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Research Article
Inhibitory Effects of Propylthiouracil on Corticosterone Production in Rat Zona Fasciculata-Reticularis Cells

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Abstract

Background and Objective: To examine the acute effects of propylthiouracil (PTU) on corticosterone release by rat adrenal Zona Fasciculata Reticularis (ZFR) cells. **Materials and Methods:** Male rats were infused with alkaline saline or PTU (10 mg kg^{-1}) via right jugular catheter. The blood samples were collected at 0, 30, 60, 120, 180 and 240 min after infusion. Rat ZFR cells were treated with PTU (12 mM) in the presence or absence of adrenocorticotropic hormone (ACTH) (10^{-9} M). Media were collected for corticosterone RIA and cells were lysed for analyzing the protein expressions of Steroidogenic Acute Regulatory (StAR) protein and P450scc. The ZFR cells were also incubated with PTU (3-30 mM) plus trilostane (a 3ß-hydroxysteroid enzyme inhibitor, 10^{-6} M) or both trilostane and 25-OH-cholesterol (10^{-7}~10^{-4} M) for 1 h and the concentration of pregnenolone in media was measured. **Results:** Propylthiouracil infusion decreased plasma corticosterone concentrations. Both basal and ACTH-stimulated corticosterone release as well as StAR protein expression in ZFR cells were attenuated by PTU treatment. The Electrophoretic Mobility Shift Assay (EMSA) results showed that the binding activity into Steroidogenic Factor-1 (SF-1) binding site was descreased by PTU. The elevated binding activity in response to ACTH treatment was diminished after PTU treatment. The pregnenolone production from ZFR cells was suppressed by PTU. Administration of PTU (10 mM) decreased Vmax by 46%. **Conclusion:** Propylthiouracil acutely diminished corticosterone production by affecting the early rate-limiting steps of steroidogenesis including the reduction of the enzyme activity of P450scc, and the expression of StAR protein.

Key words: PTU, corticosterone, ZFR cells, pregnenolone, StAR, P450scc, steroidogenic factor-1

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Propylthiouracil (PTU) is a thionamide drug, which suppresses the synthesis of thyroid hormone in thyroid and conversion of T_4 to T_3. Propylthiouracil (PTU) is commonly used to treat patients with hyperthyroidism caused by Grave's disease. It has been reported that patients treated with PTU resulted in transient leukopenia[1]. Moreover, patients who were clinically treated with PTU developed jaundice, hepatodisfunction, and hepatomegaly. The PTU is recognized as the cause of hepatotoxicity which is associated with hepatocellular necrosis then with cholestasis[2]. Administration of PTU acutely increases the release of Gastric Inhibitory Polypeptide (GIP)[3], but decreases the production of gonadal steroid hormones[3,4,5] in rats.

The previous studies indicated that the elevated plasma corticosterone levels were observed in T_4-induced hyperthyroidism and attenuated by PTU-induced hypothyroidism in postnatal rats[6]. Clinical reports indicated that adrenocortical function was activated in primary hyperthyroidism and reduced in hypothyroidism[7,8,9]. These data suggested that T_4 activated the Hypothalamus Pituitary Adrenal (HPA) axia and PTU suppressed the plasma corticosterone through the inhibition of plasma T_4 concentration. However, PTU-induced hypothyroidism suppressed the Corticotrophin Releasing Hormone (CRH) gene expression in the paraventricular nucleus in rats[10]. Meanwhile, PTU also has a direct inhibitory effect on the release of progesterone from rat granulosa cells and that of aldosterone from rat zona glomerulosa cells via the inhibition of post-cAMP and intracellular calcium pathways[5,11]. Sanchez-Franco *et al.*[9] reported that the plasma ACTH level was not altered in PTU-treated rats, although the decreased plasma corticosterone concentration was observed[9]. Mice treated with PTU for 10 weeks altered the morphology of mitochondria and induced "Brown degeneration" in adrenocortical cells[12]. These results implied that the suppressed plasma corticosterone concentration developed in PTU-induced hypothyroidism appeared to be associated with the direct pharmacological effects of PTU on the adrenal gland. It is demonstrated that PTU exhibited the pharmacological effects on corticosterone secretion by a direct attenuation of the activity of 11-hydroxylase and cAMP production in ZFR cells[13].

In adrenal glands, the biosynthesis of corticosterone involves the activation of the Steroidogenic Acute Regulatory (StAR) protein, P450scc, 3β-hydroxysteroid dehydrogenase (3β-HSD), 21-hydroxylase and 11β-hydroxylase[14,15]. During the steroidogenesis, the conversion of cholesterol to pregnenolone is the early rate-limiting step. The P450scc, located in the matrix of the inner mitochondrial membrane, catalyzes the reaction of cleavage side chain of cholesterol to form pregnenolone[16]. Transferring of cholesterol from outer to inner mitochondrial membrane is considered to be the rate-limiting step[17,18,19]. The protein responsible for the rate-limiting step is identified as a 30 kDa protein named Steroidogenic Acute Regulatory (StAR) protein[20,21]. Therefore, both StAR and P450scc play an important role in the regulation of steroidogenesis.

The regulation of StAR gene transcription is not fully understood. However, Steroidogenic Factor-1 (SF-1) is considered to be potentially important in the regulation of the StAR gene[22]. The upstream of StAR gene contains binding sites for SF-1 which has phosphorylation-dependent transcriptional activities[22,23]. Recent studies indicated that the activity of SF-1 is exerted by phosphorylated form and binds to the promoter of StAR gene. Then, the transcription of StAR gene would be further accelerated and the production of steroids could be increased[22]. In addition to StAR, the enzymes that regulated the steroidogenesis including cytochrome P450scc, aromatase and 3β-HSD are also the target genes of SF-1[24].

Since, the role of PTU in regulating the *in vivo* secretion of corticosterone and the key proteins responsible for early rate-limiting step of steroidogenesis remains unclear, this study was undertaken to investigate the plasma level of corticosterone, the expression of StAR protein, the DNA binding activity of SF-1 and the enzyme activity of P450scc under the interference of PTU.

MATERIALS AND METHODS

Animals: Male rats of the Sprague-Dawley strain weighing 300-350 g were housed in a temperature-controlled room (22±1°C) with 14 h of artificial illumination daily (0600-2000). Food and water were given *ad libitum.* All animal experimentation has been conducted humanely and in conformance with the policy statement of the Committee of National Yang-Ming University.

Materials: ACTH1-24, Bovine Serum Albumin (BSA), collagenase, Hank's Balanced Sodium Salt (HBSS), 25-OH-cholesterol, β-actin antibodies and pregnenolone were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The T4 RIA kit was purchased from DiaSorin Inc (Stillwater, MN, USA). The antipregnenolone antiserum was purchased from Biogenesis Inc. (Sandown, NH, USA). The rabbit polyclonal antibodies and mouse monoclonal antibodies were from ICN

Pharmaceuticals, Inc. (Aurora, OH, USA). The ECL detection kits and [3H]-pregnenolone were obtained from Amersham Life Science Limited (Buckinghamshire, UK). The liquid scintillation fluid was from Beckman Inc. (Fullerton, CA, USA). The polyvinylidene difluoride (PVDF) membranes were purchased from NEN Life Science Products Inc. (Boston, MA, USA).

Effects of PTU on plasma corticosterone and T_4 concentration in rats: Male rats were separated into two groups with 9 rats in each group. Animals were anesthetized with ether and catheterized via the right jugular vein[25]. After 20 h, rats were infused with 1 mL of alkaline saline (pH 9.5) or PTU (10 mg kg^{-1}, pH 9.5) via a peristaltic pump for 30 min. The blood samples were collected from jugular catheter at 0, 30, 60, 120, 180 and 240 min following infusion.

The plasma samples were collected by centrifugation at 10000×g for 1 min and the concentrations of plasma T_4 were measured by a radioimmunoassay kit[26]. In order to measure the plasma corticosterone concentration, 0.1 mL of plasma was mixed with 1 mL diethyl ether and shaken for 30 min. The plasma samples were centrifuged at 1000×g for 5 min and then quickly frozen in a mixture of acetone and dry ice. The organic phase was collected, dried and reconstituted with a buffer solution (0.1% gelatin in phosphate-buffered saline, PBS, pH 7.5). The concentrations of corticosterone in reconstituted extracts were determined by RIA.

Preparation of Zona Fasciculata-Reticularis (ZFR) cells: An adrenocortical preparation enriched with ZFR cells for culture was performed following a method described elsewhere[27] with minor modification[13,28]. The normal male rats were decapitated and then the adrenal glands were removed, encapsulated and separated into inner zone (mainly zona fasciculata and zona reticularis). The inner zone of adrenals was incubated with collagenase (2 mg mL^{-1}) at 37°C in a vibrating water bath for 60 min. The ZFR cells were dispersed by the repeated pipetting and filtered through a nylon mesh. The cells were washed twice with Krebs-Ringer bicarbonate buffer (K^+ 3.6 mmol L^{-1}, glucose 11.1 mmol L^{-1}) with 0.2% BSA medium (KRBGA), pH 7.4 and then centrifuged at 200×g for 10 min. The cell pellets were washed with 9 mL deionized water to disrupt the erythrocytes and the osmolarity immediately restored with 1 mL of 10× Hank's Balanced Salt Solution (HBSS, pH 7.4). After centrifugation, the supernatant was discarded and the pellets were resuspended in 3 mL of KRBGA medium. The cell concentration and viability were determined by the use of a hemacytometer and trypan blue exclusion method. The viability of isolated ZFR cells was 70-75%. Cells in the culture medium were further diluted to a concentration of 2.5×10^5 cells mL^{-1} and divided into the plastic test tubes.

Effects of PTU on corticosterone release and protein expressions of StAR and P450scc protein in ZFR cells: The prepared ZFR cells (2.5×10^6 cells per tube) were preincubated at 37°C under 95% O_2-5% CO_2 for 1 h prior to incubate with 10 mL medium with or without PTU at 12 mM in the presence or absence of $ACTH^{1-24}$ (1 nM) for 1 or 2 h. At the end of incubation, 5 mL ice-cold KRBGA medium were added and immediately followed by centrifugation at 100×g for 10 min at 4°C. The supernatant fluid was stored at -20°C, until analyzed for corticosterone by RIA and the cells were collected and lysed to determine the protein expression of StAR and P450scc by Western blot.

Effects of PTU on 25-OH-cholesterol-stimulated pregnenolone production in ZFR cells: For studying the influence of PTU on the enzyme activities of P450scc, an inhibitor of 3β-HSD, trilostane was used. The ZFR cells (5×10^4 cells mL^{-1}) were primed with trilostane (10 μM) for 30 min and then incubated with trilostane or trilostane plus 25-OH-cholesterol (0.1~100 μM) in the presence of PTU (3~30 mM) for 1 h. The media were collected and the concentrations of pregnenolone were measured by RIA.

Preparation of cell fractions: The isolated ZFR cells (2.5×10^6 cells per tube) were incubated for 1 or 2 h with or without PTU at 12 mM in the presence or absence of $ACTH^{1-24}$ (1 nM). The treated cells pellets were resuspended into 200 μL of cold buffer-A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mg mL^{-1} leupeptin, 1 mg mL^{-1} aprotinin and 0.5 mM phenylmethylsulfonyl fluoride) and incubated for 15 min on ice. After addition of 25 mL of buffer-A containing 2.5% NP-40, lysed cells were centrifuged (1,500×g for 6 min at 4°C) and the supernatants (cytosolic fraction) were collected and stored at -80°C. Nuclear pellets were resuspended with buffer C (20 mM HEPES, pH 7.9, 0.45 M NaCl and 1 mM EDTA plus protease inhibitors) and subjected to constant shaking for 20 min at 4°C, followed by centrifugation (20,800×g for 10 min at 4°C). The collections of the supernatants were assigned as the nuclear fraction.

Gel-shift analysis: Electrophoretic Mobility-Shift Assays (EMSA) were performed to assess the binding activity of

nuclear proteins to DNA fragment derived from the promoter of rodent StAR gene. The following sequence of the StAR promoter region was used[29]: SF-1 (-135/-83), 5′-CTCCCTCCCACCTTGGCCAGCACT-3′. The DNA fragments were 3′-end-radiolabeled by polynucleotide kinase. The treated ZFR cells were fractionated into nuclear fractions. Five-microgram nuclear extracts were incubated with 5000 cpm of radiolabeled DNA probe at room temperature for 20 min in a binding buffer described by Lin *et al.*[30]. Binding reactions were then electrophoresed through 6% nondenaturing polyacrylamide gels at 150 V for 2 h at 4°C. Gels were dried and radioactive signals were visualized by autoradiography.

Corticosterone RIA: The concentrations of corticosterone in both plasma and medium were determined by RIA as described elsewhere[13]. With anti-corticosterone No. PSW# 4-9, the sensitivity of corticosterone was 5 pg per assay tube. The intra- and interassay coefficients of variation were 3.3% (n = 5) and 9.5% (n = 4), respectively.

Pregnenolone RIA: The anti-pregnenolone antiserum was purchased from Biogenesis Inc (Sandown, NH, USA). Cross-reactivities were 67% with pregnen-36-OL-20-ONE sulphate; 19% with progesterone and <3% with 17a-hydroxypregnenolone, cholesterol, 17a-OH-progesterone, 20a-diOH-progesterone, cortisol, deoxycorticosterone, corticosterone, aldosterone androstenedione, testosterone, estradiol, estrone or estriol. For the RIA system, a known amount of unlabeled pregnenolone or an aliquot of rat ZFR cell medium, adjusted to a total volume of 0.3 mL by a buffer solution [0.1% gelatin Phosphate Buffered Saline (PBS), pH 7.5] was incubated with 0.1 mL pregnenolone antiserum diluted with 0.1% gelatin-PBS plus 0.1 mL [^{3}H]-pregnenolone at 4°C for 24 h. Duplicated standard curves of pregnenolone were incubated in each assay. An adequate amount (0.1 mL) of dextran-coated charcoal (0.5%) was added and further incubated in an ice bath for 15 min. After incubation the assay tubes were centrifuged at 1500×g for 40 min. The supernatant was mixed with 3 mL liquid scintillation fluid before the radioactivity was counted in an automatic beta counter (Beckman Coulter, Inc., Fullerton, CA, USA). The sensitivity of the pregnenolone RIA was 16 pg per assay tube. The inhibition curves produced by ZFR cell medium samples were parallel with those produced by pregnenolone. The intra- and interassay coefficients of variation were 2.5% (n = 4) and 3.9% (n = 5), respectively.

Western blotting: The ZFR cells were collected and washed three times with saline. The cells were lysed in lysis buffer consisting of 1.5% Na-lauroylsarcosine, 2.5 mM Tris-base, 1 mM EDTA, 0.68% phenylmethylsulfonyl fluoride (PMSF) and 2% proteinase inhibitors, pH 8.0 and disrupted by sonication in an ice-bath. The cell lysate was centrifuged for 12 min at 10000×g and the supernatant (cell extract) was collected. The total protein was determined by Braford protein assay[31]. The proteins were denatured by boiling for 10 min in SDS buffer (0.125 M, 12% sucrose, 0.15 M dithiothreitol)[32,33]. Western blotting and gel electrophoresis were performed as previously described[33,34,35] and the proteins were separated by 12% SDS-PAGE and electro-transferred to polyvinylidene difluoride (PVDF) membrane. The membranes were washed with TBS-T buffer (0.8% NaCl, 0.02 M tris-base and 0.3% tween-20, pH 7.6) for 5 min, then blocked for 60 min in blocking buffer (TBS-T buffer containing 5% nonfat dry milk) at room temperature. Then, the membranes were immunoblotted with primary antibodies (rabbit polyclonal antibodies to P450scc 1:1000, mouse monoclonal antibodies to StAR 1:1000 and b-actin 1:8000). Primary antibodies were detected using anti-rabbit and anti-mouse horseradish peroxides-conjugated secondary antibodies in 5% nonfat dry milk of TBS-T buffer. The membranes were washed with TBS-T buffer and then the bands of StAR or P450scc were visualized by chemiluminescence detection.

Statistical analysis: All values are given as the Mean±SEM. In some cases, the treatment means were tested for homogeneity by the analysis of variance (ANOVA) and the difference between specific means was tested for significance by Duncan's multiple-range test[36]. In other cases, Student's t-test was employed. A difference between two means was considered statistically significant when $p<0.05$.

RESULTS

Effects of PTU on plasma corticosterone and T4 in rats: The plasma levels of corticosterone were gradually increased from 30-120 min following alkaline saline infusion ($p<0.01$, Fig. 1, lower panel). In PTU-infused group, the plasma corticosterone concentrations were also increased from 30-60 min ($p<0.01$, Fig. 1, lower panel). As compared with saline group, the lower plasma corticosterone concentrations were observed at 60, 120, 180 and 240 min after PTU infusion ($p<0.05$ or $p<0.01$, Fig. 1, lower panel). The plasma T_4 level was also examined following PTU infusion. As compared with saline group, the plasma T_4 concentrations were unchanged by acute treatment of PTU in rats (Fig. 1, upper panel).

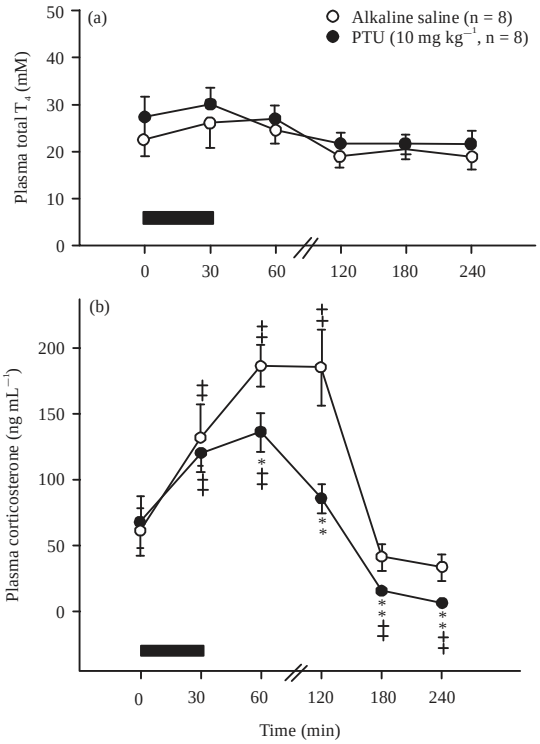

Fig. 1(a-b): Effects of PTU treated *in vivo* on plasma concentration of T_4 and corticosterone in male rats. Rats were infused with alkaline saline or PTU for 30 min (black horizontal bar), each value represents the Mean±SE mean, *,**p<0.05 and p<0.01 compared to alkaline saline group; ++p<0.01 compared with time at 0 min

Effetcts of PTU on corticosterone release in ZFR cells: To investigate the acute effects of PTU on steroidogenesis in vitro, rat ZFR cells (2.5×10^6 cells) were challenged with or without ACTH (1 nM) in combination with PTU (12 mM) for 1 or 2 h (Fig. 2). Following 1 or 2 h incubation, administration of PTU significantly reduced the corticosterone release compared with control group (p<0.05). The PTU resulted in a 46% (1 h incubation) and 70% (2 h incubation) decrease in corticosterone release from ZFR cells, respectively (Fig. 2). The ACTH at the dose of 10^{-9} M markedly (p<0.01) increased corticosterone release by ZFR cells in a time-dependent manner (Fig. 2). Administration of PTU for 1 or 2 h attenuated the ACTH-stimulated corticosterone production by 93.6% and 97.5%, respectively (Fig. 2).

Effect of PTU on protein expression of StAR and P450scc and the SF-1 binding site: The protein expressions of StAR and

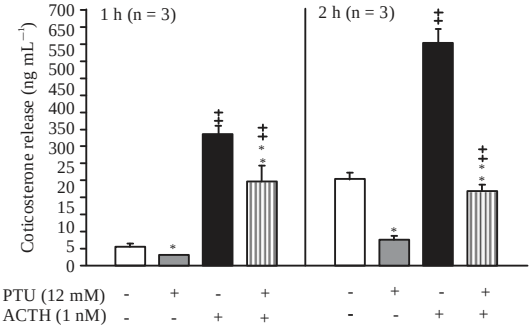

Fig. 2: Effects of PTU on basal and ACTH-stimulated corticosterone release in ZFR cells. Each column represents the Mean±SEM, *,**p<0.05 and p<0.01 compared to vehicle group (PTU = 0 M), respectively; ++p<0.01 versus corresponding basal release (ACTH = 0 M)

P450scc were analyzed by Western blotting. Signals at 54 kDa (P450scc) and 30 kDa (StAR protein) molecular weight were detected in rat ZFR cells. The signal of β-actin detected by appearance of the 45 kDa was used as an internal control. Application of ACTH (1 nM) for 2 h markedly enhanced the protein expression of StAR (Fig. 3a, p<0.05). The PTU (12 mM) significantly inhibited the basal and ACTH-stimulated protein expression of StAR (Fig. 3a, p<0.05). Neither ACTH nor PTU altered the P450scc protein expression (Fig. 3b). Since the PTU abolished the protein expression of StAR protein, the previous references showed that SF-1 potentially regulated the gene expression of StAR. Therefore, we synthesized a 24 bp DNA fragment corresponding to the SF-1 binding site (SF-1 binding site, from -135/-86) of StAR promoter. Our results demonstrated that PTU treatment of ZFR cells attenuated the binding of ZFR cell nuclear proteins to synthetic double-stranded oligonucleotides corresponding to SF-1 as shown in Fig. 4. The enhanced binding activity was observed after treatment of ACTH for 1 h. Administration of PTU decreased the ACTH-induced binding activity to SF-1 binding site (Fig. 4).

Effects of PTU on enzyme activities of P450scc: The conversion of cholesterol to pregnenolone is catalyzed by P450scc, which is the rate-limiting step in steroid biosynthesis. The intracellular pregnenolone is metabolized by 3b-HSD. An inhibitor of 3β-HSD, trilostane was used to accumulate pregnenolone production. Incubation of ZFR cells with trilostane (10 μM) increased the accumulation of pregnenolone production (Fig. 5, top, p<0.01). Administration

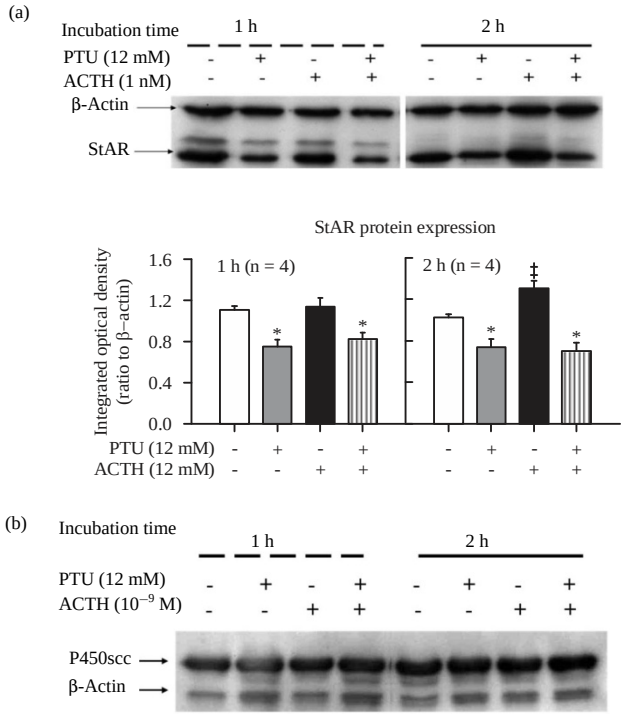

Fig. 3(a-b): Protein expression of steroidogenic acute regulatory protein (StAR) and P450scc. The ZFR cells were homogenized and total protein concentrations were determined by Bradford assay. Sample proteins were separated by SDS-PAGE and immunoblotted with anti-StAR, anti-P450scc and anti-β-actin antibodies, (a) To indicate StAR protein levels after challenging with PTU (12 mM) in the presence or absence of ACTH (1 nM) and (b) To detect P450scc expression at 1 and 2 h after treatment. Each column represents the Mean±SEM. *p<0.05 compared to vehicle group (PTU = 0 M), respectively; ++p<0.01 versus corresponding basal release (ACTH = 0 M)

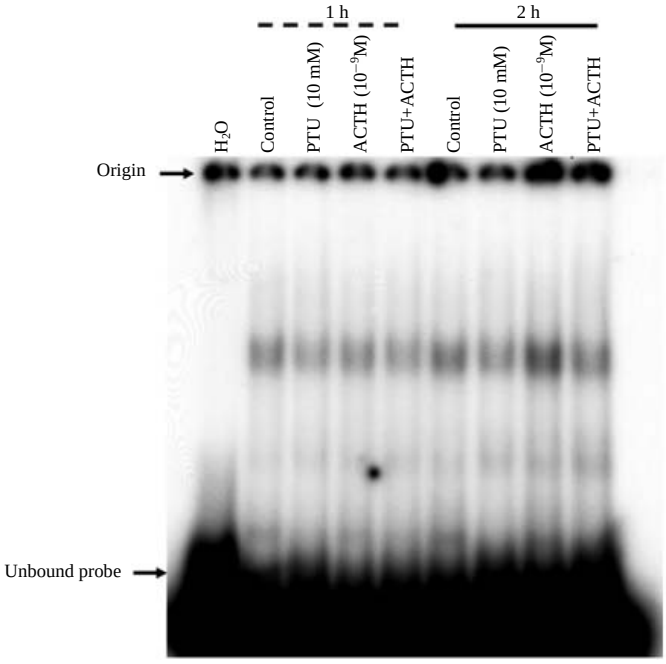

Fig. 4: Electrophoretic mobility shift analysis of binding activity to SF-1 binding sites within the StAR promoter. The nuclear fractions of ZFR cells were subjected to gel-shift analysis to detect their interactions to SF-1 binding site. Bracket indicates protein-DNA complex formation, PTU: Propylthiouracil, ACTH: Adrenocorticotropic hormone

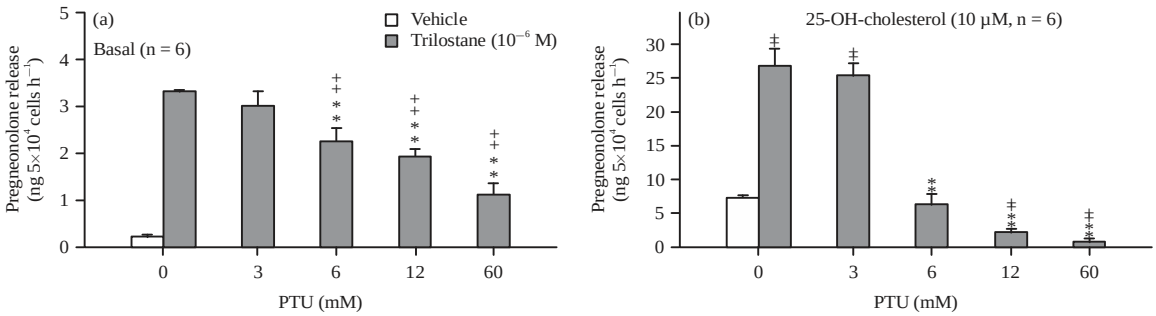

Fig. 5(a-b): Effect of PTU on pregnenolone secretion in respone to 25-OH-cholesterol (b) by ZFR cells in the presence or absence of trilostane. Each column represents the Mean±SEM. **p<0.01 compared with PTU = 0 M, ++p<0.01 versus corresponding basal release (a)

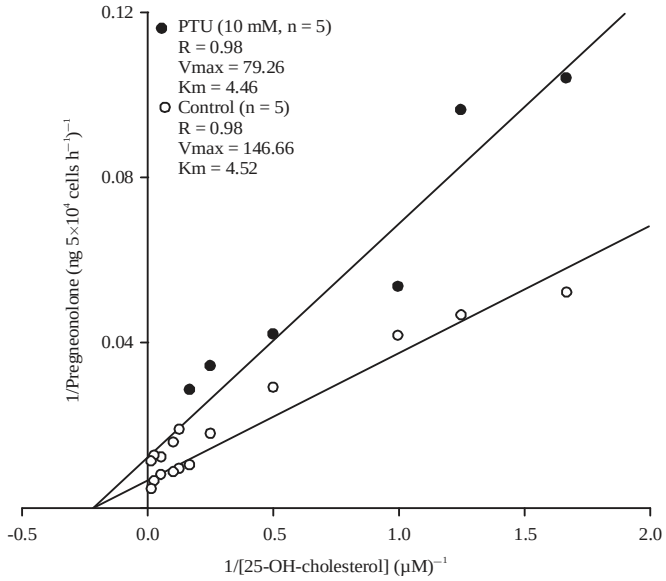

Fig. 6: Kinetic analysis of PTU inhibition on P450scc function. Double reciprocal plots of data were obtained from cultured ZFR cells challenged with 25-hydroxy-cholesterol (0.1-100 μM). The Vmax of the PTU-treated group (79.26 μM) was less than that of the control group (146.66 μM). The Km value of the both groups were almost the same

of PTU (6~30 mM) dose-dependently decreased the pregnenolone accumulation caused by trilostane (Fig. 5, top, p<0.05 and p<0.01). Application of trilostane in combination with 25-OH-cholesterol caused a 3.6-fold rise in pregnenolone production (Fig. 5, bottom, p<0.01). Administration of PTU (3~30 mM) lowered pregnenolone production in response to 25-hydroxy-cholesterol by a dose-dependent manner (p<0.01).

Furthermore, enzymatic studies for determining the Km and Vmax of P450scc in vehicle and PTU-treated groups were performed. The activity was determined by analyzing the amount of pregnenolone produced from 25-OH-cholesterol. The kinetic parameters, Km and Vmax were determined from Lineweaver-Burk plots in Fig. 6. In the presence of PTU (10 mM), 46% decrease in the Vmax as compared with control (PTU group 79.26 ng. 5×10^{4} cells^{-1} h^{-1}, versus control group 146.66 ng 5×10^{4} cells^{-1} h^{-1}) was found. The Michaelis constants (Km) were almost the same in control (4.52 mM) and in PTU group (4.46 mM).

DISCUSSION AND CONCLUSION

Prophlthiouracil (PTU) is known to block the synthesis of thyroid hormone by preventing the uptake and utilization of iodide in thyroid glands. Previous studies indicated that the chronic treatment of PTU influenced the Hypothalamus Pituitary Adrenal (HPA) axis. These data suggested that the lowered plasma level of corticosterone in PTU-induced

hypothyroidism was caused by the deficiency of circulating T_3 level rather than the direct effect of PTU[6,7,8,9]. However, PTU-induced hypothyroidism caused a significant reduction of CRH mRNA expression in paraventricular nucleus[10]. The plasma ACTH concentration was unchanged in PTU-treated animals although the lower pituitary content of ACTH was observed[9]. Furthermore, a morphological change was found in adrenal cortex in PTU-treated animals[12]. These data suggested that PTU might exert a direct effect on adrenal function. Previously, the direct effects of PTU on adrenal corticosterone synthesis *in vitro*[28] was confirmed. In addition, PTU directly regulated the secretory capacity of isolated testicular interstitial cells and Leydig cells[4,5]. In the present study, it is investigated that how the possible effects of PTU on StAR protein and P450scc were responsible for rate-limiting step of steroidogenesis.

In the animal study, the effective dose of PTU to induce hypothyroidism might be via oral intake (0.5%) or intraperitoneal injection (20 mg per day[1])[9,37]. Neither the level of free PTU nor the accumulation of PTU in tissues after treatment is detectable. In order to control the dosage effect of PTU, the animals were treated with PTU by intravenous infusion. According to the *in vivo* results, the elevated plasma pH value and plasma corticosterone concentration was observed following infusion with alkaline saline (pH 9.5). The enhanced plasma pH might be a stimulant for corticosterone release. As compared with alkaline saline treated group, PTU infusion acutely reduced the plasma level of corticosterone. The inhibitory effects of PTU on plasma corticosterone persisted for at least 180 min. However, the plasma T_4 concentration was not altered by acute infusion of PTU. These results indicated that the lower plasma corticosterone concentration was associated with the direct effects of PTU on adrenal gland and was independent of the secretion of thyroid hormones.

Steroid hormones secreted by the adrenal gland, testis, ovary and plancenta are the important factors in regulating tissue differentiation, development and homeostasis[15]. In all species, the first rate-limiting reaction in the biosynthesis of steroid hormones is the conversion of cholesterol to pregnenolone[38]. The enzyme, cytochrome P450scc, catalyzes the cleavage of the C20-C21-carbon and carbon bond of sterol to produce an obligatory intermediate named pregnenolone[39]. The P450scc locates in the matrix side of the inner mitochondrial membrane and is activated only when it is bound to the inner membrane of mitochondrion[16]. Therefore, transferring of cholesterol from the outer cholesterol-rich mitochondrial membrane to inner mitochondrial membrane is considered to be the rate-limiting step in the acute control of steroidogenesis[17,18,38]. The candidate protein for cycloheximide -sensitive, short-acting acute mediator of cholesterol transport between mitochondrial membranes is identified as a 30 kDa protein named steroidogenic acute regulatory (StAR) protein[20,21]. Both StAR and P450scc play the important roles in regulating steroidogenesis. Our *in vitro* (Fig. 2 and 3) data showed that both basal and ACTH-stimulated secretion of corticosterone were inhibited by PTU treatment. Although, 21-hydroxylase and 11β-hydroxylase were involved in the inhibitory effects of PTU on corticosterone production in rat ZFR cells[28], the changes in StAR and P450scc including protein expression and/or enzyme activity caused by PTU is still unknown. Therefore, effect of PTU on protein expression of StAR in ZFR cells was investigated in this study. Based on the results of Western blot, StAR protein was highly expressed in the presence of ACTH. After treatment with PTU, both the basal and the ACTH-stimulated expressions of StAR protein were diminished. The results indicated that PTU markedly impaired adrenal steroid hormone synthesis at least in part due to an inhibition on the protein expression of StAR.

Several factors participated in StAR gene transcription have been reported. In spite of a number of studies, the regulation of StAR gene transcription is not fully understood. The upstream of StAR gene contains binding sites for transcriptional regulators such as SF-1, DAX-1 (dosagesensitive sex reversal-adrenal hyperplasia congenital critical region on the X-chromosome) and AP-1 (activator protein-1). However, SF-1 is considered to be potentially important in the regulation of the StAR gene[22,40]. The SF-1 has been shown to have critical role not only in the regulation of steroidogenesis but also the development of the gonads and adrenal glands[23,41]. Several SF-1 consensus binding sites have been found in the StAR promoter. In rats, five SF-1 sites have been demonstrated[42] and one additional site has been identified in human beings and mice[43,44]. Based on the EMSA results, high affinitive SF-1 binding site was formed at position -106 to -97 within the rat StAR promoter[42]. Therefore, a DNA probe was synthesized corresponding to the SF-1 binding site from -135/-86 to detect if the binding activity is influenced by treatment of PTU. Our results showed that PTU diminished the binding activity to SF-1 binding site following 30 min treatment. The enhanced binding activity in response to ACTH was attenuated by PTU administration. Therefore, a transcriptional repression mechanism might play an important role in mediating PTU's inhibitory effect on StAR expression.

Numerous studies have shown that the expression and enzyme activity of P450scc was influenced by drug treatment[26,45]. Following PTU treatment, the expression of P450scc was unchanged. Therefore, the ability of PTU to act on

the enzyme activity of P450scc in ZFR cells was tested in this study. The trilostane, an inhibitor of 3β-HSD, accumulated the pregnenolone production. Both the accumulated and 25-OH-cholesterol-evoked pregnenolone productions were reduced dose-dependently by PTU treatment. These findings suggested that PTU-reduced corticosterone secretion was associated with attenuation of P450scc enzyme activity. The P450scc activity was further examined in this study by enzyme kinetic analysis. The data showed that the inhibitory effect of PTU was due to a decrease of Vmax rather than a change in Km. The PTU did not compete for binding the active site, but prevented the catalytic event. Therefore, we found that PTU acted as a noncompetitive inhibitor of cholesterol side chain cleavage reaction. However, our previous study showed that PTU resulted in a 2.2-fold increase in Km value in rat Leydig cells[4]. This was consistent with a competitive inhibition mechanism. Prophylthiouracil could influence the complex formation of P450 scc and cholesterol in Leydig cells[4]. The contrast effect of PTU on P450scc from adrenal cells and Leydig cells might be associated with tissue-specific mechanisms. Taken together, we found that PTU acted acutely and directly on adrenal glands to modulate the biosynthesis of steroid hormones. Prophylthiouracil caused the dysfunction of adrenal gland through the regulation of StAR and P450scc that are responsible for the first and rate-limiting step of steroidogenesis.

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REFERENCES

1. Cooper, D.S., 1984. Antithyroid drugs. N. Engl. J. Med., 311: 1353-1362.
2. Levy, M., 1993. Propylthiouracil hepatotoxicity: A review and case presentation. Clin. Pediatr., 32: 25-29.
3. Chiao, Y.C., H. Lin, S.W. Wang and P.S. Wang, 2000. Direct effects of propylthiouracil on testosterone secretion in rat testicular interstitial cells. Br. J. Pharmacol., 130: 1477-1482.
4. Chiao, Y.C., W.L. Cho and P.S. Wang, 2002. Inhibition of testosterone production by propylthiouracil in rat Leydig cells. Biol. Reprod., 67: 416-422.
5. Chen, J.J., S.W. Wang, E.J. Chien and P.S. Wang, 2003. Direct effect of propylthiouracil on progesterone release in rat granulosa cells. Br. J. Pharmacol., 139: 1564-1570.
6. D'Agostino, J. and S.J. Henning, 1982. Role of thyroxine in coordinate control of corticosterone and CBG in postnatal development. Am. J. Physiol.-Endocrinol. Metab., 242: E33-E39.
7. Bigos, S.T., E.C. Ridgway, I.A. Kourides and F. Maloof, 1978. Spectrum of pituitary alteration with mild and severe thyroid impairment. J. Clin. Endocrinol. Metab., 46: 317-325.
8. Gallagher, T.F., K. Yoshida, H.D. Roffwarg, D.K. Fukushima, E.D. Weitzman and L. Hellman, 1973. ACTH and cortisol secretory patterns in man. J. Clin. Endocrinol. Metab., 36: 1058-1068.
9. Sanchez-Franco, F., L. Fernandez, G. Fernandez and L. Cacicedo, 1989. Thyroid hormone action on ACTH secretion. Hormone Metab. Res., 21: 550-552.
10. Shi, Z.X., A. Levy and S.L. Lightman, 1994. Thyroid hormone-mediated regulation of corticotropin-releasing hormone messenger ribonucleic acid in the rat. Endocrinology, 134: 1577-1580.
11. Yu, P.L., W.H. Lee, W.J. Huang and P.S. Wang, 2011. Adaptation of the secretion of gastric acid and gastric inhibitory peptide in response to propylthiouracil. Adapt. Med., 3: 106-111.
12. Moore, N.A. and G. Callas, 1975. Observations on the fine structure of propylthiouracil-induced brown degeneration in the zona reticularis of mouse adrenal cortex. Anatom. Rec., 183: 293-302.
13. Lo, M.J., S.W. Wang, M.M. Kau, J.J. Chen and Y.H. Chen *et al*., 1998. Pharmacological effects of propylthiouracil on corticos terone secretion in male rats. J. Invest. Med., 46: 444-452.
14. Lieberman, S., N.J. Greenfield and A. Wolfson, 1984. A heuristic proposal for understanding steroidogenic processes. Endocr. Rev., 5: 128-148.
15. Kallen, C.B., F. Arakane, L.K. Christenson, H. Watari, L. Devoto and J.F. Strauss III, 1998. Unveiling the mechanism of action and regulation of the steroidogenic acute regulatory protein. Mol. Cell. Endocrinol., 145: 39-45.
16. Yago, N. and S. Ichii, 1969. Submitochondrial distribution of components of the steroid 11β-hydroxylase and cholesterol sidechain-cleaving enzyme systems in hog adrenal cortex. J. Biochem., 65: 215-224.
17. Crivello, J.F. and C.R. Jefcoate, 1980. Intracellular movement of cholesterol in rat adrenal cells. Kinetics and effects of inhibitors. J. Biol. Chem., 255: 8144-8151.
18. Privalle, C.T., J.F. Crivello and C.R. Jefcoate, 1983. Regulation of intramitochondrial cholesterol transfer to side-chain cleavage cytochrome P-450 in rat adrenal gland. Proc. Natl. Acad. Sci. USA., 80: 702-706.
19. Miller, W.L., 1995. Mitochondrial specificity of the early steps in steroidogenesis. J. Steroid Biochem. Mol. Biol., 55: 607-616.

20. Clark, B.J., J. Wells, S.R. King and D.M. Stocco, 1994. The purification, cloning and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). J. Biol. Chem., 269: 28314-28322.
21. Stocco, D.M., 1999. Steroidogenic acute regulatory (StAR) protein: What's new? BioEssays, 21: 768-775.
22. Stocco, D.M., 2000. Intramitochondrial cholesterol transfer. Biochimica Biophysica Acta (BBA)-Mol. Cell Biol. Lipids, 1486: 184-197.
23. Wooton-Kee, C.R. and B.J. Clark, 2000. Steroidogenic factor-1 influences protein-deoxyribonucleic acid interactions within the cyclic adenosine 3',5'-monophosphate-responsive regions of the murine steroidogenic acute regulatory protein gene. Endocrinology, 141: 1345-1355.
24. Achermann, J.C., J.J. Meeks and J.L. Jameson, 2001. Phenotypic spectrum of mutations in DAX-1 and SF-1. Mol. Cell. Endocrinol., 185: 17-25.
25. Tsai, S.C., Y.C. Chiao, C.C. Lu, M.L. Doong and Y.H. Chen *et al*., 1996. Inhibition by amphetamine of testosterone secretion through a mechanism involving an increase of cyclic AMP production in rat testes. Br. J. Pharmacol., 118: 984-988.
26. Kisselev, P., R. Wessel, S. Pisch, U. Bornscheuer, R.D. Schmid and D. Schwarz, 1998. Branched phosphatidylcholines stimulate activity of cytochrome P450SCC (CYP11A1) in phospholipid vesicles by enhancing cholesterol binding, membrane incorporation and protein exchange. J. Biol. Chem., 273: 1380-1386.
27. Purdy, S.J., B.J. Whitehouse and D.R.E. Abayasekara, 1991. Stimulation of steroidogenesis by forskolin in rat adrenal zona glomerulosa cell preparations. J. Endocrinol., 129: 391-397.
28. Lo, M.J., M.M. Kau, J.J. Chen, J.Y. Yeh, H. Lin, S.W. Wang and P.S. Wang, 1999. Age-related differences in corticosterone secretion in female rats. Metabolism, 48: 535-541.
29. Gyles, S.L., C.J. Burns, B.J. Whitehouse, D. Sugden, P.J. Marsh, S.J. Persaud and P.M. Jones, 2001. ERKs regulate cyclic AMP-induced steroid synthesis through transcription of the Steroidogenic Acute Regulatory (*StAR*) gene. J. Biol. Chem., 276: 34888-34895.
30. Lin, S., W. Wang, G.M. Wilson, X. Yang, G. Brewer, N.J. Holbrook and M. Gorospe, 2000. Down-regulation of cyclin D1 expression by prostaglandin A_2 is mediated by enhanced cyclin D1 mRNA turnover. Mol. Cell. Biol., 20: 7903-7913.
31. Zor, T. and Z. Selinger, 1996. Linearization of the Bradford protein assay increases its sensitivity: Theoretical and experimental studies. Anal. Biochem., 236: 302-308.
32. Hu, M.C., I.C. Guo, J.H. Lin and B.C. Chung, 1991. Regulated expression of cytochrome *P*-450scc (cholesterol-side-chain cleavage enzyme) in cultured cell lines detected by antibody against bacterially expressed human protein. Biochem. J., 274: 813-817.
33. Kau, M.M., J.J. Chen, S.W. Wang, W.L. Cho and P.S. Wang, 1999. Age-related impairment of aldosterone secretion in zona glomerulosa cells of ovariectomized rats. J. Invest. Med., 47: 425-432.
34. Chen, J.J., P.S. Wang, E.J. Chien and S.W. Wang, 2001. Direct inhibitory effect of digitalis on progesterone release from rat granulosa cells. Br. J. Pharmacol., 132: 1761-1768.
35. Chen, J.J., E.J. Chien and P.S. Wang, 2002. Progesterone attenuates the inhibitory effects of cardiotonic digitalis on pregnenolone production in rat luteal cells. J. Cell. Biochem., 86: 107-117.
36. Steel, G.D. and J.H. Torrie, 1960. Principles and Procedures of Statistics with Special Reference to Biological Sciences. McGraw-Hill, New York, USA.
37. Hwang, J.C., P.H. Li and W.C. Wan, 1974. Effect of induced hypothyroidism on pituitary Luteinizing Hormone (LH) concentration in female rats. Taiwan Yi Xue Hui Za Zhi, 73: 227-231.
38. Miller, W.L. and J.F. Strauss III, 1999. Molecular pathology and mechanism of action of the steroidogenic acute regulatory protein, StAR. J. Steroid Biochem. Mol. Biol., 69: 131-141.
39. Lieberman, S. and Y.Y. Lin, 2001. Reflections on sterol sidechain cleavage process catalyzed by cytochrome P450scc. J. Steroid Biochem. Mol. Biol., 78: 1-14.
40. Parker, K.L., D.A. Rice, D.S. Lala, Y. Ikeda and X. Luo *et al*., 2002. Steroidogenic factor 1: An essential mediator of endocrine development. Recent Progr. Hormone Res., 57: 19-36.
41. Beuschlein, F., C.E. Keegan, D.L. Bavers, C. Mutch and J.E. Hutz *et al*., 2002. SF-1, DAX-1 and acd: Molecular determinants of adrenocortical growth and steroidogenesis. Endocrine Res., 28: 597-607.
42. Sandhoff, T.W., D.B. Hales, K.H. Hales and M.P. McLean, 1998. Transcriptional regulation of the rat steroidogenic acute regulatory protein gene by steroidogenic factor 1. Endocrinology, 139: 4820-4831.
43. Caron, K.M., Y. Ikeda, S.C. Soo, D.M. Stocco, K.L. Parker and B.J. Clark, 1997. Characterization of the promoter region of the mouse gene encoding the steroidogenic acute regulatory protein. Mol. Endocrinol., 11: 138-147.
44. Sugawara, T., M. Kiriakidou, J.M. McAllister, C.B. Kallen and J.F. Strauss, 1997. Multiple steroidogenic factor 1 binding elements in the human steroidogenic acute regulatory protein gene 5'-flanking region are required for maximal promoter activity and cyclic AMP responsiveness. Biochemistry, 36: 7249-7255.
45. Tuckey, R.C., A.J. McKinley and M.J. Headlam, 2001. Oxidized adrenodoxin acts as a competitive inhibitor of cytochrome P450scc in mitochondria from the human placenta. Eur. J. Biochem., 268: 2338-2343.