TOXICOLOGICAL SCIENCES **118(2)**, 667–674 (2010)
doi:10.1093/toxsci/kfq302
Advance Access publication October 5, 2010

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

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Received April 26, 2010; accepted September 10, 2010

Propylthiouracil (PTU) is a common and effective clinical medicine for the treatment of hyperthyroidism. Our previous study demonstrated that short-term treatment with PTU inhibits progesterone production in rat granulosa cells. However, our present results indicate that a 16-h treatment with PTU was able to stimulate pregnenolone production in rat granulosa cells, although progesterone production was diminished by PTU through inhibition of 3β-hydroxysteroid dehydrogenase. Notably, we found that PTU treatment enhanced the conversion of cholesterol into pregnenolone, whereas the protein level of the cytochrome P450 side-chain cleavage enzyme (P450scc, which is the enzyme responding to this conversion) was not affected. Interestingly, the levels of steroidogenic acute regulatory protein (StAR) in both total cell lysate and the mitochondrial fraction were significantly increased by PTU treatment. Furthermore, the binding of steroidogenic factor-1 (SF-1) to the *StAR* promoter region was also enhanced by PTU treatment, which suggests that PTU could upregulate *StAR* gene expression. In addition to SF-1 regulation, we found that mitogen-activated protein (MAP) kinase kinase activation is an important regulator of PTU-stimulated StAR protein expression, based on the effects of the MEK inhibitor PD98059. In conclusion, these results indicate that PTU plays opposite roles in the production of progesterone and its precursor, pregnenolone. The regulation of negative feedback on speeding the cholesterol transportation and pregnenolone conversion after a 16-h PTU treatment may be the mechanism explaining PTU's inhibition of progesterone production in rat granulosa cells.

Key Words: **PTU; P450SCC; StAR; pregnenolone.**

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

both *in vivo* and *in vitro* through attenuating the activity of 11β-hydroxylase and 3′-5′-cyclic adenosine monophosphate (cAMP) production in rat zona fasciculata-reticularis cells (Lo *et al*., 1998). PTU also diminishes testosterone production by Leydig cells through inhibition of the cytochrome P450 side-chain cleavage enzyme (P450scc) and the steroidogenic acute regulatory (StAR) protein (Chiao *et al*., 2000, 2002).

Progesterone is one of the main steroid hormones produced in the ovaries. It is responsible for maintaining pregnancy and progestational activity (Hadley, 2000). Transportation of cholesterol across mitochondrion intermembrane space by StAR protein is the rate-limiting step in progesterone production. P450scc and 3β-hydroxysteroid dehydrogenase (3β-HSD) are responsible for the conversion of cholesterol to pregnenolone and pregnenolone to progesterone, respectively (Hadley, 2000).

Although there is no direct evidence indicating potential harmful effects of PTU on human reproductive health, our previous study revealed that PTU acutely decreases both the release of progesterone and pregnenolone by granulosa cells via a thyroid-independent mechanism involving the inhibition of the post-cAMP pathway. Administration of PTU for 2 h diminishes the hCG-stimulated protein levels of StAR and consequently inhibits the enzyme activities of both P450scc (conversion of 25-hydroxy-cholesterol to pregnenolone) and 3β-HSD (conversion of pregnenolone to progesterone) (Chen *et al*., 2003). In the present study, we further investigated the long-term effects of PTU on steroidogenesis in rat granulosa cells. The results show that a 16-h PTU treatment enhanced the conversion of cholesterol to pregnenolone, although progesterone production was decreased. The enhanced StAR protein expression and mitogen-activated protein (MEK) activation by PTU treatment may be the possible mechanism by which PTU induces pregnenolone accumulation.

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-hydroxyethylpiperazine-NO-2-ethanesulfonic acid (HEPES), penicillin G, streptomycin sulfate, insulin, L-glutamine, 8-bromo-cAMP (8-Br-cAMP), 25-hydroxy-cholesterol (25-OH-cholesterol), pregnenolone, phenylmethylsulfonyl fluoride (PMSF), and PTU (propylthiouracil; CAS Number: 51-52-5) were purchased from Sigma Chemical Co. (St Louis, MO). Cell culture plastic wares were obtained from Falcon Labware (Lincoln Park, NJ). The antipregnenolone antiserum was purchased from Biogenesis Inc. (Sandown, NH). The peroxidase-conjugated IgG fraction to mouse IgG and peroxidase-conjugated IgG fraction to rabbit IgG were purchased from ICN Pharmaceuticals, Inc. (Aurora, OH). The anti-P450scc antibody and anti-StAR antibody were kindly provided by Dr B.C. Chung (Hu *et al*., 1991) and Dr D.M. Stocco (Lin *et al*., 1998), respectively.

Isolation and culture of granulosa cells. The preparation of granulosa cells was modified from a previously described procedure (Chen *et al*., 2001; Hwang *et al*., 1996; Tsai *et al*., 1999). Immature female rats, 25–27 days of age, were sc injected with PMSG (25 IU per rat) to stimulate the maturation of ovarian follicles. After 48–60 h, rats were cervical dislocated and ovaries were excised and submerged into cold harvest medium (sterile DMEM/Ham's F12 1:1 containing 0.1% fatty acid–free BSA, 20mM HEPES, 100 IU/ml penicillin G, and 100 μg/ml streptomycin sulfate, pH 7.4). After trimming free the fat and connective tissues, follicles were punctured with a 26-gauge needle to release granulosa cells. The harvested cells were centrifuged at 200 × g for 10 min at 4°C and resuspended in growth medium (DMEM/Ham's F12 containing 10% fetal calf serum, 2 $\mu g/ml^1$ insulin, 100 IU/ml penicillin G, and 100 μg/ml streptomycin sulfate). The viability of granulosa cells was quantified by trypan blue staining, and cells were subsequently plated in 24-well plates at 2×10^5 viable cells per well and incubated at 37°C with 5% CO_2 and 95% air for 2 days.

MTT assay. A modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay was used to quantify the proliferation of rat granulosa cells (Chen *et al*., 2010; Kuo *et al*., 2009). Yellow MTT compound (M5655; Sigma Chemical Co.) was converted by living cells to blue formazen, which is soluble in dimethyl sulfoxide (D8418; Sigma Chemical Co.). The intensity of blue staining in the culture medium proportionally represents the number of living cells and was measured by an optical density reader (Anthos-2001, Austria) at 570 nm (Lin *et al*., 2007).

Radioimmunoassay of progesterone. The concentration of progesterone in the culture medium was determined by radioimmunoassay (RIA) as previously described (Chen *et al*., 2001; Kan *et al*., 2003). Using an antiprogesterone serum provided by our laboratory (No. W5), the sensitivity of progesterone RIA was 5 pg per assay tube. The cross-reactivities of this antiprogesterone antibody were 8% with pregnenolone; 8% with testosterone; 8% with androstenedione; 2.5% with 5α-dihydrotestosterone; 2% with 17α-hydroxy-progesterone; and less than 0.3% with estriol, cortisone, hydrocortisone, and cholesterol. The intra- and interassay coefficients of variability were 4.8% ($n = 5$) and 9.5% ($n = 4$), respectively.

ELISAof 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, 2.5mM Tris base, 1mM EDTA, and 0.1% PMSF, pH 7.8). Protein concentration was determined by the Bradford protein assay method with modifications (Bradford, 1976). The aliquots of cell lysate were boiled in SDS sample buffer (0.06M Tris base, 2% SDS, 0.0005% bromophenol blue, 6% sucrose, and 50mM 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-β-actin (1:8000). 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 $MgCl_2$, 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 $MgCl_2$, 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 (20mM HEPES, 0.45M NaCl, and 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′-CTCCCTCC**CACCTT**GGCCAGCACT-3′, which contains the SF-1 binding site, was labeled with [γ-^{32}P-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 $MgCl_2$, 2.5mM EDTA, 2.5mM DTT, 250mM NaCl, and 50mM Tris-HCl), 1 µg poly (dI): poly (dC), and 50,000 cpm of ^{32}P-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 (V_{max}) and Michaelis constants (K_m) for vehicle and PTU groups were estimated using SigmaPlot.

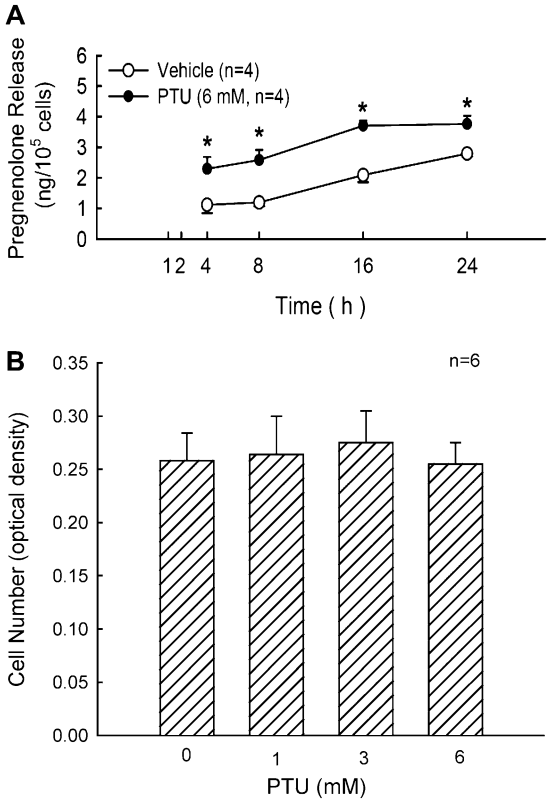

FIG. 1. The stimulatory effects of PTU on pregnenolone production in rat granulosa cells. (A) Rat granulosa cells were treated with or without PTU (6mM) for different periods of time. Pregnenolone production in the culture medium was measured by ELISA described in the ''Materials and Methods'' section ($n = 4$). *$p < 0.05$ compared with the vehicle group. (B) Different dosages of PTU (0–6mM) were applied to rat granulose cells for 16 h. The effect of PTU on cell proliferation was measured by the MTT assay as described in the ''Materials and Methods'' section ($n = 6$).

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 dependently 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

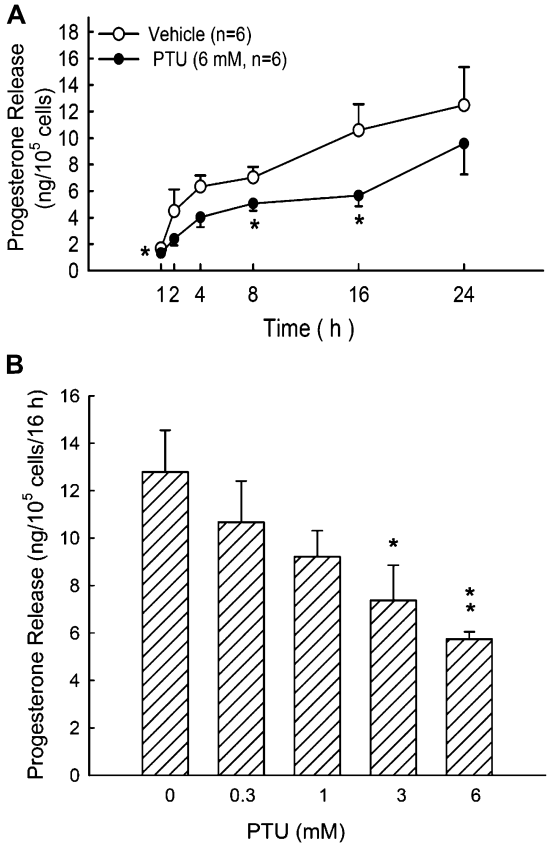

FIG. 2. The inhibitory effects of PTU on progesterone production in rat granulosa cells. (A) Rat granulosa cells were treated with or without PTU (6mM) for different periods of time ($n = 6$). (B) Different doses of PTU were applied to cells for 16 h ($n = 5$). Progesterone production in the culture medium was measured by RIA as described in the ''Materials and Methods'' section. $*p < 0.05$ and $**p < 0.01$ compared with the vehicle or PTU = 0 group.

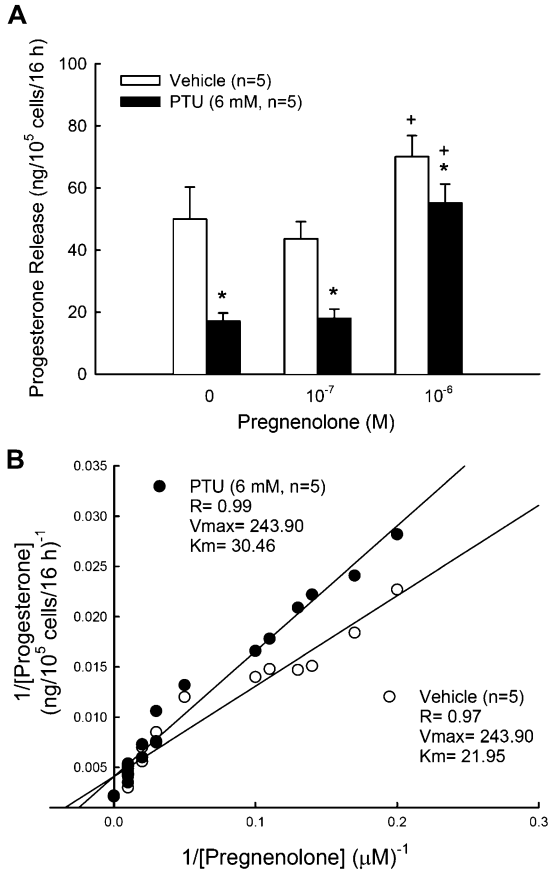

FIG. 3. The inhibitory effects of PTU on 3β-HSD function in rat granulosa cells. (A) Different dosages of pregnenolone (10^{-6} and 10^{-7}M) were administered with or without PTU (6mM) for 16 h. Progesterone production in the culture medium was measured by RIA as described in the ''Materials and Methods'' section ($n = 5$). $*p < 0.05$ compared with the vehicle group. $+p < 0.05$ compared with the pregnenolone = 0 group. (B) Double reciprocal data plots were obtained for cultured granulosa cells challenged with pregnenolone (10^{-5} to 10^{-7}M) ($n = 5$). The V_{max} for both groups was nearly the same (243.9 ng per 10^5 cells 16 per h). The K_m for the PTU-treated group (30.46μM) was greater than the vehicle group (21.95μM).

progesterone production in the presence or absence of pregnenolone supplement (10^{-7} to 10^{-6}M) (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^{-7}M). The maximum velocities (V_{max}) for the vehicle group and the PTU group were nearly the same (243.9 ng per 10^5 cells per 16 h). The Michaelis constant (K_m) for the PTU-treated group (30.46μM) was greater than that of the vehicle group (21.95μM) (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 trilostane (10^{-5}M) 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^{-5} to 10^{-7}) for 16 h in the presence of trilostane (10^{-5}M). 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).

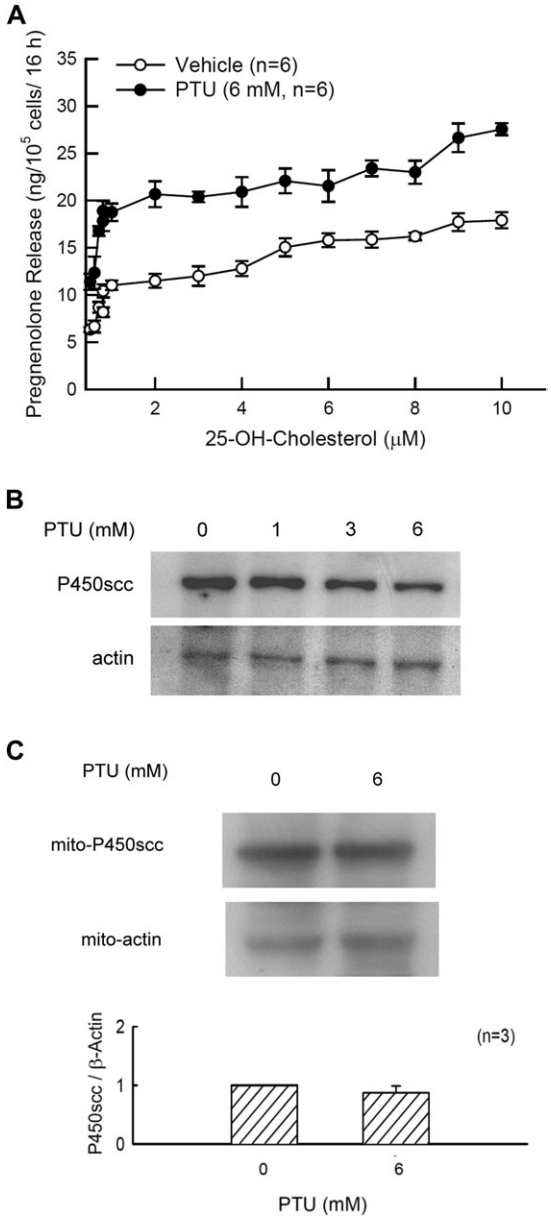

FIG. 4. Effects of PTU on the conversion of cholesterol to pregnenolone and protein expression of P450scc. (A) Granulosa cells were challenged with 25-OH-cholesterol (10^{-5} to 10^{-7}M), and the pregnenolone production in the culture medium was measured by ELISA ($n = 6$). The effects of PTU treatment on P450scc protein expression in total cell lysate (B) and mitochondrial fractions (C) were detected by Western blotting. Actin was used as an internal control. The quantitative results are also shown ($n = 3$).

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.*

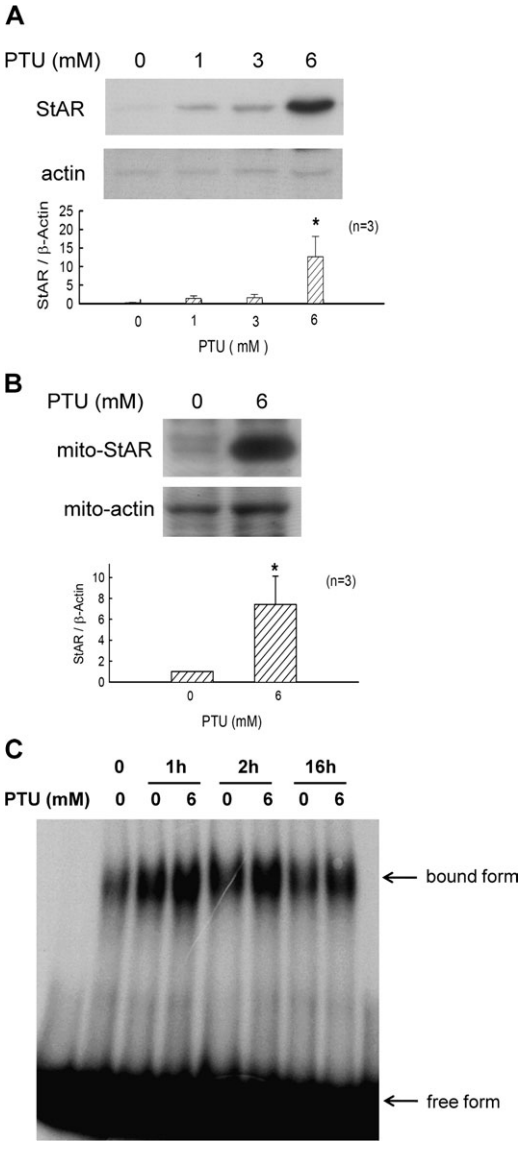

FIG. 5. The stimulatory effects of PTU on StAR protein expression. The effects of PTU treatment on StAR protein expression in total cell lysate (A) and mitochondrial fractions (B) were detected by Western blotting. Actin was used as an internal control. The quantitative results are also shown ($n = 3$). (C) The binding of SF-1 with the *StAR* promoter region after PTU treatment (6mM for indicated times) was evaluated by an EMSA as described in the ''Materials and Methods'' section.

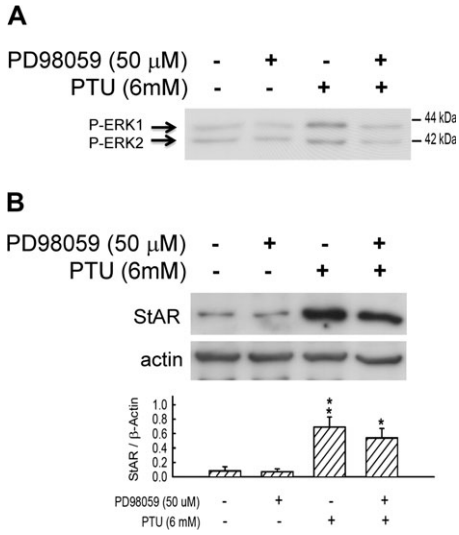

FIG. 6. Effects of PTU on MEK-dependent StAR protein expression. The MEK inhibitor, PD98059 (50μM), was added with or without PTU (6mM) to the culture medium of rat granulosa cells for 16 h. ERK1/2 phosphorylation (A) and StAR protein expression (B) were determined by Western blotting, as described in the ''Materials and Methods'' section. Actin was used as an internal control. The quantitative results are shown ($n = 2$–3). *$p < 0.05$ and **$p < 0.01$ compared with the vehicle group.

(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 $3.1 \pm 0.82 \times 10^{-5}$M in normal subjects ($n = 6$) and $2.8 \pm 1.4 \times 10^{-5}$M in hyperthyroidism patients ($n = 7$). Another report showed serum concentrations of PTU ranging from 1.6 to 7.5 μg/ml (0.94 to 4.4×10^{-5}M) 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 3β-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 3β-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 3β-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.

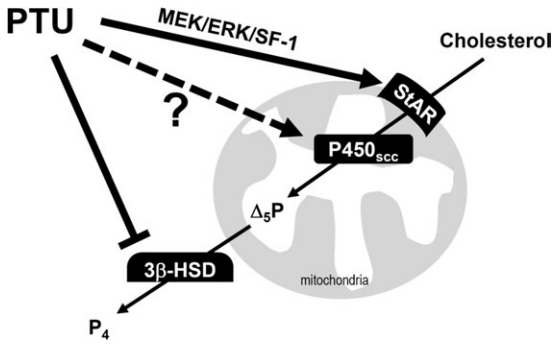

FIG. 7. Schematic representation demonstrating the novel mechanisms of PTU effects on steroid production in rat granulosa cells.

The protein level of P450scc, 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 P450scc 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 P450scc enzyme localizes to the inner membrane of mitochondria, cholesterol has to penetrate across the mitochondrial outer membrane in order to associate with P450scc. 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.

FUNDING

National Science Council (NSC 97-2320-B- 010-017-MY3 to P.S.W., National Yang-Ming University).

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