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Regulatory mechanism of *Cordyceps sinensis* mycelium on mouse Leydig cell steroidogenesis

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Abstract **We demonstrate the mechanism by which *Cordyceps sinensis* (CS) mycelium regulates Leydig cell steroidogenesis. Mouse Leydig cells were treated with forskolin, H89, phorbol 12-myristate 13-acetate, staurosporine, or steroidogenic enzyme precursors with or without 3 mg/ml CS; then testosterone production was determined. H89, but not phorbol 12-myristate 13-acetate or staurosporine, decreased CS-treated Leydig cell steroidogenesis. CS inhibited Leydig cell steroidogenesis by suppressing the activity of P450scc enzyme, but not 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, 20α-hydroxylase, or 17β-hydroxysteroid dehydrogenase enzymes. Thus, CS activated the cAMP–protein kinase A signal pathway, but not protein kinase C, and attenuated P45scc enzyme activity to reduce human chorionic gonadotropin-stimulated steroidogenesis in purified mouse Leydig cells.**

Key words: Testosterone; Mouse; Leydig cell; Steroidogenesis; *Cordyceps sinensis*

1. Introduction

In the male reproductive system, luteinizing hormone (LH) will associate with the receptors on Leydig cells to activate adenylate cyclase through G proteins. The activation results in an increase of intracellular cAMP, which in turn activates protein kinase (PK) A [1]. PKA will phosphorylate some proteins and/or induce de novo synthesis of proteins [2]. These proteins will facilitate the transfer of cholesterol into the inner mitochondrial membrane, where P450scc enzyme converts cholesterol to pregnenolone [3]. Steroidogenic enzymes in the smooth endoplasmic reticulum, including 3β-hydroxysteroid dehydrogenase (3β-HSD), 17α-hydroxylase, 20α-hydroxylase, and 17β-hydroxysteroid dehydrogenase (17β-HSD), will then process pregnenolone to testosterone, a hormone necessary for male reproduction [4].

Cordyceps sinensis (CS), a fungal parasite on the larvae of Lepidoptera, is a time-honored herbal medicine in many East Asian cultures [5]. Studies have shown its pharmacological activities, including modulation of immune response [6,7], inhibition of tumor growth [8,9], decrease of blood pressure [10,11], increase of hepatic energy metabolism and blood flow [12], improvement of bioenergy in the liver [13], induction of cell apoptosis [14], and secretion of adrenal hormone [15]. It has also been shown that CS can enhance reproductive activity and restore impaired reproductive functions [16]. How CS regulates Leydig cell steroidogenesis, however, remains elusive.

We have previously demonstrated that CS alone could stimulate steroid production in both normal and tumor mouse Leydig cells [17–19]. However, testosterone production with human chorionic gonadotropin (hCG) treatment was suppressed by CS in purified normal mouse Leydig cells [19]. In the present study, we examine whether CS exerts direct action on sites along the PKA or PKC pathways, or on steroidogenic enzymes in normal mouse Leydig cells. Cells were treated with *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride (H89, PKA inhibitor), staurosporine (PKC inhibitor), or phorbol 12-myristate 13-acetate (PMA, PKC activator) with or without CS to examine whether CS activated the PKA or PKC signal transduction pathways. Also, maximal doses of forskolin (stimulator of adenylate cyclase), 22R-hydroxycholesterol (substrate for P450scc), pregnenolone (substrate for 3β-HSD), progesterone (substrate for 17α-hydroxylase), 17α-hydroxyprogesterone (substrate for 20α-hydroxylase), and androstenedione (substrate for 17β-HSD) were added to cells with or without CS to determine whether CS affected enzyme activity.

2. Materials and methods

2.1. Chemicals

M199 medium, fetal bovine serum, Dulbecco's phosphate-buffered saline, and gentamicin sulfate were purchased from Gibco (Grand Island, NY, USA). Tissue culture grade sodium bicarbonate, bovine serum albumin (BSA), HEPES, penicillin, Percoll gradient solution, streptomycin, β-nicotinamide adenosine dinucleotide, H89, dehydroepiandrosterone, nitroblue tetrazolium, testosterone, ether, PMA, charcoal, forskolin, and staurosporine were purchased from Sigma Chemical (St. Louis, MO, USA). [^{3}H]Testosterone used for radioimmunoassay was purchased from DuPont-New England Nuclear (Boston, MA, USA). Antiserum to testosterone was a kind gift from Dr. Paulus S. Wang (National Yang Ming University, Taipei, Taiwan).

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Abbreviations: LH, luteinizing hormone; CS, *Cordyceps sinensis*; BSA, bovine serum albumin; PKA, protein kinase A; PKC, protein kinase C; 3β-HSD, 3β-hydroxysteroid dehydrogenase; 17β-HSD, 17β-hydroxysteroid dehydrogenase; H89, *N*-(2-[*p*-bromocinnamylamino]ethyl)-5-isoquinolinesulfonamide hydrochloride; PMA, phorbol 12-myristate 13-acetate

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2.2. Animals

Male B6 (C57BL/6NCrj) mice, 5–6 weeks old, were purchased from National Cheng Kung University Animal Center (Tainan, Taiwan). B6 mice should be less than 8 weeks old for experiments to exclude the exposure of Leydig cells to LH. All animals were housed in groups of four in 29×18×13-cm polyethylene cages. The animal room was maintained at 22–24°C under a constant 12-h light/dark cycle. Purina mouse chow (Ralston-Purina, St. Louis, MO, USA) and water were always available.

2.3. Leydig cell isolation

Testes were removed from the killed mice and decapsulated in M199 containing 4 mM $NaHCO_3$, 25 mM HEPES, 0.06 g penicillin, 0.05 g streptomycin and 0.2% BSA, pH 7.35. After decapsulation, the testes were incubated in a shaking water-bath (120 cycles/min) at 37°C in M199 containing 1% BSA and 100 U/ml collagenase (type II; Worthington Biochemical, Lakewood, NJ, USA) for 10 min. After incubation, cold M199 was added to stop the action of the collagenase. Seminiferous tubules were separated from interstitial cells by gravity sedimentation. Cells were then collected by centrifugation (300×*g* for 6 min) and resuspended in 2 ml of M199 containing 0.1% BSA. This suspension, which did not contain seminiferous tubules and was composed of interstitial cells, contained 20–30% Leydig cells. This interstitial cell preparation was layered onto a Percoll gradient and then centrifuged at 800×*g* at 4°C for 20 min. The gradient, which was preformed by centrifugation at 25 000×*g* for 30 min, contained 10 ml of isotonic Percoll solution and 15 ml M199 plus 0.1% BSA and 25 mM HEPES [20]. A 1-ml fraction of gradient was collected from the top. Mouse Leydig cells were mainly distributed in fractions 23–25. The total number of cells and the percentage of 3β-HSD-positive cells were determined in this Leydig cell preparation [20]. The purity of the Leydig cells was 80–85%.

2.4. Cell culture

Cells were maintained at 37°C in a humidified environment containing 95% air and 5% CO_2 for all of the following experiments. Approximately 5×10^4 cells/100 μl M199 were plated into each well of 96-well plates. After 2 h, cells were washed twice with medium without any serum and then treated with 3 mg/ml CS with or without various reagents for 3 h. At the end of the incubation, the media were withdrawn and testosterone levels were determined by radioimmunoassay [19].

2.5. Radioimmunoassay

Media from cultures with different treatments were collected and diluted with medium to fall within the standard curves for the respective assays. Twenty-five μl of diluted sample was withdrawn into a glass tube and 100 μl each of testosterone antiserum and [^{3}H]testosterone were added. An equilibrium reaction occurred at room temperature for 2 h and was stopped by putting the tubes in ice. Charcoal was added and incubated for 15 min at 4°C and then centrifuged for 10 min to spin down the charcoal bound with free [^{3}H]testosterone. The supernatant was poured into 3 ml of scintillation fluid and samples were counted in a β-counter for 2 min [19,21].

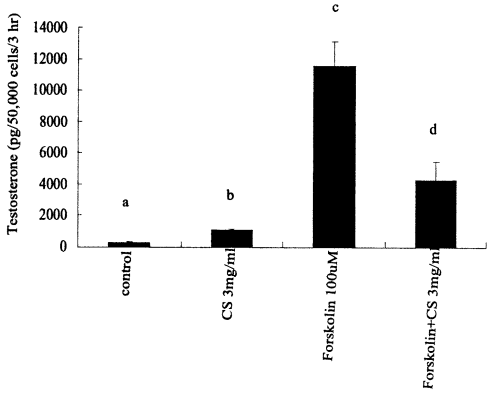

Fig. 1. Effects of CS with forskolin treatment on testosterone production in mouse Leydig cells. Cells were stimulated with a maximal concentration of forskolin (100 μM) with or without CS (3 mg/ml) for 3 h. Different letters above the bars indicate significant differences.

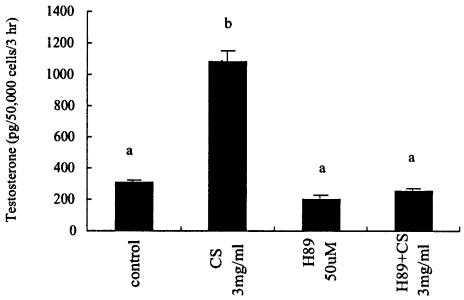

Fig. 2. Effects of CS with H89 treatment on testosterone production in mouse Leydig cells. Cells were stimulated with maximal concentrations of H89 (50 μM) with or without CS (3 mg/ml) for 3 h. Different letters above the bars indicate significant differences.

2.6. Statistical analysis

Each data point in the figures represents the mean ± S.E.M. of testosterone production in three separate experiments with triplicates of each treatment. Statistically significant differences between treatments and controls were determined by one-way ANOVA and the Fisher PLSD multiple comparison procedure. Statistical significance was set at $P<0.05$.

3. Results

3.1. The effects of CS with forskolin treatment on testosterone production in mouse Leydig cells

To determine whether CS affects the cAMP–PKA signal transduction pathway in mouse Leydig cells, an optimal concentration (100 μM) of forskolin was added with or without CS (3 mg/ml) for 3 h (Fig. 1). CS treated with forskolin significantly reduced testosterone production ($P<0.05$). The suppressive effect was about 60%, which indicates that CS might directly influence the cAMP–PKA signal transduction pathway to regulate mouse Leydig cell steroidogenesis.

3.2. The effect of CS with H89 treatment on testosterone production in mouse Leydig cells

To further confirm whether CS affects the cAMP–PKA signal transduction pathway in mouse Leydig cells, an optimal concentration (50 μM) of H89, a PKA inhibitor, was added

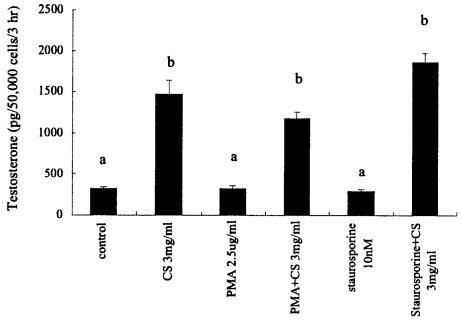

Fig. 3. Effects of CS with PMA or staurosporine treatments on testosterone production in mouse Leydig cells. Cells were stimulated with maximal concentrations of PMA (2.5 μM), or staurosporine (10 nM), respectively, with or without CS (3 mg/ml) for 3 h. Different letters above the bars indicate significant differences.

with or without CS (3 mg/ml) for 3 h. Fig. 2 illustrates that the stimulatory effect of CS on mouse Leydig cell steroidogenesis was completely blocked by H89 ($P < 0.05$). This result strongly suggests that CS activated the PKA pathway to regulate mouse Leydig cell functions.

3.3. The effect of CS with PMA and staurosporine treatments on testosterone production in mouse Leydig cells

To determine whether CS activates the PKC signal transduction pathway in mouse Leydig cells, PMA (2.5 μM), a PKC activator, and staurosporine (10 μM), a PKC inhibitor, were added separately, with or without CS (3 mg/ml), for 3 h (Fig. 3). Testosterone production was not reduced with the treatment of CS plus PMA or staurosporine ($P > 0.05$). These results indicate that CS could not activate the PKC signal pathway to influence steroidogenesis in mouse Leydig cells.

3.4. The effects of CS with 22R-hydroxycholesterol treatment on testosterone production in mouse Leydig cells

To determine the effect of CS on the activity of P450scc enzyme in normal mouse Leydig cells, an optimal concentration (50 μM) of 22R-hydroxycholesterol was added with or without CS (3 mg/ml) for 3 h (Fig. 4). CS significantly reduced testosterone production with the treatment of 22R-hydroxycholesterol ($P < 0.05$). The suppressive effect was 30%. This result indicates that CS might have a direct inhibitory effect on P450scc enzyme.

3.5. The effects of CS with pregnenolone, progesterone, 17α-hydroxyprogesterone, or androstenedione treatments on testosterone production in mouse Leydig cells

To determine the effect of CS on 3β-HSD, 17α-hydroxylase, 20α-hydroxylase, and 17β-HSD enzymes on mouse Leydig cells, pregnenolone (7.95 μM), progesterone (7.95 μM), 17α-hydroxyprogesterone (7.95 μM), or androstenedione (7.95 μM) were added separately with or without CS (3 mg/ml) for 3 h. CS did not reduce testosterone production when combined with pregnenolone (Fig. 5A), progesterone (Fig. 5B), 17α-hydroxyprogesterone (Fig. 5C), or androstenedione (Fig. 5D) ($P > 0.05$). This result indicates that CS has no effect on 3β-HSD, 17α-hydroxylase, 20α-hydroxylase, or 17β-HSD enzymes in mouse Leydig cells.

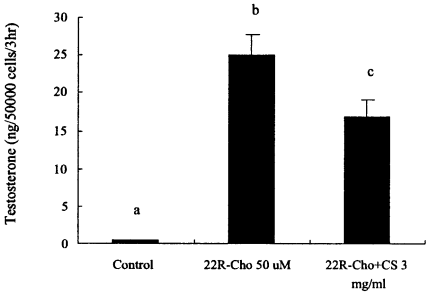

Fig. 4. Effects of CS with 22R-hydroxycholesterol treatment on testosterone production in mouse Leydig cells. Cells were stimulated with a maximal concentration of 22R-hydroxycholesterol (50 μM) in the absence of presence of CS (3 mg/ml) for 3 h. Different letters above the bars indicate significant differences.

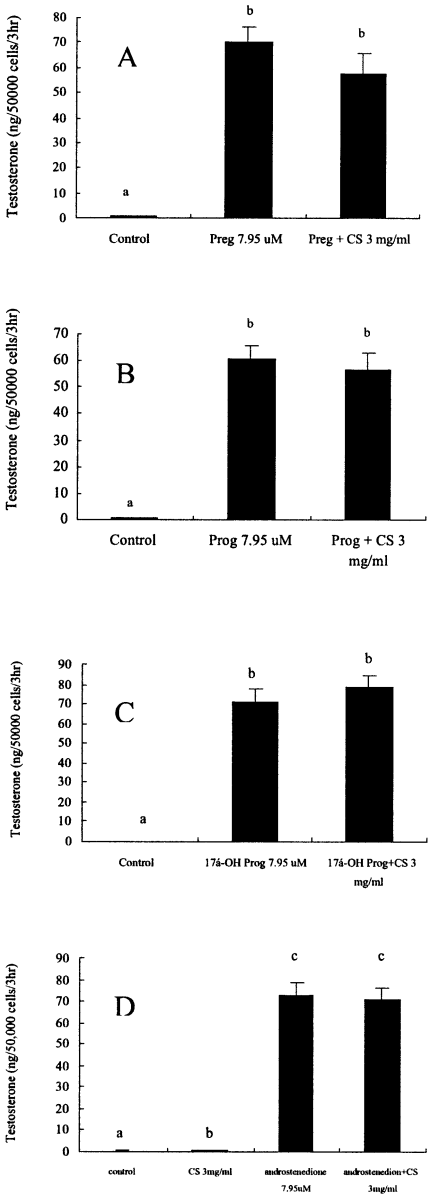

Fig. 5. Effects of CS with pregnenolone, progesterone, 17α-hydroxyprogesterone, or androstenedione treatments on testosterone production in mouse Leydig cells. Cells were stimulated with maximal concentrations of pregnenolone (A), progesterone (B), 17α-hydroxyprogesterone (C), or androstenedione (D) with or without CS (3 mg/ml) for 3 h. Different letters above the bars indicate significant differences.

4. Discussion

The present study demonstrates that CS activates the PKA signal transduction pathway, but not the PKC signal pathway, to regulate steroidogenesis in normal mouse Leydig cells. In addition, CS directly attenuates P450scc enzyme activity, but not 3β-HSD, 17α-hydroxylase, 20α-hydroxylase, or 17β-HSD to suppress hCG- or cAMP-stimulated steroidogenesis in mouse Leydig cells.

In the present study, H89 completely abolished the effect of CS on Leydig cell steroidogenesis, which suggests that CS may stimulate only the cAMP–PKA signal transduction pathway. It is well known that LH interacts with the receptors on Ley-

dig cells to activate adenylate cyclase through G proteins and that intracellular cAMP will increase to further activate PKA [1]. PKA will phosphorylate proteins and induce de novo synthesis of proteins to facilitate steroidogenesis [2,3]. Thus, that CS regulates cAMP–PKA signal transduction for steroidogenesis in mouse Leydig cells is not extraordinary. Moreover, in the present study, PKC activator or inhibitor did not affect testosterone production in the presence of CS, which demonstrates that the PKC pathway was not involved. Although it has been shown that the PKC pathway may also play some roles in steroidogenesis [22,23], it is clear that CS induced only the cAMP–PKA signal transduction pathway, but not the PKC pathway, to regulate mouse Leydig cell steroidogenesis in the present study.

Some studies have shown that many factors can inhibit the activities or expression of steroidogenic enzymes such as P450scc, 3β-HSD, 17α-hydroxylase, 20α-hydroxylase, and 17β-HSD, and that this inhibition correlates with the low production of testosterone by Leydig cells [24–26]. CS might have adverse effects on one or more of these enzymes, thereby reducing testosterone production in mouse Leydig cells. In the present study, CS significantly suppressed the activity of P450scc enzyme in Leydig cells, which correlated with the low production of testosterone in the presence of a testosterone production stimulator. In addition to suppressing the activity of P450scc, it is also possible that CS might similarly affect the expression of other steroidogenic enzymes and, therefore, steroidogenesis, which has been shown in many studies [27–29]. Indeed, CS reduced the activity of P450scc enzyme only by about 30%, which was not consistent with the 60% inhibition with forskolin treatment in the present study or with hCG treatment in our previous report [19]. These results strongly indicate that CS had an inhibitory effect on something other than P450scc. It has been shown that StAR protein plays an essential role in steroidogenesis [2,3,30]. CS might also affect StAR protein, a possibility that warrants further investigation.

Moreover, it has been shown that there are at least seven classes of chemical constituents of CS – proteins, polysaccharides, sterols, nucleoside, fatty acids, vitamins, and inorganics – and that some of these components do have pharmacological properties [5,16]. The peptide and carbohydrate moieties of ligands play an important role in recognizing receptors that activate signal pathways for various functions [31]. In the present study, we found that CS could activate the cAMP–PKA signal transduction pathway to regulate mouse Leydig cell testosterone production. However, we cannot rule out the possibility that different components of the polysaccharides and/or glycoproteins in CS may also activate other pathways to influence steroidogenesis. This might explain the 30% difference in the inhibitory effects of forskolin-treated CS on steroidogenesis and untreated CS on P450scc enzyme activity. Thus, the search for the finer components of CS that affect steroidogenesis and the involved signal pathways is worth further investigation.

In conclusion, CS stimulated the cAMP–PKA signal transduction pathway to regulate mouse Leydig cell testosterone production. Moreover, the suppression of hCG-stimulated testosterone production in mouse Leydig cells by CS might be due to the CS-induced inhibition of P450scc enzyme activity.

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