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Chronic Effects of Propylthiouracil on Metabolism and Secretion of Adrenocorticotropin and Corticosterone in Male Rats

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Background: Propylthiouracil (PTU) is a thioamide drug for treating hyperthyroidism. We have already found that chronic administration of PTU decreases the level of plasma corticosterone and attenuates both the activities of 11β-hydroxylase and the generation of cyclic adenosine monophosphate (cyclic AMP) in rat zona fasciculata-reticularis (ZFR) cells. The present study aimed to investigate the chronic effects of PTU on metabolism, adrenocorticotropic hormone (ACTH) and corticosterone secretion. **Methods:** Male rats were randomly divided into four groups and injected subcutaneously with saline, PTU, thyroxine (T_4) or PTU plus T_4 once daily for 2 weeks. For metabolism studies, the body weight, food intake, water intake, urine volume, and feces weight of every rat were recorded daily. The rats were sacrificed after treatment, and the plasma thyroid stimulating hormone (TSH), ACTH, and corticosterone were measured. ZFR cells were separated from adrenal glands and then stimulated by forskolin (an activator of adenylyl cyclase, 10^{-5} M), 8-bromo-cAMP (8-Br-cAMP, a permeable analogue of cAMP, 10^{-5} M), ACTH (a stimulator to increase corticosterone secretion, 10^{-9} M), and deoxycorticosterone (DOC, a steroidogenic precursor of corticosterone, 10^{-5}M) in order to investigate the alternation of corticosterone secretion and enzyme activity. **Results:** Chronic administration of PTU decreased food intake and feces weight but increased urine volume. The concentration of plasma TSH increased after chronic PTU treatment. PTU also suppressed release of ACTH and decreased production of corticosterone. After the isolation of ZFR cells from PTU and PTU plus T_4-treated rats, both basal and evoked levels of corticosterone release were enhanced. **Conclusion:** Chronic administration of PTU inhibited pituitary gland from releasing ACTH, and this reaction led to decrease in corticosterone production in vivo. PTU treatment might upregulate downstream messengers of ACTH signal pathway in ZFR cells, thus enhancing release of corticosterone in vitro.

Key words: PTU, metabolism, ACTH, corticosterone

INTRODUCTION

Hyperthyroidism is a pathological syndrome, which causes the excess of thyroid hormone in cells[1]. Patients usually suffer fatigue, nervousness, anxiety, weight loss, palpitations, and heat sensitivity. The most common case of this syndrome is Graves' disease. The traditional treatments for hyperthyroidism include antithyroid drugs, radioactive iodine, and surgery. Since antithyroid drugs were launched in the 1940's, both thionamide propylthiouracil (PTU) and methimazole (MMI) have been the major treatment for Graves' disease in the United States.[1] Thionamide inhibits thyroid hormone synthesis by interfering with the oxidation and organic binding of iodide into thyroglobulin.[2] In addition, PTU inhibits deiodination of thyroxine (T_4) from being its active form, triiodothyronine (T_3), in peripheral tissues.[3] The usual daily dose of propylthiouracil ranges between 250 and 450 mg in three separate doses.[1,4] Clinical studies have reported that the most common side effect of PTU treatment in hyperthyroid patients is transient leucopenia;[2] and in many cases, PTU has been found to induce several toxic effects on liver,[5,6] including jaundice, severe hepatocellular dysfunction, and hepatomegaly. Apart from those on toxic effects of PTU on liver, clinical reports on effects of PTU use on endocrine system are rare.

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It has been documented that the reproductive endocrine systems in both male and female animals are affected by PTU-induced hypothyroidism. In male rats, PTU-induced neonatal hypothyroidism increases adult testicular size, number of Sertoli cells, and daily sperm production in adult rats and mice,[7-9] and these effects are attributed to increase in proliferation of immature Leydig cells[10,11] and decrease in production of testosterone from Leydig cells.[12] In female prepubertal rats, administration of PTU leads to reduction of ovarian weight and decrease in number of primordial, multilaminar, and Graafian follicles.[13] We previously showed that PTU acts directly on Leydig cells to inhibit steroidogenesis by reducing the function of P450 side-chain cleavage (P450scc) enzyme and the steroidogenesis acute regulatory (StAR) protein[14,15] and it also reduces release of progesterone from rat granulose cells by inhibiting P450scc and 3β-hydroxysteroid dehydrogenase activities.[16] In addition to the effects of PTU on the steroidogenesis of reproductive system, the changes of glucocorticoid secretion in PTU-induced hypothyroidism have also been studied.[17,18] We have demonstrated that chronic administration of PTU decreases plasma corticosterone level by attenuating both activities of 11β-hydroxylase and cAMP production in rat zona fasciculata-reticularis (ZFR) cells[19] and corticosterone production is suppressed after direct PTU treatment on ZFR cells in vitro. However, the chronic effects of PTU on metabolism and whether the inhibitory effects of PTU on plasma corticosterone level are due to the changes in secretion of ACTH remain unclear.

In the present study, the effects of chronic administration of PTU on metabolism, adrenocorticotropic hormone (ACTH) and corticosterone secretion were investigated. Male Sprague-Dawley rats were injected subcutaneously with saline, PTU, thyroxine (T_4) or PTU plus T_4 once daily for two weeks, and plasma thyroid stimulating hormone (TSH), ACTH and corticosterone were measured after rats were sacrificed. ZFR cells were isolated from adrenal cortex and challenged with forskolin (an activator of adenylyl cyclase,10^{-5} M), 8-bromo-cAMP (8-Br-cAMP, a permeable analogue of cAMP, 10^{-5} M), ACTH (a stimulator to increase corticosterone secretion, 10^{-9} M) and deoxycorticosterone (DOC, a steroidogenic precursor of corticosterone, used to examine the activity of 11β-hydroxylase, 10^{-5}M) to investigate the action mechanism involving several pathways of PTU action on corticosterone secretion. This study was undertaken to evaluate the effects of PTU treatment on hormone production from non-thyroid glands, especially the anti-inflammation and stress hormone cortisol. This report might give medical information for hyperthyroid patients using PTU.

MATERIALS AND METHODS

Animals

Male Sprague-Dawley rats weighing 250-300 g were housed in a temperature-controlled room (22±1 °C) with 14 hours of automatic illumination daily (0600-2000). Food and water were given *ad libitum*. Animal care was in accordance with the Guidelines for Animal Care of National Yang-Ming University.

Chronic Effects of PTU and T_4 on Metabolism

Male rats were randomly divided into four groups and then injected subcutaneously with saline, PTU (20 mg/ml/kg body wt, Sigma, St. Louis, MO, USA), T_4 (25 μg/ml/kg body wt, Sigma) or PTU plus T_4, respectively, once daily for 2 weeks. All rats were housed in Nalgene metabolic cages (Nalge Company, Rochester, NY, USA). The body weight, food intake, water intake, urine volume, and feces weight of all rats were recorded daily.

Chronic Effects of PTU and T_4 on Concentrations of Plasma ACTH, TSH and Corticosterone

After 2 weeks, the saline-, PTU-, T_4 -, and PTU plus T_4-treated male rats were sacrificed between 0900-1000 in the morning. The trunk blood was collected and plasma samples were separated by centrifugation and stored at -20 °C. The concentrations of ACTH, TSH, and corticosterone were measured by radioimmunoassay (RIA). To measure corticosterone, plasma was mixed with diethyl ether (5×volume), shaken for 30 min, and then quickly frozen in a mixture of acetone and dry ice. The organic phase was collected, dried, and reconstituted in a buffer solution (0.1% gelatin in phosphate-buffered saline (PBS), pH 7.5) before the concentration of corticosterone was measured by RIA.

Preparation of Zona Fasciculata-Reticularis Cells for Cell Culture

An adrenocortical preparation enriched with zona fasciculata-reticularis (ZFR) cells for culture was performed according to the method described by Purdy et al.[20] with minor modifications.[21] After decapitation, the adrenal glands were rapidly excised, cleaned, and then stored in ice-cold 0.9% NaCl solution. The encapsulated glands were separated into outer-zone (mainly zona glomerulosa) and inner-zone (mainly zona fasciculata-reticularis) fraction with forceps. The fractions of inner zone from 6-8 adrenals were incubated with collagenase (10 mg/ml,

Sigma) vibrantly (100-110 strokes per minute) in water bath at 37 °C for 60 min. The collagenase was dissolved in 2-4 ml of Krebs-Ringer bicarbonate buffer (3.6 mmol K^+/l, 11.1 mmol glucose/l) with 0.2% BSA medium (KRBGA), pH 7.4. Then ZFR cells were dispersed by being repeatedly pipetted and filtered through a nylon mesh. After centrifugation at 200×g for 10 min, the cells were washed in KRBGA medium and centrifuged again. Erythrocytes were eliminated from ZFR cells by being washed with 9 ml of distilled water for 10 seconds, and ZFR cells were then quickly mixed with 1 ml of 10×Hanks' balanced salt solution (HBSS, pH 7.4). After centrifugation at 200×g for 10 min, the supernatant was discarded, and the pellet was resuspended in 3 ml of KRBGA solution. An aliquot (20 μl) was used for cell counting in a hemocytometer after being stained with 0.04% trypan blue. Cells in culture medium were further diluted to a concentration of 5×10^4 cells/ml and divided among the test tubes.

In Vitro Experiments

ZFR cells were incubated with or without hormones dissolved in 1 ml/tube of KRBGA medium for 60 min at 37 °C under 95% O_2-5% CO_2. To measure the effects of forskolin, 8-Br-cAMP, ACTH, and deoxycorticosterone on production of corticosterone from male rat ZFR cells, the cells were first preincubated for 60 min with KRBGA medium. Following preincubation, the cells were incubated in the tubes containing 0.5 ml of medium, forskolin (10^{-5} M), 8-Br-cAMP (10^{-5} M), ACTH (10^{-9} M) or deoxycorticosterone (DOC, 10^{-5} M, Sigma). The concentration of medium corticosterone was measured by RIA.

RIA of Plasma ACTH

The concentration of plasma ACTH was determined by RIA. The anti-h-ACTH was provided by the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK-NIH, Bethesda, MD, USA). The standard of ACTH was purchased from Sigma. The h-ACTH was radioiodinated by chloramine-T method. To measure the concentration of ACTH, each plasma sample was adjusted to a total volume of 300 μl by assay buffer (960 ml EDTA-PBS pH 7.4, 20 ml aprotinin, 1 ml triton X-100, make up to 1 liter) and incubated with 50 μl anti-h-ACTH (1:1600) at 4 °C overnight. On the next day, 100 μl of ^{125}I-ACTH (10000 cpm/0.1 ml) was added to each tube and the tubes were further incubated for another night at 4 °C. On the third day, 100 μl of sheep anti-gamma globulin (ARGG, 1:20 dilution) was added into each tube and the tubes were continually incubated for another 48 hours at 4 °C. At the end of the incubation, the assay tubes were centrifuged at 1000×g for 30 min at 4 °C. The supernatant was discarded and the radioactivity remaining in the precipitate was counted by the gamma counter (1470 Wallac Wizard Gamma Counter, PerkinElmer, MA, USA).

RIA of Plasma TSH

The concentration of plasma TSH was determined by RIA. The anti-rat-TSH and the rat TSH-RP-3 that served as standard preparations were provided by the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK-NIH, Bethesda, MD, USA). Rat TSH-I-8 was used for radioiodination. To measure the TSH level, each plasma sample was adjusted to a total volume of 500 μl by 0.1% gelatine-PBS and incubated with 200 μl of anti-rat-TSH (1:1000 dilution) at 4 °C overnight. On the next day, 50 μl of ^{125}I-labled TSH (10000 cpm/0.1 ml) was added into each tube and the tubes were incubated at 4 °C for 24 h. On the third day, 150 μl of sheep anti-rabbit-gamma globulin (ARGG, 1:20 dilution) was added into each tube and the tubes were continually incubated at 4 °C for another 48 hours. At the end of the incubation, the assay tubes were centrifuged at 1000×g for 30 min at 4 °C. The supernatant was discarded and the radioactivity remaining in the precipitate was counted by the gamma counter (1470 Wallac Wizard Gamma Counter, PerkinElmer, MA, USA).

RIA of Corticosterone

The concentration of corticosterone in the media was determined by RIA as described previously.[19] An antiserum to the corticosterone was generated by immunizing rabbits with 4-pregnen-11β, 21-diol-3, 20-dione 3-carbozymethyloxime-BSA conjugate (Steraloids Inc., Wilton, NH, USA). With this antiserum (PSW4-9), an RIA was established for the measurement of plasma corticosterone levels. In this RIA system, a known amount of unlabeled corticosterone, an aliquot of plasma extract, or media samples adjusted to a total volume of 0.2 ml by a buffer solution (0.1% gelatin-PBS, pH 7.5) were incubated with 0.1 ml of corticosterone antiserum (1:3000 dilution) diluted with 0.1% gelatin-PBS and 0.1 ml of [^{3}H] corticosterone (~8,000 cpm, Amersham International, Amersham, UK) at 4 °C for 24 h. Duplicate standard curves with 5 points ranging from 20 to 4,800 pg of corticosterone were included in each assay. An adequate amount (0.1 ml) of 0.25% dextran-coated charcoal (charcoal, Sigma Chemical) was then added for further incubation in an ice bath for 15 min. At the end of incubation,

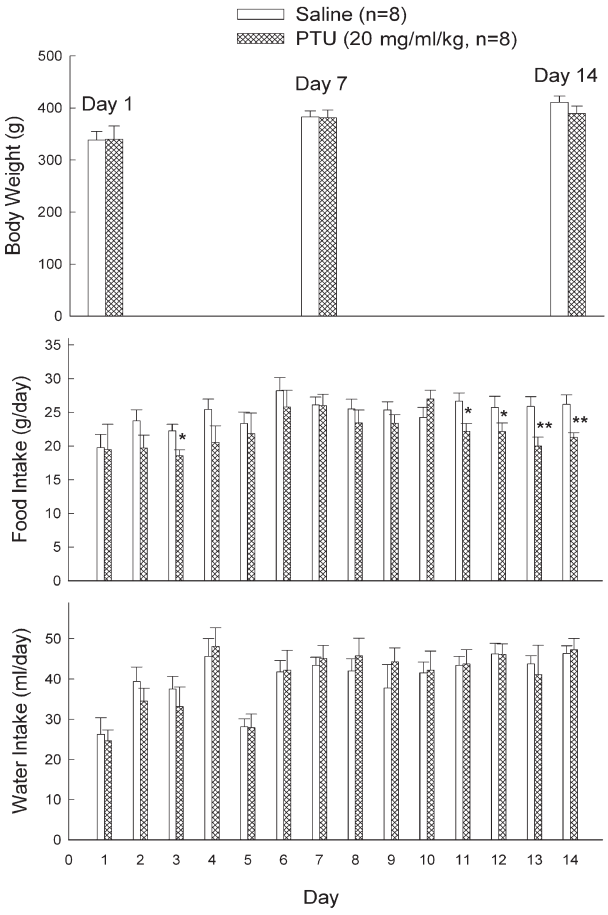

Fig. 1 The metabolic data of body weight, food intake and water intake in saline- and PTU-treated male rats. *, **, $P< 0.05$ and $P< 0.01$ as compared with alkaline saline-treated rats, respectively. Each value represents the mean±SEM.

the assay tubes were centrifuged at 1,000×g for 30 min. The supernatant was mixed with 3 ml of liquid scintillation fluid (Ecoscint A, National Diagnostics, Atlanta, GA, USA) before the radioactivity was counted in an automatic beta counter (LS6500, Beckman, Fullerton, CA, USA).

Statistical Analysis

Data were expressed as mean ±standard error of the mean (SEM). The treatment means for homogeneity were tested using an ANOVA, and the difference among specific means for significance was tested using Duncan's multiple-range test.[22] In Figs. 1 and 2, Student's *t*-test was used. The difference between two means was considered statistically significant when *P* was less then 0.05.

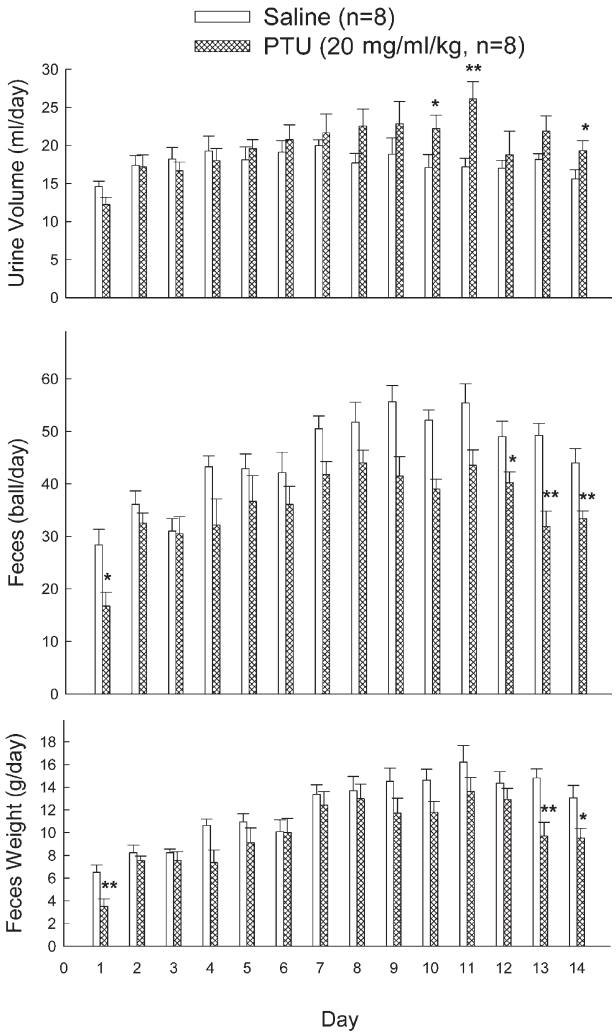

Fig. 2 The metabolic data of urine volume, feces balls and feces weight in saline- and PTU-treated male rats. *, **, $P< 0.05$ and $P< 0.01$ compared with alkaline saline- treated rats. Each value represents the mean ±SEM.

RESULTS

Chronic Effects of PTU and T_4 on Metabolism in Rats

Nalgene metabolic cages were employed to examine the effects of PTU-induced hypothyroidism and T_4 on metabolism. The food intake and feces weight were decreased while the urine volume was increased by PTU treatment for 12-14 days as compared with those after saline treatment (Figs. 1 and 2). There was no significant change in T_4- and PTU plus T_4-treated rats (data not shown). These data showed that PTU-induced hypothyroidism suppressed metabolism in rats.

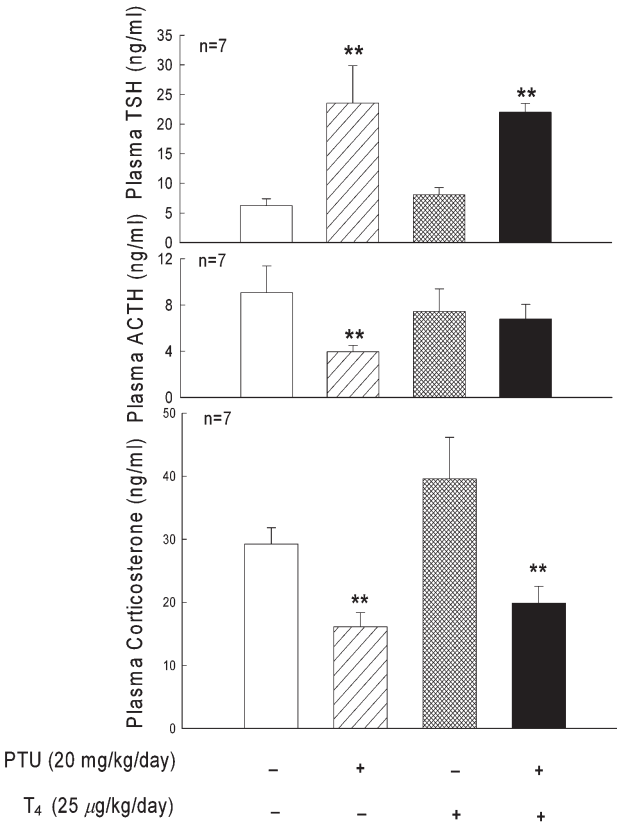

Fig. 3 Concentrations of plasma TSH (upper panel), ACTH (central panel) and corticosterone (lower panel) in saline-, PTU-, T_4-, PTU plus T_4-treated male rats. **, $P< 0.01$ as compared with saline-treated rats, respectively. Each value represents the mean±SEM.

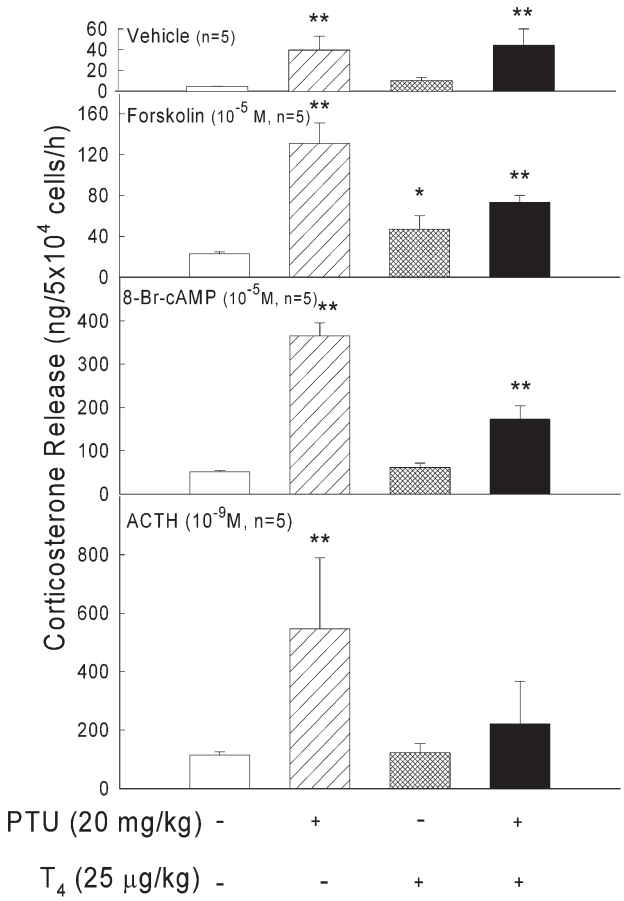

Fig. 4 Effects of forskolin, 8-Br-cAMP and ACTH on corticosterone production by rat ZFR cells from saline-, PTU-, T_4-, PTU plus T_4-treated male rats. *, **, $P< 0.05$ and $P< 0.01$ as compared with alkaline saline-treated rats, respectively. Each value represents the mean±SEM.

Chronic Effects of PTU and T_4 on Plasma TSH in Rats

To determine whether TSH level increases after PTU-induced hypothyroidism, plasma TSH was measured by RIA. The level of plasma TSH increased ($P < 0.01$) in PTU- and PTU plus T_4-treated rats as compared with that in saline-treated rats. Administration of T_4 did not alter the concentration of plasma TSH (Fig. 3). The data showed that T_4 replacement did not compensate the effect of PTU on TSH release in male rats.

Chronic Effects of PTU and T_4 on Plasma Corticosterone in Rats

A previous study showed that PTU could suppress corticosterone production after PTU treatment.[19] In the present study, the levels of plasma corticosterone were also decreased by PTU ($P < 0.01$) and PTU plus T_4 ($P < 0.01$). In addition, T_4 replacement did not compensate the inhibitory effect of PTU on corticosterone secretion.

Chronic Effects of PTU and T_4 on Plasma ACTH in Rats

Plasma ACTH level was examined to determine whether the decrease in PTU induced corticosterone was due to the suppression of ACTH release. The level of plasma ACTH decreased ($P < 0.01$) in PTU-treated rats (Fig. 3). Administration of PTU plus T_4 and T_4 did not alter the concentration of plasma ACTH (Fig. 3). These data showed that the decrease in corticosterone production was partly due to decrease in ACTH level.

Effects of PTU and T_4 on Evoked Release of Corticosterone by ZFR Cells In Vitro

To investigate directly the ability of ZFR cells to

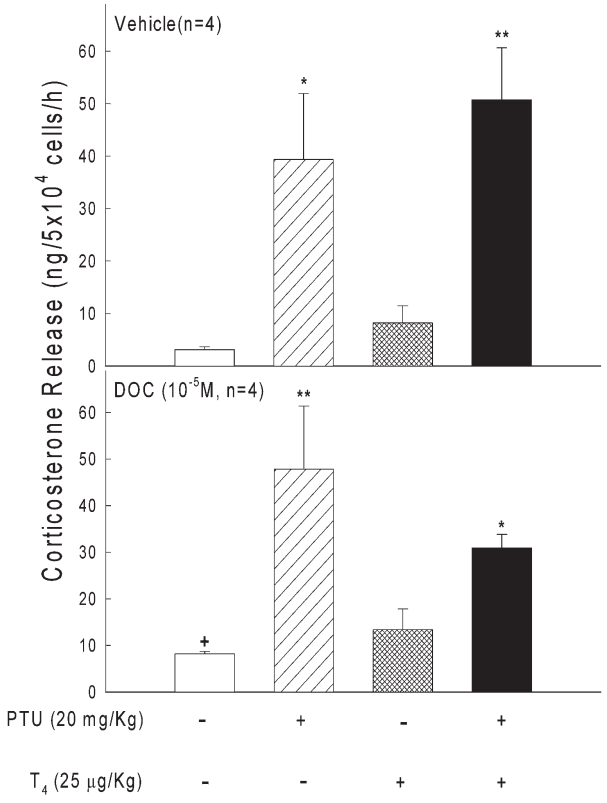

Fig. 5 Effects of deoxycorticosterone (DOC) on corticosterone production by rat ZFR cells from saline-, PTU-, T_4-, PTU plus T_4-treated male rats. *, **, $P<$ 0.05 and $P<$ 0.01 as compared with saline-treated rats, respectively. +, $P<$ 0.05 as compared with basal level. Each value represents the mean±SEM.

produce corticosterone, cells were isolated from adrenal gland after PTU administration for 2 weeks. The concentration of corticosterone released from ZFR cells in response to forskolin, 8-Br-cAMP, and ACTH increased ($P < 0.01$) after chronic PTU treatment as compared with that of the saline-treated group (Fig. 4). T_4 treatment increased the level of corticosterone released from ZFR cells in response to forskolin (Fig. 4). T_4 replacement did not reduce completely the stimulatory effects of PTU on both basal and evoked release of corticosterone in vitro (Fig. 4).

Effects of PTU and T_4 on Activity of 11β-Hydroxylase in ZFR Cells

Deoxycorticosterone (DOC) is a precursor of corticosterone, and is converted to corticosterone by 11β-hydroxylase. To determine whether the increase in corticosterone production after PTU administration is induced by the activity of 11β-hydroxylase, ZFR cells were treated by DOC (10^{-5} M), and the release of corticosterone was measured. The corticosterone released from rat ZFR cells of PTU- and PTU plus T_4-treated rats increased ($P < 0.01$) after DOC stimulation in vitro. However, there was no significant difference between basal and DOC-stimulated level, showing that chronic PTU treatment-induced corticosterone production from ZFR cells was not due to the activity of 11β-hydroxylase (Fig. 5).

DISCUSSION

The dose of PTU used in the present study followed that in previous research.[19] It could reduce peripheral T_4 and T_3 level and evaluate plasma TSH. In addition, in the clinical protocol, the dose of PTU ranges from 250 to 450 mg/day.[2,4] For a patient whose weight is 60 kg, the dose is approximately 4-7.5 mg/kg. The dose of PTU used in this study was 20 mg/kg, which is higher than the clinical dose. However, it usually takes 4 to 12 weeks for most patients to improve considerably or achieve normal thyroid function.[2] In order to examine the effect of PTU within the shorter therapeutic course, a higher dose of PTU was employed in the present study. The normal serum concentration of T_4 is 0.043-0.125 μg/ml, and the T_4 concentration used in the present study was 25 μg/ml. Therefore, the serum concentration of T_4 is approximately 0.2 μg/ml, which is relatively close to the normal concentration.

The present results indicated that chronic administration of PTU decreased food intake and feces weight but increased urine volume. These results, which are consistent with clinical observations, might be attributed to the inhibitory effects of PTU on T_4 production and decreased metabolism in rats.[23-25] The increase in urine volume caused by PTU administration might be due to inappropriate inhibition of PTU on antidiuretic hormone (ADH) secreted from pituitary gland,[26] or damaged renal function.[27]

Chronic administration of PTU increased TSH concentration in rats but decreased the levels of plasma corticosterone and ACTH as compared with the saline-treated group. Apparently, the stimulating effect of chronic administration of PTU on TSH secretion was due to negative feedback, and the decreased corticosterone secretion was mostly caused by inhibition of ACTH release. These results were consistent with previous findings, suggesting that PTU could reduce pituitary secretion.[28-30] The inhibitory effect of PTU on corticosterone production was not altered by the replacement with T_4. This result suggested

that the deficit of T_4 or ACTH is not the only reason for hyposecretion of corticosterone in hypothyroidism caused by PTU. There might be another inhibitory effect of PTU on corticosterone secretion in vivo, which merits further investigation.

In the in vitro study, we found that both basal and evoked release of corticosterone from ZFR cells of PTU- and PTU plus T_4-treated rats were higher than those in the control group, and instead the corticosterone level was suppressed in vivo. In the in vivo study, the down-regulatory effects of PTU on corticosterone secretion might be through the suppression of ACTH level or another effect on ZFR cells. However, in the in vitro study, ZFR cells were separated from the control of ACTH and PTU and showed a higher capability for the secretion of corticosterone. The possible mechanism for this discrepancy is as follows. In the in vivo condition, ZFR cells tried to reverse the low level of corticosterone. Therefore, ZFR cells might upregulate downstream messengers of ACTH signal pathway in ZFR cells. Once these ZFR cells were separated from the control of ACTH and PTU, they would show higher ability in secretion of corticosterone than the control group. This might account for the difference in level of corticosterone after the treatment of PTU between in vivo and in vitro studies. However, we cannot exclude the direct effect of PTU on ZFR cells. In our previous study,[19] the secretion of corticosterone in ZFR cells was decreased after direct treatment of PTU.

There was no change in corticosterone release from ZFR cells in T_4-treated group. However, in the PTU plus T_4-treated group, corticosterone release from ZFR cells did not increase after ACTH stimulation as compared with the saline-treated group, but there was no significant difference between PTU- and PTU plus T_4-treated groups, which needs further confirmation and investigation. Since there was no increase in corticosterone secretion from PTU- and PTU plus T_4-treated ZFR cells after DOC stimulation, it would imply that chronic administration of PTU did not alter the activity of 11β-hydroxylase. However, the detailed mechanism of increase in corticosterone secretion from ZFR cells that alters chronic administration of PTU in vivo remains unclear.

Our finding suggested that chronic administration of PTU in rats could reduce metabolism by suppressing food intake and then decreased feces weight. PTU administration suppressed ACTH release and resulted in lower corticosterone level. Furthermore, corticosterone release increased after chronic PTU administration, and it might be due to upregulate downstream messengers of ACTH signal pathway in ZFR cells. Future studies on molecular level in ZFR cells will shed light on the detailed mechanism. These results suggested that the alteration of PTU on glucocorticoid production in vivo and in vitro might raise more considerations when using PTU in those patients with abnormal adrenal functions or disorders of cortisol production.

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