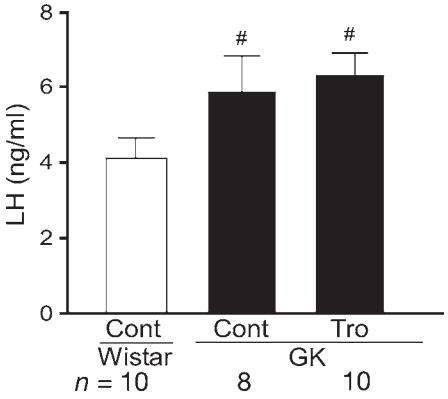

Fig. 2. Effect of Tro on serum LH levels in eCG-treated Tx rats. GK and Wistar rats were treated and bled as described in Fig. 1. Each value is the mean ± SE of 8–10 rats. $^{\#}p < 0.05$ vs Wistar control.

eCG treatment was determined. As expected, the testosterone and LH serum levels were higher in the eCG-treated Tx GK rats than in the eCG-treated Tx Wistar (control : background strain of GK) rats (Figs. 1 and 2). When the eCG-treated Tx GK rats were treated with Tro (100 mg kg^{-1}), their testosterone levels decreased to those observed in the eCG-treated Tx Wistar rats (Fig. 1). Tro had no effect on the testosterone levels of eCG-treated Tx Wistar rats (data not shown). Treatment with Pio (10 mg kg^{-1}), another insulin-sensitizing agent, also tended to decrease the testosterone levels, although this did not reach statistical significance (Fig. 1). In contrast, neither agent affected the high LH serum levels in eCG-treated Tx GK rats (Fig. 2).

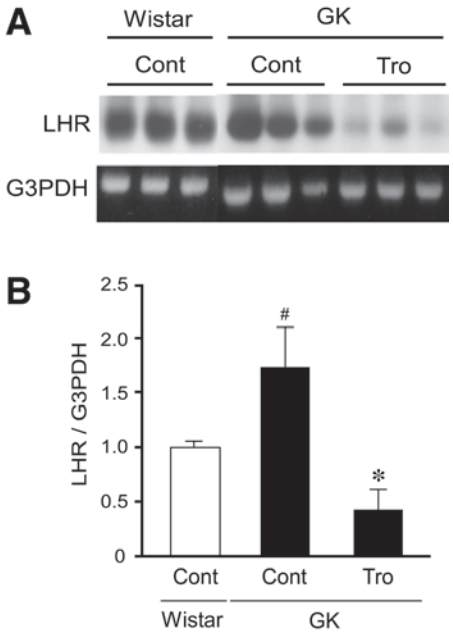

Fig. 3. Effect of Tro on ovarian LHR mRNA expression in eCG-treated Tx GK rats. GK and Wistar rats were treated as described in Fig. 1, sacrificed after the blood sample was taken and their ovaries were harvested. (**A**) LHR mRNA levels were determined by semiquantitative RT-PCR analysis using a DIG-labeled LHR cDNA probe. Each lane shows data obtained from an individual animal. (**B**) Quantitation of the LHR mRNA levels shown in A by using NIH Image. These values were normalized relative to G3PDH mRNA levels and expressed as a ratio relative to the density of the band in Wistar control. Each value is the mean ± SE of 6 rats. $^{\#}p < 0.05$ vs Wistar control; $^{*}p < 0.05$ vs untreated GK control.

Effect of Tro on the Elevated Ovarian LHR mRNA Expression in eCG-Treated Tx GK Rats

As observed previously, the ovaries of eCG-treated Tx GK rats showed up-regulated LHR mRNA expression compared to eCG-treated Tx Wistar rat ovaries (Fig. 3). Tro treatment (100 mg kg^{-1}) clearly decreased these LHR mRNA levels in eCG-treated Tx GK rats to the levels observed in eCG-treated Tx Wistar rats. Tro had no significant effect on the LHR mRNA levels in eCG-treated Tx Wistar rats, although it tended to decrease the levels.

Serum Insulin Levels in eCG-Treated Tx GK Rats and Effect of Tro on These Levels

When the serum insulin levels of eCG-treated Tx Wistar and GK rats were compared, no significant differences were observed (Fig. 4). Moreover, Tro (100 mg kg^{-1}) did not affect the insulin levels in the eCG-treated Tx GK rats.

Effect of Tro on Testosterone Secretion and LHR Expression by Cultured Theca Cells Obtained From eCG-Primed GK Rats

To examine whether Tro directly acts on ovarian cells, theca cells were isolated from eCG-primed GK rats and cultured. The production of testosterone by the cells increased

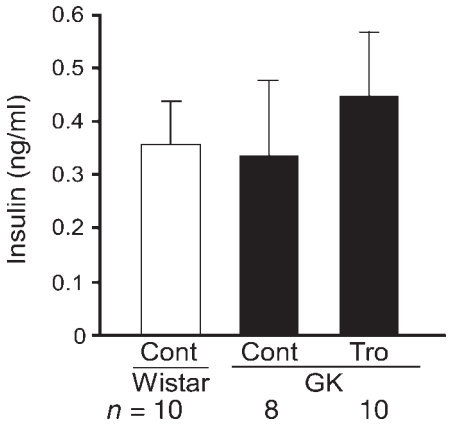

Fig. 4. Effect of Tro on the serum insulin levels in eCG-treated Tx GK rats. GK and Wistar rats were treated and bled as described in Fig. 1. Each value shows the mean ± SE of 8–10 rats.

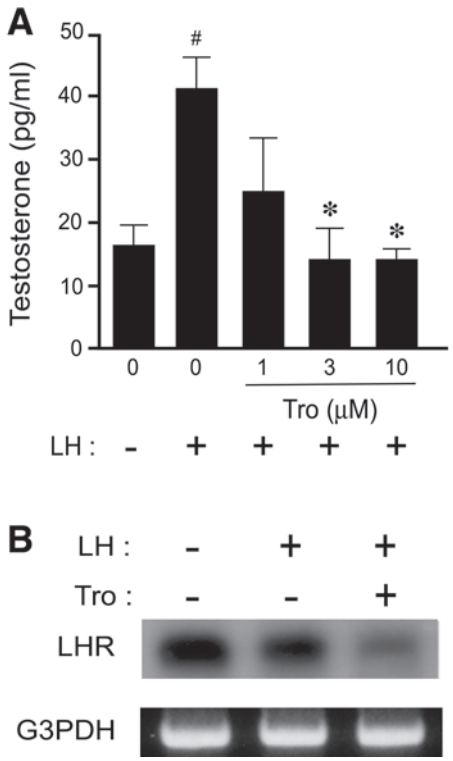

Fig. 5. Effect of Tro on testosterone and LHR mRNA production by cultured theca cells obtained from eCG-primed GK rats. The cells were cultured for 24 h with LH (100 ng mL^{-1}) and Tro (1, 3, 10 μ*M*). (**A**) Serum testosterone levels were determined by RIA. Each value is the mean ± SE of 5–6 cultures. $^{\#}p < 0.05$ vs LH (–) control; $^{*}p < 0.05$ vs the LH (+)-only control. (**B**) The expression of LHR mRNA was detected by semiquantitative RT-PCR using a DIG-labeled LHR cDNA probe.

significantly in the presence of LH (100 ng ml^{-1}) (Fig. 5A) and treatment with between 1 and 10 μ*M* Tro lowered these levels in a dose-dependent manner.

Figure 5B shows the effects of Tro (10 μ*M*) on the LHR mRNA expression in the cultured theca cells. LHR mRNA expression was suppressed by the treatment with Tro.

Discussion

We have shown previously that thyroidectomy in rats followed with eCG treatment significantly elevates their serum levels of testosterone and LH, and that this was markedly more pronounced in insulin-resistant GK rats as compared to Wistar rats *(27)*. Because PCOS patients show similar elevations of serum testosterone and LH levels, these observations suggested that eCG-treated Tx rats may be a new animal model of PCOS and as such may be useful for studying the pathophysiology of PCOS. The present study evaluated the effects of an insulin-sensitizing agent, Tro, on the elevated LH and testosterone secretion in eCG-treated Tx GK rats to determine whether the changes in testosterone and LH levels observed in GK rats that arise from thyroidectomy and eCG treatment are due to insulin resistance.

Insulin resistance, which is a characteristic of type 2 diabetes, is known to cause the ovary to function abnormally *(8,29)*. Supporting this, the insulin-sensitizing thiazolidinedione derivatives Tro, Pio, and rosiglitazone modulate sex hormone levels in women *(3)*. Moreover, PCOS, which is characterized by chronic anovulation, hyperandrogenism, and an elevated LH/FSH ratio, is highly associated with insulin resistance *(8,29)*. Furthermore, treatment with Tro raises the rate of ovulation in PCOS patients and improves their hyperandrogenism and other symptoms *(2,3,9)*. The present study shows that Tro can normalize abnormal ovarian hormone levels in eCG-treated Tx GK rats, perhaps by acting directly on the pathway of reproductive hormone production. We found that Tro decreased the testosterone levels in eCG-treated Tx GK rats to almost the same levels seen in eCG-treated Tx Wistar rats, although neither Tro nor Pio affected the enhanced LH levels observed in eCG-treated Tx GK rats. These observations are consistent with a report showing that Tro treatment lowers the serum levels of androgen in male rats with obesity *(10)*. Tro was shown to lower androgen levels in women with PCOS as described above. Based upon a possible hypothesis of PCOS proposed by Slowey *(25)*, there is a possibility that the Tro-induced decrease in testosterone levels may result from a reduction in serum insulin concentrations, which augment ovarian steroidogenesis. Further, Tro significantly reduced insulin levels in women with PCOS *(13)* and lowered the fasting insulin and hemoglobin A_{1c} levels, but not glucose levels in GK rats *(16)*. However, our study here challenges this notion since we did not observe any differences in the insulin levels of these eCG-treated Tx Wistar and GK rats, and Tro did not alter the serum insulin levels of these rats. Thus, in our model, it appears that Tro improves testosterone levels without affecting the serum insulin levels. As the glucose levels were not influenced by Tro treatment (data not shown), a reduction in glucose levels may not be involved in the elevation of insulin sensitivity. We found that Tro suppressed the enhanced ovarian LHR mRNA expression observed in eCG-treated Tx GK rats. This suggests

that the Tro-induced reduction of the testosterone levels in eCG-treated Tx GK rats may be mediated by down-regulating ovarian LHR expression, which in turn reduces the excessive stimulation of LH in these rats.

To investigate whether Tro acts directly in enhancing ovarian physiology, we examined the effect of Tro on testosterone secretion by rat theca cells. These cells were studied because it has been reported previously that Tro impairs androgen biosynthesis in theca cells *(24,28)* and inhibits progesterone production in granulosa cells in swine *(11)*. Moreover, Tro decreases both basal and gonadotropin-stimulated androsterone production by rat theca interstitial cells and progesterone production by human granulosa lutein cells *(21)*. We found that Tro decreased the theca cell testosterone secretion that is induced by LH in vitro. Furthermore, Tro also diminished the LHR mRNA levels in these LH-treated cells. These effects were similar to those observed by our in vivo studies and suggest that Tro acts directly on ovarian cells to elicit its activities. It is well known that the effects of thiazolidinedione derivatives like Tro and Pio are partially mediated by stimulating peroxisome proliferator-activated receptor-γ (PPARγ) *(5)*. Schoppee et al. *(24)* detected PPARγ mRNA expression in primary cultures of porcine theca cells and found that Tro or 15- deoxy-delta^{12,14}-prostaglandin J_2 (15d-PGJ_2), a putative endogenous ligand for PPARγ, decreases their androgen production by impairing the ability of CYP17 to synthesize androgen from progestin. However, it remains unclear whether the inhibitory effect of Tro is mediated by its ability to directly activate PPARγ. Indeed, Tro has only low affinity for PPARγ compared to Pio or rosiglitazone *(5)*. In the present study, we found Pio did not reduce the elevated testosterone levels as well as Tro did. Thus, the ability of Tro to inhibit ovarian function may involve mechanisms other than stimulating PPARγ. With regard to these alternative mechanisms, Veldhuis et al. *(28)* demonstrated that, in porcine theca cells, Tro inhibits testosterone production and the mRNA expression of p450c17, an enzyme that is essential for the production of testosterone. Moreover, interestingly, Arlt et al. *(1)* reported that this Tro-induced reduction of testosterone production is mediated by its direct inhibition of P450c17 and 3β-hydroxysteroid dehydrogenase (3β-HSD). There is no evidence showing that P450c17 and 3β-HSD activation is linked to PPARγ stimulation, nor has it been reported that LHR expression may be related to PPARγ activation. Thus, it is possible that Tro inhibits ovarian LHR mRNA expression in a manner that does not involve PPARγ activation. Instead, Tro may act in an inhibitory manner on many steps of androgen biosynthesis, including inhibiting LHR expression and inactivating p450c17 and 3β-HSD.

Several reports indicate that steroid hormones are involved in regulating insulin sensitivity *(17)*. For example, androgen acts directly on peripheral tissues to lower their receptivity to insulin, thereby increasing their insulin resistance *(7)*. Moreover, glucose tolerance, an indicator of insulin resistance, is decreased in women when testosterone or its derivatives is administered; this results in hyperinsulinism. In addition, women taking contraceptive agents, namely, a combination of estrogen and progesterone, showed accelerated insulin resistance. Thus, steroid hormones may elevate insulin resistance. We have found that normal GK rats have higher baseline serum levels of steroid hormones, especially progesterone and estradiol, than normal Wistar rats *(27)*. This might imply that these steroids contribute to the greater susceptibility of intact GK rats to acquire insulin resistance.

Mechanisms that induce insulin resistance include decreasing the autophosphorylation of the insulin receptor, decreasing the expression of the insulin-response glucose transporter and its translocation, and deleting downstream molecules of the insulin-signaling pathway. It is important to determine how these molecules are linked to changes in the insulin resistance of the ovary in GK mice, as this will improve our understanding of the relationship between insulin resistance and ovarian functions.

In conclusion, Tro decreases testosterone production and LHR expression by ovarian cells in GK rats both in vivo and in vitro. These effects of Tro may be related to the ameliorating effects of Tro on the symptoms of PCOS.

Materials and Methods

Animals and Experimental Schedule

Six-week-old female Wistar and GK rats (Charles River Japan, Inc., Kanagawa, Japan) were maintained in air-conditioned rooms (temperature 23 ± 1°C and humidity 55 ± 5%) under controlled lighting (12-h light/day schedule) with free access to food and water. All animal-handling protocols and surgical procedures 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 at 6 wk of age, as previously described in detail *(14)*. The Tx Wistar and GK rats were then injected subcutaneously at 10:00 am with 5 IU eCG upon diestrus about 1 wk (6–8 d) after the thyroidectomy; these injections continued once daily for 5 d. Tro and pioglitazone (Pio) were suspended, respectively, at 20 mg/0.5 mL and 2 mg/0.5 mL in 0.5% carboxymethyl cellulose and the eCG-treated Tx GK and Wistar rats were injected with these preparations just after each daily eCG treatment at 100 mg kg^{-1} and 10 mg kg^{-1}, respectively. Blood was collected 24 h after the final injection of eCG plus or minus Tro/Pio and the serum levels of LH, testosterone, and insulin were measured. The ovaries were homogenized with RNA extraction buffer and used to determine LHR mRNA levels by semiquantitative reverse transcription polymerase chain reaction (RT-PCR) analysis *(27)*.

Radioimmunoassay of LH and Testosterone

The serum LH concentrations were measured with a NIDDK RIA kit (provided by Dr. A. F. Parlow, Director, Pituitary Program and Antisera Center, Harbor-UCLA Medical Center, Torrance, CA, USA) using antisera against LH (S-10) *(14)*. The results were calculated by comparison with a standard curve using LH-RP-3. The levels of testosterone in serum or culture medium were assayed by RIA using antisera against testosterone (GDN#250) provided by Prof. G. D. Niswender (Colorado State University, Fort Collins, CO, USA) *(14)*. The intra- and interassay coefficients of variation were less than 10% for all RIA assay data.

Measurement of Insulin

Serum insulin concentrations were determined by using the enzyme-linked immunosorbent assay in the Revis insulin kit (Shibayagi, Wako, Tokyo, Japan).

Analysis of LHR mRNA Levels

Poly $(A)^+$ RNA was isolated with the QuickPreps micro mRNA purification kit (Amersham Pharmacia Biotech) and RT-PCR analysis of LHR mRNA expression was performed as described previously *(27)*.

Preparation of Rat Ovarian Theca Cells and Culture

Theca cells were isolated from the preovulatory ovary of eCG-primed immature GK rats as described previously *(19)*. Briefly, whole ovary was digested with collagenase (1 mg mL^{-1} type I) and DNase (0.5 μg mL^{-1}) and the resulting cell suspension was passed through a cell strainer (70 μm Nylon, BD Bioscience, MA, USA) to remove cell debris. The theca cells were then purified by using a Percoll density gradient, washed twice with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline, and cultured for 24 h in 24-well culture dishes at a density of 5.0×10^5 cell $well^{-1}$ in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 0.1 mg mL^{-1} gentamicin, 0.1 mg mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin. The cells were then incubated for an additional 24 h in serum-free DMEM. Thereafter, the cells were treated for 24 h with Tro (1, 3, 10 μ*M*) in the presence of LH and the culture medium was collected to measure the testosterone production, while the cells were used to isolate poly $(A)^+$ RNA for analyzing the LHR mRNA levels. Tro was dissolved with dimethyl sulfoxide and then diluted with culture media (final concentration, < 0.1%).

Reagents

Equine CG was purchased from Teikoku Hormone Mfg Co., Tokyo, Japan. Tro was kindly donated by Sankyo Co., Ltd. (Tokyo, Japan) and Pio was a gift from Takeda Chemical Industries, Ltd. (Osaka, Japan). Carboxymethyl cellulose and dimethyl sulfoxide, which were used to suspend and dissolve Tro, respectively, were purchased from Wako Pure Chemical Co. (Tokyo). Collagenase type I and DNase were purchased from Sigma Chemical Co. (St Louis, Mo, USA).

Data Analysis and Statistics

Data are represented as means ± SEM. The significance of differences between the data was determined by *t*-test and ANOVA. Differences with $p < 0.05$ were considered significant. The RT-PCR picture shows a representative of the several experiments.

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