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Upregulation of Steroidogenic Enzymes and Ovarian 17β-Estradiol in Human Granulosa-Lutein Cells by *Cordyceps sinensis* Mycelium[1]

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ABSTRACT

There is increasing evidence that 17β-estradiol (E_2) directly influences the quality of maturing oocytes and thus the outcome of assisted reproduction treatment. Although *Cordyceps sinensis* (CS) mycelium, a Chinese herbal medicine, is believed to enhance libido and fertility in both sexes, the mechanism of its effect in women has not been determined. The aim of the present study was to evaluate the effects of CS on steroidogenic enzyme expression and E_2 biosynthesis in human granulosa-lutein cells (GLC). We found that CS induced E_2 production by GLC in a dose- and time-dependent manner and that a 3-h treatment with CS induced increased levels of mRNAs coding for the P450 side chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), and aromatase. Western blot analysis demonstrated that, after treatment with CS for 3 h, protein levels of steroidogenic acute regulatory protein (StAR) and aromatase were upregulated while P450scc and 3β-HSD levels showed no substantial change. New protein synthesis was required for CS-induced E_2 production because it was abrogated by cycloheximide pretreatment. Addition of 22(R)-hydroxycholesterol, thus bypassing the need for StAR protein, did not induce as much E_2 production as CS treatment, indicating that upregulation of StAR protein was not the only factor contributing to CS-induced steroidogenesis. Cotreatment of GLCs with CS and aminoglutethimide, an aromatase inhibitor, completely abolished CS-induced E_2 production. In conclusion, treatment of GLCs with CS results in increased E_2 production due, at least in part, to increased StAR and aromatase expression. These data may help in the development of treatment regimens to improve the success rate of in vitro fertilization.

estradiol, granulosa cells, mechanisms of hormone action, ovary, steroid hormones

INTRODUCTION

One important factor in determining success rates in assisted reproduction treatment is oocyte quality. Despite improved results due to several important innovations, the pregnancy rate per collected oocyte remains far too low. One of the most critical factors determining the success of in vitro fertilization (IVF)/embryo transfer is the quality of the collected oocytes. By producing numerous paracrine factors, granulosa cells have a critical impact on the development, maturation, and quality of oocytes. 17β-estradiol (E_2) is the most important granulosa-derived hormone affecting the properties of oocytes and there is increasing evidence that it directly influences the quality of maturing oocytes. For example, addition of E_2 to oocyte maturation medium increases the fertilization and cleavage rates of in vitro-matured oocytes [1]; large follicles with meiotically competent oocytes have higher E_2 concentrations than those containing meiotically incompetent oocytes [2]; the quality of oocytes, as assessed by morphology, is associated with the E_2/testosterone and E_2/progesterone ratios in the follicular fluid [3]; and elevated plasma E_2 levels result in greater implantation rates by increasing the number of oocytes and embryos and high-grade embryos for transfer/cryopreservation [4].

Cordyceps sinensis (CS) is a fungus that parasitizes Lepidoptera larvae. It attacks the caterpillars in late autumn, and by early summer of the following year, has killed the caterpillar, and the fruiting body protrudes from its head. Because of its life cycle, in Mandarin, it is called the Dong-Chong-Xia-Cao (winter-worm and summer-plant) [5]. In traditional Chinese medicine, CS is used to treat weakness after sickness, lung- and kidney-associated diseases and, in particular, sexual dysfunction [6]. Recently, the pharmacological effects of CS have been evaluated in many studies, which have shown that it modulates immune responses [7–11], inhibits tumor cell proliferation [12–14], enhances hepatic function [15], regulates insulin sensitivity [16, 17], decreases plasma cholesterol levels [18], and has hypotensive and vasorelaxant activity [19]. In addition, it modulated steroidogenesis. Rat adrenal cells treated with CS show increased corticosterone production, and this effect is mediated through protein kinase C [20]. In addition, steroidogenesis in both the MA-10 Leydig cell tumor line and primary mouse Leydig cells is induced by CS treatment [21–23]. However, the effects of CS on the female reproductive system have never been studied.

The aim of this study is to evaluate the effect of CS on steroidogenesis in human granulosa-lutein cells (GLCs). We hypothesized that CS enhances the fecundity of women by modulating the E_2-producing capacity of granulosa cells. The ultimate goals of this study were to determine how CS modulates ovarian steroidogenesis and whether it could be used clinically to improve the success rate of IVF.

MATERIALS AND METHODS

Preparation of CS Extract

The extracted mycelium of *C. sinensis* was kindly supplied by Dr. Peter Fang (Herbal Tech, Edmonton, AB, Canada). Preparation of the extracted

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TABLE 1. Primers used.

Gene		Sequence	Accession no.	PCR length (bp)
StAR	Forward	GACTTTGTGAGCGTGC	AF035277	103
	Reverse	TGATGACACCCTTCTGC		
P450scc	Forward	AGCGATTCATTGATGCC	M14565	155
	Reverse	CTGGGTGTATATGTCAGCTTTA		
3β-HSD	Forward	ACACTTGTGCGTTAAGAC	M77144	129
	Reverse	CTGGGTTGACTGTAGAGAA		
CYP17	Forward	GTGGTTAAATGGACCCTG	NM_000102	102
	Reverse	CGAAGCACCTCTCGGA		
Aromatase	Forward	GTGACCAATGAATCGGG	M28420	154
	Reverse	TGAGGAGAGCTTGCCA		
18S	Forward	GTGTGCCTACCTACG	X03205	114
	Reverse	TGACCCGCACTTACTG		

CS was as follows: 30 g of cultured *C. sinensis* mycelium powder was extracted with 240 ml of water in a water bath of 100°C for 3 h with reflux. Eighty milliliters of the water extract was then lyophilized to yield 2.9 g of the dry powder, which was 29% of the original mycelium powder. The rest of the water extract (160 ml) was mixed with 160 ml of absolute ethanol for extraction. This 50% alcoholic fraction was dried to yield 3.7 g of the dry powder, which was 18% of the original mycelium powder. The combination of both aqueous and alcohol extracts was then used in the present study. The concentration indicated in this study is the net weight of extracted CS dissolved in culture medium.

Preparation of GLCs

GLCs were obtained from women undergoing assisted reproduction treatment at the National Cheng Kung University Hospital; the cells, provided as coded samples, were a by-product of the IVF/embryo transfer procedure. This study was approved by the Clinical Research Ethics Committee at the National Cheng Kung University Medical Center and informed consent was obtained from each patient. The patients received various regimens of Metrodin (Serono, Randolph, MA) and Pergonal (Serono) and all received 10 000 IU of human chorionic gonadotrophin (hCG) 36 h prior to follicular aspiration. Approximately 1.0 ml of modified human tubal fluid medium (Irvine Scientific, Santa Ana, CA) containing Hepes buffer, antibiotics, and heparin was added to each follicular fluid aspirate during the oocyte-retrieval procedure. After removal of the oocytes and cumulus masses, the follicular fluid containing the granulosa cells was transported to the laboratory for GLC isolation. Granulosa cells from a single individual were pooled, but those from different subjects were not.

The GLCs were prepared as described previously [24]. In brief, the follicular fluid from each subject was divided equally into 15-ml disposable, sterile centrifuge tubes and centrifuged at 400 × *g* for 10 min at room temperature, creating a firm layer of GLCs on top of the red blood cell pellet. Each GLC layer was removed by aspiration and resuspended in 2 ml of Hanks solution containing 50 μg/ml of DNAse I and 2 mg/ml of type IV collagenase (Worthington Biochemical Co., Lakewood, NJ), then the samples were combined in a sterile 50-ml centrifuge tube and a single cell suspension prepared by shaking at 125 rpm for 30 min at 37°C. After centrifugation at 400 × *g* for 10 min at room temperature, the pellet was resuspended in 6 ml of Dulbecco modified Eagle medium (DMEM)/F12 and the suspension layered onto 4.0 ml of Ficoll-Paque in 15-ml centrifuge tubes and centrifuged at 600 × *g* for 20 min. The cell layer was removed from each Ficoll-Paque column and the cells washed twice with 10 ml of DMEM/F12, then suspended in 5 ml of culture medium (DMEM/F12 supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin sulfate, 0.625 μg/ml of fungizone, and 10% fetal bovine serum), counted on a hemocytomer, and adjusted to a concentration of 1 × 10^5 cells/ml in culture medium. Cell viability was determined using 0.04% Trypan blue and 1 × 10^5 viable cells were plated in each well of 24-well culture plates. After attachment of the cells for 16–18 h, the debris was removed by washing with Hanks solution, then low serum culture medium consisting of DMEM/F12 supplemented with 100 U/ml of penicillin, 100 μg/ml of streptomycin sulfate, 0.625 μg/ml of fungizone, 2% fetal bovine serum, and 1× GlutaMAX (Gibco/Invitrogen, Carlsbad, CA) was added to each well. This was designated as Day 0 of culture.

Cell Culture and Treatments

To minimize residual effects of the hCG administered at the time of induced ovulation, the cells were cultured for 2 days [24], incubated with 1 ml fresh medium for 2 h, and subjected to the various treatment regimens. In the first experiment, the cells were treated with 1 ml of medium containing 0, 0.01, 0.1, 1, or 10 mg/ml of CS extract (net weight/ml medium) or 10 IU/ml of hCG and incubated for 1, 3, and 6 h, then the culture medium was collected to measure the E_2 concentration. To determine the effect of CS on steroidogenic enzyme expression, the GLCs were treated for 3 h with CS (0.01, 0.1, or 1 mg/ml) or control medium, lysed in the well using RNA lysis buffer (4 M guanidinium isothiocyanate, 10 mM Tris-HCl, pH 8.0, 0.5% SDS, and 1% dithiothreitol), and RNA isolated as described previously [24–27]. For Western blot analysis, 1 × 10^6 cells cultured in 30-mm Petri dishes were treated for 3 h with control medium with or without 1 mg/ml of CS, then harvested in Tris-sucrose-EDTA buffer (10 mM Tris, 250 mM sucrose, and 0.1 mM EDTA, pH 7.4). To determine whether new protein synthesis was required for, and the effect of aromatase inhibitors on, CS-induced E_2 production, GLCs were treated for 3 h with control medium or CS in the presence or absence of 1 μg/ml of cycloheximide or 50 μM aminoglutethimide, a selective aromatase inhibitor [28], respectively, then the culture medium was collected for E_2 assay.

Quantification of mRNAs Using Real-Time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The gene-specific primers (Table 1) were designed according to sequences deposited in GenBank using the Light Cycler built-in primer design program (Roche, Mannheim, Germany). Total RNA (0.5 μg) was denatured at 70°C for 10 min and reverse transcribed for 60 min at 42°C in the presence of 100 pmoles of random hexamer, 2.5 mM $MgCl_2$, 0.4 mM deoxy-NTPs, 10 mM dithiothreitol, and 10 U RNAse H^- reverse transcriptase in a total volume of 20 μl (SuperScript II RT, Invitrogen), then the sample was heated to 95°C for 10 min and 2 μl of the resulting cDNA used as a template for real-time PCR. Real-time PCR was carried out on the LightCycler System (Roche) using the double-strand DNA-binding dye, Syber Green I, for the detection of PCR products. The PCR reaction mixture contained 400 nM of the primer pair (Table 1) and FastStart DNA Master Mix (Roche), and the reaction conditions were 95°C for 10 min, followed by 45 cycles of 95°C for 30 sec, 59°C for 30 sec, and 72°C for 30 sec. The fluorescence intensity of the Syber Green I was read on the LightCycler at 85°C after the end of each extension step. The data are expressed as the number of cycle thresholds (*Ct*), the PCR cycle number at which the fluorescent signal in each reaction reached a preset threshold above background. A dissociation curve was created using the built-in melting curve program of the LightCycler to confirm the presence of a single PCR product. Ribosomal 18S RNA was used as the internal control for each sample and the *Ct* value for each sample normalized against that for 18S RNA.

Western Blots

The detailed procedure for Western blotting has been described previously [24, 26, 29]. In brief, 20 μg of protein was resolved by SDS-PAGE and transferred to a polyvinylidene fluoride membrane (Millipore Co., Bedford, MA), which was then blocked with 5% skimmed milk and

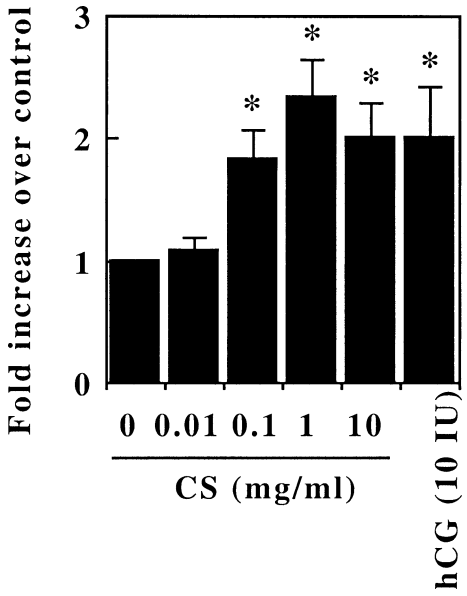

FIG. 1. Dose-dependent effect of CS on E_2 production by human granulosa-lutein cells. Cells were incubated for 3 h with various concentrations of CS (0, 0.01, 0.1, 1, and 10 mg/ml) or 10 IU/ml of hCG, then E_2 production was measured by ELISA. Each data point was normalized to the 0 CS control at 3 h and represents the mean ± SEM (n = 9). The asterisks indicate a statistically significant difference between the control and the CS group ($P < 0.05$).

incubated with specific antibodies. After washing and incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies, bound antibody was detected using the enhanced chemiluminescence system (PerkinElmer Life Sciences Inc., Boston, MA). The blots were then stripped with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, and 62.5 mM Tris-HCl, pH 6.7) and reprobed with different antibodies.

17β-Estradiol Assay

E_2 levels in the culture medium were measured by competitive ELISA as described previously [25]. In brief, sheep anti-E_2 polyclonal antibody (The Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, CO) was added for 90 min at room temperature to a 96-well plate precoated with rabbit anti-sheep IgG antibodies (CalBiochem, San Diego, CA), then, after washing off excess primary antibody, the samples were added to the plate and incubation continued for another 90 min at room temperature. Fifty microliters of HRP-conjugated E_2 (a kind gift from Dr. M.C. Wiltbank, University of Wisconsin-Madison) was added to each well for 90 min at room temperature, then the plate was washed four times with washing buffer (20 mM MOPS and 0.05% Tween 20, pH 7.2) before 125 μl of substrate solution (50 mM sodium acetate, pH 4.4, 0.5 M H_2O_2, and 20 mg/ml of 3,3′,5,5′-tetramethyl benzidine) was added to each well and the plate incubated at 37°C for 10 min with shaking. Color development was terminated by adding 50 μl of 0.5 M H_2SO_4 to each well, then the absorbance at 450 nm was read in a plate reader. The sensitivity (80% bound) of the E_2 assay was 14.6 pg/ml and the intra- and interassay coefficients of variation were 4.2% and 7.6%, respectively.

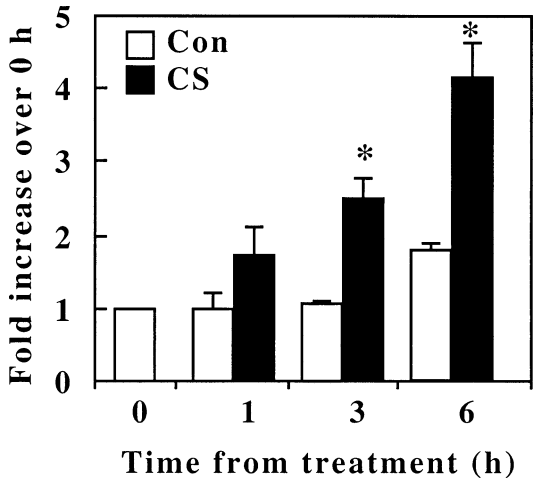

FIG. 2. Time dependency of the effect of CS on E_2 production by human granulosa-lutein cells. Cells were incubated for 0, 1, 3, or 6 h with 1 mg/ml of CS, then E_2 production was measured by ELISA. Each data point was normalized to the 0-h control and represents the mean ± SEM (n = 7). The asterisks indicate a statistical significant difference from the control result at the same time point ($P < 0.05$).

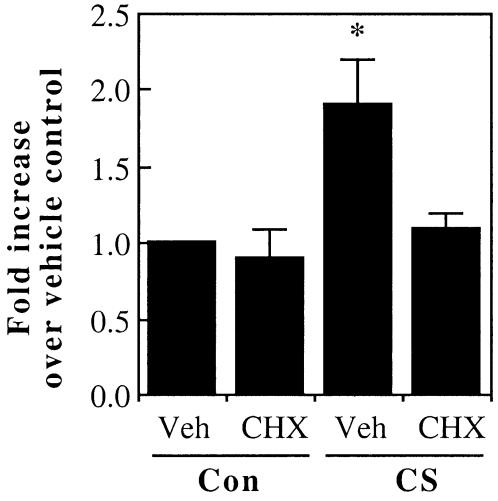

FIG. 3. New protein synthesis is required for CS-induced E_2 production by human GLCs. Cells were pretreated with cycloheximide (CHX) or vehicle (Veh) 5 min prior to incubation for 3 h with 1 mg/ml of CS or control medium (Con), then the medium was collected for E_2 measurement. Each data point was normalized to the Veh-/Con-treated sample and represents the mean ± SEM (n = 6). The asterisk indicates a statistically significant difference ($P < 0.05$) from the other values.

Statistical Analyses

Data were analyzed using the general linear model of the Statistical Analysis System [30]. Differences between specific mRNA or E_2 concentrations were analyzed by one-way ANOVA followed by Duncan multiple range test if significant differences were found.

RESULTS

Dose-Dependent and Time-Dependent Effects of CS on E_2 Production by Human GLCs

To evaluate whether CS modulated steroidogenesis, human GLCs were treated with various concentrations of CS for 3 h or with 1 mg/ml of CS for different times and E_2 production measured. The basal E_2 concentration of cells treated with control medium for 3 h (Con) was 138.4 ± 20.2 pg/ml. Because of great variations among different subjects, the data were then expressed as fold change over control throughout the current report. As shown in Figure 1, treatment for 3 h with 0.1, 1, or 10 mg/ml of CS caused a significant 1.7- to 2.3-fold increase in E_2 production, comparable with that induced by 10 IU/ml of hCG. The maximal effect of CS on E_2 production was seen at 1 mg/ml, the effect at 10 mg/ml not being significantly different. Using 1 mg/ml of CS, E_2 production significantly increased after treatment for 3 or 6 h to 220% or 230%, respectively, of the control levels at the same time point (Fig. 2). Prolonged treatment (24 h) with CS did not result in any further increase in E_2 production, indicating it is a rapid and

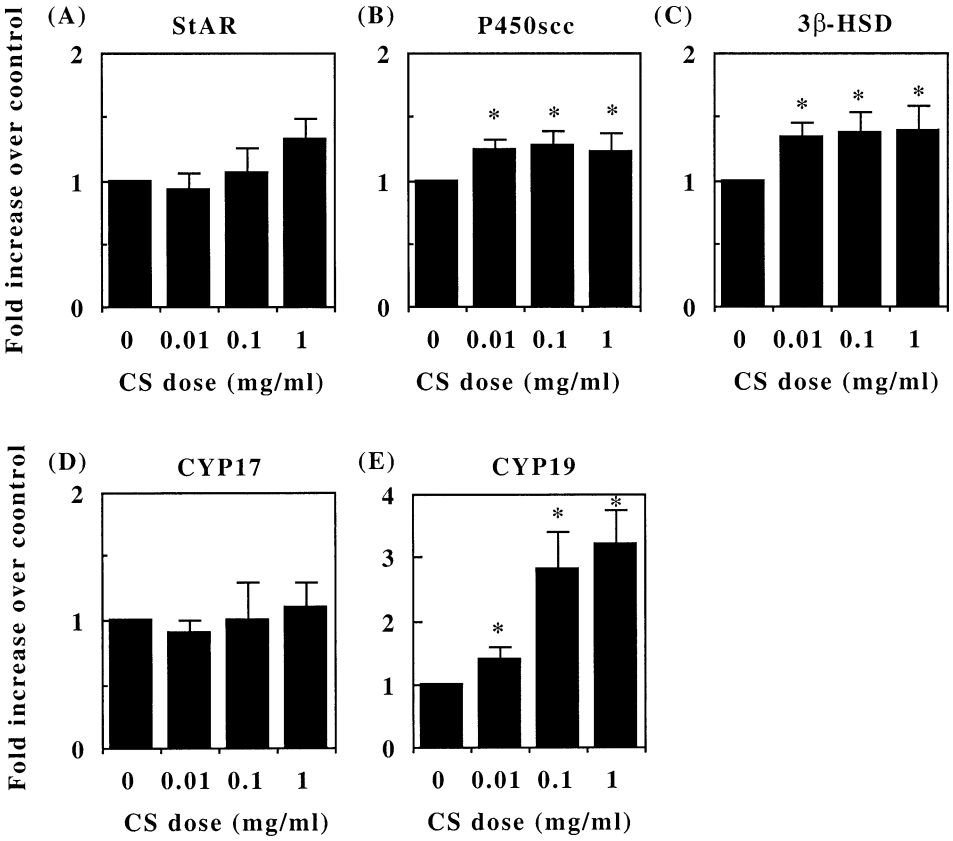

FIG. 4. Effects of CS on steroidogenic enzyme mRNA levels in human granulosa-lutein cells. Cells were treated for 3 h with different concentrations of CS (0, 0.01, 0.1 and 1 mg/ml), then levels of mRNAs coding for StAR (**A**), P450scc (**B**), 3β-HSD (**C**), CYP17 (**D**), or aromatase (**E**) were determined by real-time RT-PCR. Each data point was normalized to the 0 CS control at 3 h and represents the mean ± SEM (n = 12). Asterisks indicate statistical difference between the control and test group ($P < 0.05$).

transient effect (data not shown). To determine whether synthesis of new protein was required for CS-induced E_2 production, the cells were incubated for 30 min with 1 μg/ml of cycloheximide before treatment for 3 h with 1 mg/ml of CS; this pretreatment had no effect on the basal E_2 concentration, but completely inhibited CS-induced E_2 production (Fig. 3).

Effects of CS on Steroidogenic Enzyme mRNA Levels in Human GLCs

Because new protein synthesis was required for CS-induced E_2 production, we examined which steroidogenic enzymes were associated with this effect by measuring concentrations of mRNAs coding for steroidogenic acute regulatory protein (StAR), P450 side chain cleavage enzyme (P450scc), 3β-hydroxysteroid dehydrogenase (3β-HSD), CYP17, or aromatase using real-time RT-PCR with external standards. As shown in Figure 4, CS treatment had no significant effect on StAR and CYP17 mRNA levels, but upregulated P450scc and 3β-HSD mRNA levels by 25–35% and aromatase mRNA levels by over 300%.

StAR and Aromatase Protein Levels Are Upregulated by CS

When Western blotting was used to define which steroidogenic enzymes were increased at the protein level by CS treatment, the results showed that P450scc and 3β-HSD levels were not affected by 3 h of treatment with 1 mg/ml of CS (Fig. 5A). In contrast, StAR and aromatase expression was significantly increased (Fig. 5, B and C), which was inhibited by pretreatment with cycloheximide (Fig. 5D).

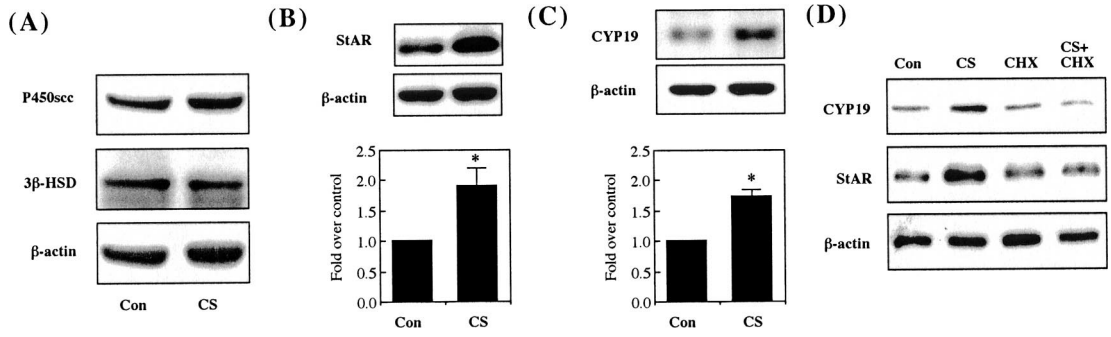

FIG. 5. Effect of CS on steroidogenic enzyme protein levels in human granulosa-lutein cells. Cells were treated for 3 h with 1 mg/ml of CS or medium, then protein levels of P450scc (**A**), 3β-HSD (**A**), StAR (**B**), and aromatase (**C**) were measured by Western blotting. In **B** and **C**, the upper panel shows a representative Western blot and the lower panel shows the mean ± SEM for four experiments normalized to the control value. The asterisks indicate a significant difference between the control and CS group ($P < 0.05$). Panel **D** is the representative Western blot, showing that expression of StAR and aromatase induced by CS treatment can be inhibited by pretreatment with cycloheximide (CHX).

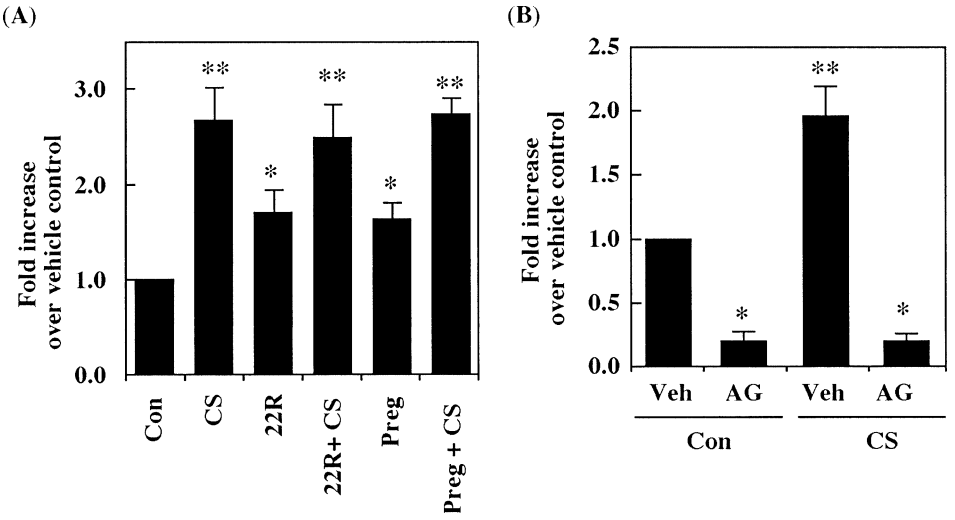

FIG. 6. Role of StAR and aromatase in E_2 production by human granulosa-lutein cells. **A**) Cells were treated for 3 h with medium (Con) or 1 mg/ml of CS in the presence or absence of 50 μM 22(R)-hydroxycholesterol (22R) or 10μM pregnenolone (Preg), then E_2 production was determined by ELISA. Each data point was normalized to the 3 h 0 CS control and represents the mean ± SEM of four experiments. **B**) Cells were treated for 3 h with medium (Con) or 1 mg/ml of CS with (AG) or without (Veh) 50 μM aminoglutethimide, then E_2 production was determined by ELISA. Each data point was normalized to the vehicle-/medium-treated control and represents the mean ± SEM of seven experiments. * Indicates that the value is significantly different from the 3-h control value; **, the value is significantly different from that marked * ($P < 0.05$).

Role of StAR and Aromatase on CS-Induced E_2 Production

StAR transports cholesterol across the mitochondrial membrane. As shown in Figure 6A, incubation of GLCs for 3 h with 22(R)-hydroxycholesterol resulted in a smaller increase in E_2 production than that induced by treatment for 3 h with 1 mg/ml of CS, and cotreatment with 22(R)-hydroxycholesterol and CS resulted in no enhancement of E_2 production compared with CS alone. Similarly, addition of pregnenolone (10 μM) as a substrate resulted in about a 60% increase in E_2 production while cotreatment with pregnenolone and CS induced similar levels of E_2 production as that treated with CS alone (Fig. 6A). When GLCs were treated for 3 h with 1 mg/ml of CS in the presence or absence of the aromatase inhibitor, aminoglutethimide, to examine the role of aromatase in CS-induced E_2 production, aminoglutethimide caused significant suppression of both basal and CS-induced E_2 production in a dose-dependent manner (Fig. 6B and data not shown).

DISCUSSION

CS can enhance libido and fertility in both sexes [5], although the underlying mechanisms are obscure. We have previously demonstrated that CS can induce male sex hormone production in primary cultures of rat Leydig cells and in the mouse Leydig cell tumor line MA-10 [21–23]. In the present study, we provided evidence that CS upregulated steroidogenesis in human GLCs via upregulation of several steroidogenic enzymes. To our knowledge, this is the first report demonstrating a stimulatory effect of CS on female sex hormone production, thus explaining the modulatory effect of CS on female reproductive function.

Steroidogenesis is a complex process that can be regulated, to differing degrees, at several levels, and the best way to control steroidogenesis is to regulate not just the rate-limiting enzyme but as many as possible of the enzymes involved. For example, insulin increased FSH-induced steroid production via differential effects on StAR, P450scc, and 3β-HSD at the mRNA and/or protein levels [31], while leptin directly regulated human ovarian steroidogenesis by interfering with either the translational or posttranslational steps of CYP17 and/or aromatase synthesis [32]. In the present study, we found that CS induced E_2 production via the modulation of several steroidogenic enzymes, in particular, the two most important proteins in the E_2 biosynthesis pathway, StAR and aromatase. StAR protein is indispensable for acute trophic hormone-stimulated steroidogenesis by the adrenal gland, testis, and ovary [33, 34]. How StAR transports cholesterol is not completely understood, but it is clear that it promotes the transport of cholesterol from the outer mitochondrial membrane to the inner membrane, where the P450scc complex is located [35–37]. Transport of cholesterol across the mitochondrial membrane is generally believed to be one of the rate-limited steps in steroid biosynthesis. In the present study, the increased expression of StAR protein induced by treatment of GLCs with CS extract correlated with increased E_2 production, clearly demonstrating that StAR plays a role in CS-induced E_2 production. However, addition of 22(R)-hydroxycholesterol or pregnenolone, thus bypassing the function of StAR, did not produce levels of E_2 comparable with those induced by CS treatment, indicating that other factors are involved. Indeed, using quantitative real-time RT-PCR analysis, we found that the levels of transcripts of other steroidogenic enzymes, namely P450scc, 3β-HSD, and aromatase, were upregulated by CS. Although StAR mRNA levels were no different in control and CS-treated GLC at 3 h posttreatment, this may be due to the nature of the acute response of StAR. It is known that StAR is an immediate early gene in response to stimulation, and upregulation of StAR mRNA by CS may have occurred at an earlier time point.

Although CS caused an increase in P450scc and 3β-HSD mRNA levels after 3 h of treatment, the levels of the proteins were not increased, indicating they may not be involved in the acute upregulation of E_2 by CS. This may be due to the fact that both P450scc and 3β-HSD are relatively stable [38] compared with other steroidogenic enzymes; therefore, the variation in mRNA levels may not be immediately reflected at the protein level. For example, a rapid decrease in P450scc and 3β-HSD transcripts after prostaglandin $F_{2\alpha}$ treatment of bovine and ovine corpus luteum did not result in a rapid change in the levels or enzyme activities of these proteins or steroid production [38, 39], indicating that the change in the mRNA levels was not a major factor in controlling steroid production. Although addition of aminoglutethimide inhibited E_2 production may argue that there is a role for P450scc in CS-induced steroidogenesis, because aminoglutethimide can inhibit both P450scc and aromatase enzyme activities. Obviously, inhibition of P450scc enzyme activity by aminoglutethimide

will contribute to dramatic suppression of E_2 production but it is not the same idea as that upregulation of P450scc involves in CS-induced acute E_2 production. Addition of pregnenolone as a substrate (bypass the steps controlled by StAR and P450scc) fails to affect E_2 production induced by CS. Moreover, pregnenolone does not cause more E_2 synthesis compared with that stimulated by 22(R)-hydroxycholesterol (bypass the step controlled by StAR only). Put together, these data indicate that P450scc does not play significant roles in CS-induced acute E_2 production. Nevertheless, whether CS-induced P450scc and 3β-HSD mRNA upregulation affects E_2 production by human GLCs after long-term incubation remains to be investigated.

In contrast, upregulation of aromatase plays a significant role in the control of E_2 production induced by CS. Although StAR and aromatase coordinately regulate E_2 production, their effects are different in terms of time and mode of action. An increase in StAR protein usually leads to acute production of steroids, whereas an increase in aromatase levels may be responsible for the later increase in steroidogenesis. Aromatase mRNA levels in human GLCs were increased by 300% after 3 h of CS treatment, whereas protein levels at the same time point only increased by 75%, suggesting that the effect of CS on upregulation of aromatase protein was not yet maximal at 3 h. Thus, the increase in E_2 levels at this time point might be due to both increased cholesterol transport into mitochondria, a step mediated by StAR protein, and increased conversion of testosterone to E_2, a reaction catalyzed by aromatase. This idea is also supported by the observations that the addition of 22(R)-hydroxycholesterol to substitute for StAR function resulted in less E_2 production than with CS and that cotreatment with 22(R)-hydroxycholesterol and CS was no more effective than treatment with CS alone. However, StAR is a labile protein, its concentration peaking about 4–8 h after stimulation [33, 40], and the increase in aromatase levels caused by CS treatment may be more important in increasing E_2 production at later time points (e.g., 24 h).

One unique characteristic of human GLC is the expression of CYP17 and production of androgen to serve as a substrate for aromatase [41–43]. It is hypothesized that CYP17 regulates another committed step of steroidogenesis in human GLC. The hypothesis was first made primarily based on the observation that only miniscule amounts of CYP17 mRNA were detected in human GLC and corpus luteum [41, 44]. Subsequent studies demonstrated that the protein levels and enzyme activity of CYP17 were also much lower than those of aromatase in cultured human GLC [42, 43], suggesting that the supply of substrate for aromatase may control the rate of E_2 production. Moreover, Moran et al. identified that 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), an environmental toxicant with the ability to disrupt ovarian function, inhibits the expression and enzyme activity of CYP17, leading to decrease in E_2 production [42]. The study provided evidence to support that the molecular target of TCDD is CYP17 but not aromatase or StAR. In the current study, however, we did not find any change in the mRNA level of CYP17 after treatment with CS for 3 h. The discrepancy may be due to the differences in duration of treatment and/or substance used. Indeed, TCDD caused CYP17 downregulation and E_2 diminution was evident only after long-term incubation (>4 days). Others examining effects of hormonal regulation of CYP17 gene expression in a shorter time period (<48 h) have failed to detect any change in response to the stimuli [45–47]. Thus, change in CYP17 expression or enzyme activity may not be responsible for acute regulation of steroidogenesis. Whether prolonged incubation with CS would affect CYP17 expression and/or enzyme activity leading to alteration of E_2 production remains as an open question and warrants further investigation.

Upregulation of ovarian steroidogenesis and thus enhancement of female reproductive function is the primary strategy for treating female infertility. Many treatment regimens aimed at improving ovarian function using gonadotropin or analogues have improved the success rate of IVF. However, adverse effects, such as ovarian hyperstimulation syndrome and gonadotropin resistance, have sometimes been reported, but the reasons for these are not completely understood. Thus, a simple effective approach to treatment with fewer adverse effects is required. The use of natural or herbal medicines to modulate ovarian activity and enhance reproductive function is an alternative approach, and many herbs have been tested and found to improve function of the female reproductive system [48–50]. In the present study, we showed that CS stimulates E_2 production in human GLCs by upregulating the expression of several key enzymes, especially StAR and aromatase, making it a good candidate to join the list of herbs with ovarian function-improving capability. However, more in-depth investigations are required to understand the actions of CS. For example, how CS activates cells, then conducts the signal into cells, is unknown. We have previously reported that CS-stimulated testosterone production by mouse Leydig cells is mediated by the protein kinase A pathway [51], but whether similar mechanisms are responsible for CS-induced E_2 production in human GLCs is not known. Thus, the characterization of the signaling pathway and the molecular mechanism of CS-induced steroidogenesis is a natural extension of this study and is currently under investigation.

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