Stimulatory Effects of Propylthiouracil on Pregnenolone Production through Upregulation of Steroidogenic Acute Regulatory Protein Expression in Rat Granulosa Cells

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Propylthiouracil (PTU) is a commonly used hyperthyroidism drug that inhibits both the synthesis of thyroid hormones (Cooper, 1984) and the conversion of thyroxine (T4) to triiodothyronine (T3) (Cooper, 1984; Yang and Gordon, 1997). For adults, the clinical dosage of PTU varies depending on the severity of the disease and ranges from 100 to 300 mg three times daily. After 4–8 weeks, the doses can be tapered down, and the optimal duration of therapy is considered to be 12–18 months (Abraham and Acharya, 2010). Clinical studies show that the PTU treatment in hyperthyroidism patients may be associated with side effects such as transient leucopenia (Cooper, 1984), hepatotoxicity (Hanson, 1984; Jonas and Eidson, 1988; Rivkees, 2010; Rivkees and Szarfman, 2010), hepatomegaly, jaundice (Chastain et al., 1999; Deidiker and deMello, 1996; Levy, 1993), and vasculitis (Chastain et al., 1999; Sorribes et al., 1999). PTU is the primary treatment choice during pregnancy because it has been reported to minimally penetrate the placenta; however, PTU is still categorized as a class D agent in the United States because of the potential for fetal hypothyroidism (Cooper, 2005). Some recent studies that employed PTU to induce hypothyroidism drop a hint of the possible effects of PTU on female reproductive function (Hapon et al., 2007, 2003; Hatsuta et al., 2004; Thrift et al., 1999). On the other hand, administration of PTU results in a marked reduction of ovarian weight and the number of primordial, multilaminar, and Graafian follicles in mice (Chan and Ng, 1995).

The effects of PTU on male reproductive function have been well described. PTU-induced neonatal hypothyroidism increases testis size and sperm production, accompanied by increased numbers of Sertoli and germ cells (Hess et al., 1993; Joyce et al., 1993). Subsequent studies demonstrated that testosterone production is reduced in parallel with decreased numbers of human chorionic gonadotropin (hCG)–binding sites in Leydig cells after PTU treatment (Hardy et al., 1993).

We previously reported that PTU also has direct effects on steroidogenesis in rats. PTU decreases corticosterone production...
MATERIALS AND METHODS

Animals. Immature female Sprague-Dawley rats were housed in a temperature-controlled room (22 ± 1°C) with 14 h of artificial illumination daily (0600–2000 h). Food and water were supplied ad libitum. The investigations were conducted in accordance with the Guide for Care and Use of Laboratory Animals (National Science Council).

Reagents. Chemicals and reagents including collagenase, hyaluronidase, pregnant mares’ serum gonadotropin (PMSG), Dulbecco’s modified Eagle’s medium (DMEM/F12), fatty acid-free bovine serum albumin (BSA), N-2-hydroxethylpiperazine-N-2-ethanesulfonic acid (HEPES), penicillin G, streptomycin sulfate, insulin, t-glutamine, 8-bromo-cAMP (8-Br-cAMP), 25-hydroxycholesterol (25-OH-cholesterol), pregnenolone, phenylmethylsulfonyl fluoride (PMSF), and PTU (propylthiouracil; CAS Number: 51-52-5) were obtained from Falcon Labware (Lincoln Park, NJ). The antipregnenolone antibodies. The signals were visualized by enhanced chemiluminescence procedures. Western blots were performed using the antibodies anti-StAR (1:1000), anti-P450scc (1:2000), antiphospho-ERK (extracellular signal-regulated kinase) (1:500), and anti-fl-actin (1:5000).

ELISA of pregnenolone. The concentration of pregnenolone in the culture medium was measured using an ELISA, as described elsewhere (Wu et al., 2010). Briefly, 96-well plates were coated with pregnenolone-BSA. Pregnenolone standards (purchased from Sigma Chemical Co.) or samples (50 μl per well), in combination with 50 μl primary antibody (1:12,800 dilution in blocking buffer), were added and incubated at 37°C for 1 h. After the incubation, plates were washed four times with washing buffer (PBS containing Tween-20, 0.05%, pH 7.3–7.4) and then incubated with conjugated secondary antibody (200 μl per well; IgG-HRP, 1:5000 dilution in blocking buffer) at 37°C for 30 min. After washing, 3,3′,5,5′-tetramethylbenzidine substrate (Sigma Chemical Co.) was supplied (200 μl per well) and cultured in the dark at room temperature for 10 min. Finally, HCl was applied to stop the reaction, and the absorbance values were measured at 450 nm (Microplate Reader; Dynatech Laboratories, Chantilly, VA).

Western blotting. To determine protein expression, Western blotting was performed as described elsewhere (Kan et al., 2004) with modifications. Briefly, after culturing with or without PTU, granulosa cells were harvested and lysed in homogenization buffer (1.5% Na-lauroylsacrosine, 2.5 mM Tris base, 1 mM EDTA, and 0.1% PMSF, pH 7.8). Protein concentration was determined using the Bradford protein assay method with modifications (Bradford, 1976). The aliquots of cell lysate were boiled in SDS sample buffer (0.6% Tris base, 2% SDS, 0.005% bromophenol blue, 6% sucrose, and 50 mM dithiothreitol (DTT)) and underwent electrophoresis with a 12% mini-gel by standard SDS-polyacrylamide gel electrophoresis procedures. Western blots were performed using the antibodies anti-StAR (1:1000), anti-P450scc (1:2000), antiphospho-ERK (extracellular signal-regulated kinase) (1:500), and anti-fl-actin (1:5000). The secondary antibodies used for Western blotting were goat anti-mouse (Promega) and goat anti-rabbit (Promega) horseradish peroxidase-conjugated antibodies. The signals were visualized by enhanced chemiluminescence detection (Amersham International, Stafford, UK).
**Electrophoretic mobility shift assay.** Electrophoretic mobility shift assays (EMSAs) were performed to investigate the effect of PTU on the interaction of steroidogenic factor-1 (SF-1) and the StAR promoter region. For nuclear extraction, cells were collected and resuspended in 50 μl lysis buffer A containing protease inhibitors (10mM HEPES, 10mM KCl, 1.5mM MgCl2, 0.1% aprotinin, and 0.1% PMSF, pH 7.9). After bathing on ice for 5 min, 6.25 μl buffer B (10mM HEPES, 10mM KCl, 1.5mM MgCl2, and 2.5% NP-40, pH = 7.9) was added and the tube was vortexed. The homogenates were centrifuged at 13,800 × g for 30 s to pellet the crude nuclear extract. The nuclear pellet was resuspended with 30 μl buffer (0.45M NaCl, 1mM EDTA, pH 7.9), followed by vigorous rocking at 4°C for 20 min. The nuclear lysate was centrifuged for 10 min at 13,800 × g, and the supernatant was assayed directly or stored at −80°C. A fragment of 5′-flanking region of the StAR gene (−144/−123), 5′-CTCCCTCCACCTTGCCACGACT-3′, which contains the SF-1 binding site, was labeled with [γ-32P-ATP] as described elsewhere (Reinhart et al., 1999). The bold and underlined sequences are the consensus sequences of zinc finger structure of SF-1 protein. The binding complex included 5 μg of nuclear protein, 4 μl of 5× binding buffer (20% glycerol, 5mM MgCl2, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, and 50mM Tris-HCl), 1 μg poly (dI): poly (dC), and 50,000 cpm of 32P-labeled SF-1 probe. After incubation at room temperature for 20 min, the samples were loaded onto a 6% polyacrylamide gel, and electrophoresis was continued at 80 volts for 15 min followed by 150 volts for 90 min. The gel was then vacuum dried and exposed to x-ray film overnight, which was then developed.

**Statistical analysis.** All values are expressed as mean ± SEM. Treatment means were tested for homogeneity by the ANOVA, and the differences between specific means were tested for significance using the Duncan’s multiple range test (Steel and Torrie, 1960). All analyses were performed by specific formulas in Microsoft Excel. The chosen levels of significant and highly significant differences were p < 0.05 and p < 0.01, respectively. The maximum velocities (Vmax) and Michaelis constants (Km) for vehicle and PTU groups were estimated using SigmaPlot.

**RESULTS**

**Stimulatory Effects of PTU on Pregnenolone Production in Rat Granulosa Cells**

Our previous study reported that PTU (1.5–12mM) acutely decreases not only the basal release of pregnenolone but also the release of pregnenolone in response to 25-OH-cholesterol at a high dose of PTU (12mM) within 2 h (Chen et al., 2003). To further characterize the temporal effects of PTU on pregnenolone production, PTU (6mM) was administered to the culture medium of rat granulosa cells for different time intervals, and the intermediate steroid, pregnenolone, was measured by ELISA. The results indicate that a longer PTU treatment could increase pregnenolone production compared with the vehicle group (Fig. 1A). The results of the MTT assay revealed that the increase in pregnenolone is not due to variation in cell number (Fig. 1B), and the constant values of cell number also indicate that PTU (0.3–6mM) is not cytotoxic to granulosa cells.

**Inhibitory Effects of PTU on Progesterone Production in Rat Granulosa Cells**

Our previous results demonstrated acute PTU inhibition of pregnenolone and progesterone production in rat granulosa cells (Chen et al., 2003). However, in this study, we found that pregnenolone was increased under a longer time period of PTU treatment. To verify whether the temporal effects of PTU on progesterone production correspond to pregnenolone production, PTU (6mM) was added to the culture medium of rat granulosa cells for different time intervals, and progesterone levels were measured by RIA. Although pregnenolone production was increased after 16 h of exposure to PTU (Fig. 1A), the release of progesterone was still inhibited compared with the vehicle group (Fig. 2A). We also found that a 16-h PTU treatment could dose dependent inhibit progesterone production (Fig. 2B).

**PTU Inhibition of 3β-HSD Activity in Rat Granulosa Cells**

To determine the mechanism of PTU inhibition of progesterone, the activity of steroidogenic enzyme, 3β-HSD, which converts pregnenolone to progesterone, was analyzed. Pregnenolone was added into the culture medium of granulosa cells as a precursor, and secreted progesterone was measured by RIA. We found that PTU (6mM for 16 h) inhibited...
progesterone production in the presence or absence of pregnenolone supplement (10⁻⁷ to 10⁻⁶M) (Fig. 3A). Subsequently, the PTU effect on 3β-HSD activity in granulosa cells was detected by kinetic analysis. Double reciprocal plots of the data were obtained from cultured granulosa cells challenged with pregnenolone (10⁻⁵ to 10⁻⁷M). The maximum velocities ($V_{\text{max}}$) for the vehicle group and the PTU group were nearly the same (243.9 ng per 10⁵ cells per 16 h). The Michaelis constant ($K_m$) for the PTU-treated group (30.46 lM) was greater than that of the vehicle group (21.95 lM) (Fig. 3B). These results indicate that a 16-h PTU treatment inhibited 3β-HSD enzyme activity in rat granulosa cells.

**PTU Stimulation of the Conversion of Cholesterol to Pregnenolone in Rat Granulosa Cells**

To further investigate the opposite effects of PTU on the production of two steroid hormones, the conversion process of cholesterol to pregnenolone was then monitored. Granulosa cells were primed for 30 min with the 3β-HSD inhibitor triolostane (10⁻⁵M) to disrupt the conversion of pregnenolone to progesterone before exposure to PTU. Pregnenolone production was measured by ELISA after administration of PTU and different concentrations of the steroidogenic precursor 25-OH-cholesterol (10⁻⁶ to 10⁻⁷) for 16 h in the presence of triolostane (10⁻⁵M). Similar to the results shown in Figure 1A, 16 h of treatment with PTU significantly increased pregnenolone production after various dosage supplements of cholesterol (Fig. 4A). To evaluate whether the increased pregnenolone is correlated to a change in the P450scc enzyme, protein analysis was performed, and we found that the P450scc protein levels in total cell lysate were even slightly decreased by PTU treatment (Fig. 4B). In addition, the protein levels of P450scc in the mitochondrial protein fraction were not affected by PTU treatment (Fig. 4C).
PTU Stimulation of StAR Protein Expression

Because PTU increased pregnenolone production but did not affect P450scc, the precursor supplement may affect pregnenolone production. Cholesterol transportation across the mitochondrial membrane is the rate-limiting step in steroid biosynthesis; StAR plays an important role in this process (Hadley, 2000). We further identified the effects of PTU on StAR protein levels, and the results indicate that PTU dose dependently increased StAR protein expression in granulosa cell lysates (Fig. 5A). Furthermore, a significant increase in mitochondrial StAR protein expression after PTU treatment was also found (Fig. 5B). To identify the mechanism by which PTU increases StAR protein expression, we performed EMSAs to evaluate whether the binding of SF-1 with the StAR promoter region was affected by PTU treatment. The results show that after treatment with PTU (6mM), the binding of SF-1 to the StAR promoter region was enhanced compared with the control groups (Fig. 5C). The data suggest that PTU was able to increase StAR-dependent cholesterol transportation and likely enhance subsequent conversion of cholesterol to pregnenolone.

PTU Stimulation of StAR Protein Expression through MEK

Several lines of evidence indicate that MEK is involved in the regulation of StAR expression (Gyles et al., 2001; Hammer et al., 1999). Here, we found that MEK inhibition by the molecular inhibitor PD98059 affected PTU-induced ERK1 phosphorylation (Fig. 6A). The data further indicate that PTU-induced StAR protein expression was slightly diminished by this MEK inhibitor (Fig. 6B), which suggests that MEK might be involved in PTU regulation of StAR expression.

DISCUSSION

Thyroid disease is frequent in women, and PTU has historically been the drug of choice for treating pregnant and breast-feeding women with this condition because of its limited transfer into the placenta and breast milk (Marchant et al., 1977; Streetman and Khanderia, 2003). Although PTU is effective against hyperthyroidism, there still exist some side effects after PTU treatment. Therefore, the effects of PTU on tissues other than the thyroid, especially on the female reproductive system, are worthy of investigation. Our previous results demonstrated that acute treatment with PTU can inhibit progesterone production by affecting P450scc activity in rat granulosa cells (Chen et al., 2003). Our present results show that a 16-h treatment with PTU could stimulate the production of pregnenolone, which is the precursor of progesterone in the process of steroidogenesis, but production of progesterone remained low. This finding implies that granulosa cells may counter the inhibitory effect of PTU on progesterone production by increasing upstream precursors in a negative feedback manner.

The dose of PTU employed in the present study (6mM), which follows our previous experiments (Chen et al., 2003; Chiao et al., 2000), is higher than the clinical dosage. However, the serum levels of PTU after oral administration of this drug vary depending on patients’ conditions (e.g., age and disease state) (Melander et al., 1977; Sato et al., 1983). The serum half-life of PTU is about 1.5 h (Cooper, 2005). Sato et al.
(1983) revealed that the maximum serum concentration of PTU after a single 200 mg oral dose was achieved within 1 h and that these concentrations were $3.1 \pm 0.82 \times 10^{-3}$ in normal subjects ($n = 6$) and $2.8 \pm 1.4 \times 10^{-5}$ in hyperthyroidism patients ($n = 7$). Another report showed serum concentrations of PTU ranging from 1.6 to 7.5 mg/ml ($0.94$ to $4.4 \times 10^{-3}$) 1 h after a single 400 mg oral dose of the drug in hyperthyroidism patients ($n = 17$) (Kampmann and Molholm Hansen, 1981). Most blood PTU is metabolized by the liver, and this drug may cause hepatotoxicity, including severe liver injury and acute liver failure (Hanson, 1984; Jonas and Eidson, 1988; Rivkees, 2010; Rivkees and Mattison, 2009). Therefore, it is possible that PTU may have accumulating effects after long periods in patients.

Our previous studies indicated that short-term (2 h) treatment with PTU could lead to the decline of progesterone production in rat granulosa cells by decreasing the protein levels of StAR and P450scc and inhibiting the enzyme activities of $3\beta$-HSD and P450scc. In the current studies, we explored the effects of PTU on steroid production by granulosa cells using a longer duration of exposure. Treatment with PTU for 16 h was performed in this study, and production of both progesterone and its precursor, pregnenolone, in culture medium were detected. Surprisingly, although progesterone production was still inhibited, pregnenolone was significantly stimulated by 16 h of PTU treatment. To understand the opposite effects of PTU on steroid production, the relevant steps of steroidogenesis were further investigated. The conversion of pregnenolone into progesterone was diminished by PTU, which suggests that $3\beta$-HSD activity was affected by the 16-h PTU treatment, as in our previous study using a short-term PTU challenge. According to the results of the enzyme kinetic analysis (Fig. 3B), we suggest that PTU acts as a competitive inhibitor of $3\beta$-HSD, based on the increasing $K_m$ value and unchanging $V_{max}$ values compared with vehicle groups.

In addition, the first cholesterol conversion step of steroidogenesis was investigated, and the data show that the conversion of cholesterol into pregnenolone was enhanced.
The protein level of P450sc, which is responsible for the conversion of cholesterol to pregnenolone, was not affected by the 16-h PTU treatment. These results imply that the cholesterol conversion may not or may only partially contribute to P450sc activation. The one remaining possibility for the stimulatory effects of PTU on pregnenolone production is the availability of cholesterol, the key steroid precursor. Because the P450sc enzyme localizes to the inner membrane of mitochondria, cholesterol has to penetrate across the mitochondrial outer membrane in order to associate with P450sc. The StAR protein plays a major role in the transport of cholesterol into the inner space of mitochondria (Hadley, 2000). We found that in both whole cell and mitochondria-enriched lysates, StAR protein was significantly upregulated by a 16-h PTU treatment.

The orphan nuclear receptor transcription factor, SF-1, can function at the StAR gene promoter site to control StAR transcription (Sandhoff et al., 1998; Sugawara et al., 1996, 1997). EMSAs were performed to investigate the transcriptional regulation of StAR by SF-1. We found that the binding of SF-1 to the StAR promoter was enhanced by PTU treatment, which supports the finding of increased protein levels. According to these findings, we suggest that PTU may acutely decrease progesterone production through inhibiting 3β-HSD activity; however, to maintain progesterone production after longer exposure to PTU, granulosa cells speed up the upstream rate-limiting step, which is cholesterol transportation, by upregulating StAR protein expression. This can explain why stimulatory effects of PTU on pregnenolone production were detected.

In the past decade, researchers have shown that the mitogen-activated protein kinase family plays important roles in steroidogenesis (Gyles et al., 2001; Manna et al., 2006). The activation of the ERKs enhances the phosphorylation of SF-1 and expression of StAR protein (Gyles et al., 2001). The results in this study show that MEK inhibition partially blocked the PTU-induced increase in StAR protein expression, which suggests that in addition to SF-1, MEK/ERK activation may be another strategy by which granulosa cells counter PTU-triggered inhibition of progesterone.

It is known that the hypothalamus-pituitary-thyroid axis is functionally associated with and interferes with the hypothalamus-pituitary-ovary axis (Doufas and Mastorakos, 2000) and that both hyper- and hypothyroidism may result in menstrual disturbances (Doufas and Mastorakos, 2000; Krassas, 2000). Although there are few reports in the literature claiming that PTU can negatively affect human reproductive health during pregnancy, PTU may interfere with the hypothalamus-pituitary-ovary axis and produce various alterations in hormonal profiles while it disrupts the synthesis and conversion of thyroid hormones. In this study, we reveal the direct function and possible regulatory mechanisms of PTU on steroidogenesis in rat granulosa cells (Fig. 7) and hope these findings will bring more knowledge to the application of PTU in the future.

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