Cordyceps sinensis and Its Fractions Stimulate MA-10 Mouse Leydig Tumor Cell Steroidogenesis

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ABSTRACT: The effects of Cordyceps sinensis (CS) and its extracted fractions on steroidogenesis in MA-10 cells were determined. Different concentrations of CS and 3 fractions of CS (F1, a water-soluble polysaccharide; F2, a water-soluble protein; and F3, a poorly water-soluble polysaccharide and protein) were added to MA-10 mouse Leydig tumor cells with or without human chorionic gonadotropin (hCG), and the production of steroid and the expression of steroidogenic acute regulatory protein (StAR) were examined. The results showed that CS alone (2–10 mg/mL) stimulated MA-10 cell progesterone production in a dose-dependent relationship. Fractions F1 and F3 (2–10 mg/mL) also had significant (P < .05) stimulatory effects on MA-10 cell steroidogenesis with a dose-dependent relationship. However, fraction F2 did not have an effect on MA-10 cells. CS and F3, but not F1, significantly induced more steroid production in hCG-stimulated MA-10 cells (P < .05). As a temporal relationship, F1 and F3 (2 mg/mL) maximally stimulated progesterone production between 1 and 3 hours after stimulation in MA-10 cells. In addition, CS and F3 significantly enhanced MA-10 cell StAR protein expression, which indicates that CS and F3 may use a cyclic adenosine monophosphate signal transduction pathway to activate MA-10 Leydig cell steroidogenesis in a manner to that of luteinizing hormone.

Key words: Human chorionic gonadotropin, steroidogenic acute regulatory protein, progesterone, fungus.


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Cordyceps sinensis (CS), a member of the Ascomycetes class of fungi, parasitizes larvae of Lepidoptera (Manabe et al, 1996). According to many ancient descriptions of Chinese herbs, CS possesses important pharmacological activities and has been used as an herbal medicine for centuries in Asian societies. Investigations have shown that CS is capable of modulating immune responses (Liu et al, 1992; Xu et al, 1992), inhibiting tumor growth (Yoshida et al, 1989; Bok et al, 1999), decreasing blood pressure (Manabe et al, 1996), reducing microorganism proliferation (White and Dawson, 1979), expanding bronchial smooth muscles (Zhu et al, 1998a), and promoting the secretion of adrenal hormones (Wang et al, 1998). Chinese people also believe that CS can restore impaired reproductive functions in both sexes, such as impotency and infertility. It has been shown that CS can enhance libido (Zhu et al, 1998b); however, there is no evidence that CS does indeed influence the reproductive system, or even directly affects sexual hormone release. Nevertheless, the supply of CS is inadequate to meet demand, particularly because it exists in high-altitude areas and is not easily harvested. However, the mycelium of the fungus has recently been cultured and a dried, powdered form of the mycelium is now commercially available.

In the male reproductive system, gonadotropin-releasing hormone (GnRH) from the hypothalamus stimulates the pituitary to release luteinizing hormone (LH). LH travels to the testis, where it activates the Leydig cell cyclic adenosine monophosphate (cAMP) signal transduction pathway. cAMP then stimulates protein kinase A (Moger, 1991), which phosphorylates proteins. Phosphorylated proteins will further phosphorylate other proteins or induce new protein synthesis, such as the steroidogenic acute regulatory (StAR) protein. The function of the StAR protein is to facilitate the transfer of free cholesterol from cytoplasm into the inner membrane of mitochondria (Stocco, 1999), where the cytochrome P450 side-chain cleavage enzyme converts cholesterol to pregnenolone (Simpson, 1979). The translocation of cholesterol into mitochondria by StAR protein is considered as a rate-limiting step in steroidogenesis (Stocco and Clark, 1996). Pregnenolone is then transported to smooth endoplasmic reticulum for further synthesis into the steroid hormone testosterone, which is essential in reproduction (Saez, 1994).

We have previously demonstrated that CS can stimulate steroid production in MA-10 mouse Leydig tumor cells.
(Huang et al, 2000; Ju et al, 2000). In the present study, the effects and mechanisms of CS and its extracted fractions on MA-10 cell steroidogenesis were further investigated. Various concentrations of crude CS and 3 fractions of CS (F1, a water-soluble polysaccharide; F2, a water-soluble protein; and F3, a poorly water-soluble polysaccharide and protein) with or without hCG were used to treat MA-10 cells. Steroid production and the expression of StAR protein were determined by radioimmunoassay (RIA) and Western blotting, respectively.

Materials and Methods

Chemicals
Cultured mycelium of Cordyceps sinensis was supplied by Simpson Biotech Company Ltd (Taipei, Taiwan, Republic of China). Waymouth MB 752/1 medium, fetal bovine serum (FBS), Dulbecco phosphate buffered saline (PBS) with calcium or magnesium (PBS+ ), gentamicin sulfate, and lyophilized trypsin-EDTA were purchased from Gibco (Grand Island, NY). Tissue culture-grade sodium bicarbonate, Hapes, Tris(HCl), mercaptoethanol, sodium dodecyl sulfate (SDS), sucrose, Tween 20, EDTA, glycerol, bromophenol blue, and hCG (10 000 IU per gram) were purchased from Sigma Chemical Company (St Louis, Mo). A Renaissance kit and ¹²⁵I-progesterone used for chemiluminescence and RIA, respectively were purchased from DuPont-New England Nuclear (Boston, Mass). Antiserum to progesterone was obtained from Holly Hills Biologicals Inc (Hillsboro, Ore) and from Dr Douglas M. Stocco (Texas Tech University Health Sciences Center, Lubbock, Tex).

CS Fraction Preparation
Crude CS (100 g) was extracted with 800 mL distilled water and shaken at 37°C for 72 hours. The solution was then centrifuged at 13 000 × g at 4°C for 30 minutes to collect the pellet, which was labeled fraction 3 (F3). The supernatant was applied to a G150 gel filtration column (3 × 100 cm) with 50 mM CH₃COONH₄ buffer at pH 6.0. Two peaks were collected and the first peak was assigned as F1 and the second peak was assigned as F2.

Cell Culture and Experiments
The MA-10 cell line was a gift from Dr Mario Ascoli (University of Iowa, Iowa City, Iowa) and was maintained with standard techniques (Ascoli, 1981). This well-studied mouse Leydig tumor cell line produces progesterone as the major steroid in response to both trophic hormones (LH and hCG) and cAMP analogues (Ascoli, 1981; Huang et al, 1995). Cells (1 × 10⁶) were plated in 100-mm diameter tissue culture dishes and grown for 24 hours in Waymouth medium containing 15% FBS. The medium was then removed and the cells were washed twice with PBS+. Various concentrations of CS and CS fractions in Waymouth medium without serum in the presence or absence of hCG were added to the cells for 3 hours. Cells were then collected for isolation of mitochondria. Cells cultured in 96-well plates were treated with hCG alone or hCG plus CS and CS fractions, and media were collected and stored at ~20°C until they were assayed for progesterone by RIA. Cells were maintained in a humidified atmosphere containing 95% air and 5% CO₂ at 37°C.

Isolation of Mitochondria
After stimulation, cells were washed twice with PBS+ and then collected from the dishes by scraping in an isolation buffer of 10 mM Tris, 0.25 M sucrose, and 0.1 mM EDTA pH 7.4. Cells were homogenized at 1000 × g for 20 passes using a motorized glass homogenizer fitted with a serrated Teflon pestle. The homogenates were centrifuged at 600 × g for 30 minutes, and the resultant supernatants were centrifuged at 12 000 × g for 30 minutes to pellet the mitochondria (Stocco and Kilgore, 1988; Huang et al, 1997a; Liu et al, 2001). Mitochondria were then resuspended in a small volume of isolation buffer. An aliquot was removed for protein determination according to the method of Lowry (Lowry et al, 1951), and mitochondria were once again collected by centrifugation at 12 000 × g. The amount of protein in the cell debris—the pellet of first centrifugation—was also determined and combined with mitochondria protein as total cellular protein.

Immunoblot Analysis
The mitochondria pellets were solubilized in a sample buffer consisting of 25 mM Tris/HCl pH 6.8, 1% SDS, 5% mercaptoethanol, 1 mM EDTA, 4% glycerol, and 0.01% bromophenol blue and loaded on 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) minigels (Mini-Protein II System; Bio-Rad, Richmond, Calif). Electrophoresis was performed at 200 V for 40 minutes using a standard SDS-PAGE running buffer (24 mM Tris/HCl, 0.19 M glycine, 0.5% SDS pH 8.3), and the proteins were electrophoretically transferred to polyvinylidene difluoride membranes (Bio-Rad) at 100 V for 2 hours in transfer buffer (20 mM Tris/HCl, 150 mM glycin, 10% methanol, 0.01% SDS). Immunodetection of the 30-kd StAR protein using rabbit polyclonal antisera to the StAR protein was performed as previously described (Ju et al, 2000, Wu et al, 2001). The appropriate protein bands of interest were quantitated using the Quantity One (PDQ, Huntington Station, NY) computer-assisted image analysis system. The data for protein quantitation were expressed as integrated optical density, which had been normalized.

Radioimmunoassay
RIA as previously described (Huang et al, 1997b) was used to perform quantification of progesterone on aliquots of the medium from control and treated cells. In brief, media from cultures with different hormone treatments were collected and diluted with medium to align with standard curves for the respective assays. Twenty-five microliters of diluted sample was withdrawn to a glass tube and 100 μL progesterone antisera and 100 μL ³H-progesterone were added. The equilibrium reaction occurred at room temperature for 2 hours and was stopped by placing the
Figure 1. The effects of various concentrations of CS on progesterone production in MA-10 cells. Different concentrations of CS without or with 50 ng/mL of hCG were added to cells for a 3-hour incubation. Media were collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean ± SEM of progesterone production of 3 experiments with triplicates in each treatment. *Statistically significant difference (P < .05) compared with controls; **statistically significant difference (P < .05) compared with hCG treatment alone.

Figure 2. The effects of 1 mg/mL CS and CS fractions without or with hCG treatment on progesterone production in MA-10 cells. CS, F1, F2, or F3 at 1 mg/mL, without or with 50 ng/mL hCG, were added to cells for a 3-hour incubation. Media were then collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean ± SEM of progesterone production of 3 experiments with triplicates in each treatment. *Statistically significant difference (P < .05) compared with control treatment; **statistically significant difference (P < .05) compared with hCG treatment alone.

Results

Effects of CS on Progesterone Production in MA-10 Cells

Various concentrations of CS (0.5–10 mg/mL) without or with hCG (50 ng/mL) were used to treat MA-10 cells for a 3-hour incubation (Figure 1). Progesterone production significantly increased with CS treatments at 2 mg/mL, 4 mg/mL, and 10 mg/mL compared with the control (P < .05). Similarly, the cells treated with CS and with 2 mg/mL, 4 mg/mL, and 10 mg/mL of hCG resulted in significantly additive increases in progesterone production compared with hCG treatment alone (P < .05).

Effects of CS Fractions on Progesterone Production in MA-10 Cells

The effects of CS and 3 water-extracted fractions of CS (F1, F2, and F3) on progesterone production in MA-10 cells were further studied. CS, F1, F2, or F3 at 1 mg/mL, without or with 50 ng/mL hCG, were added to cells for a 3-hour incubation. Media were then collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean ± SEM of progesterone production of 3 experiments with triplicates in each treatment. *Statistically significant difference (P < .05) compared with control treatment; **statistically significant difference (P < .05) compared with hCG treatment alone.

To determine the effects of CS and CS fractions on...
hCG-stimulated progesterone production in MA-10 cells, 50 ng/mL hCG plus CS, F1, F2, or F3 at 10 mg/mL were added to cells for a 3-hour incubation. Media were then collected and assayed for progesterone production by RIA. Each data point in the figure represents the mean ± SEM of progesterone production of 3 experiments with triplicates in each treatment. *Statistically significant difference (P < .05) compared with control treatment; **statistically significant difference (P < .05) compared with hCG treatment alone.

Dose Effect of F1 and F3 on Progesterone Production in MA-10 Cells

As described above, F1 and F3 fractions of CS had stimulatory effects on MA-10 cell steroidogenesis, thus the dosage effects of both fractions of CS on MA-10 cells were further investigated. Dosages ranging from 0.5 to 10 mg/mL of F1 or F3 were added to MA-10 cells for a 3-hour incubation. Figure 4 illustrates that dosages higher than 1 mg/mL of F1 resulted in a significant increase in progesterone production compared with the control (P < .05) in MA-10 cells. There were no differences in steroid production among controls and F1 at 0.5 mg/mL and 1 mg/mL treatments (P > .05).

To determine the effects of various concentrations of F1 on hCG-stimulated progesterone production in MA-10 cells, 50 ng/mL of hCG without or with 0.5–10 mg/mL of F1 were added to MA-10 cells for a 3-hour incubation. Figure 4 also illustrates that F1 at any dosage had no effect on hCG-stimulated progesterone production in MA-10 cells (P > .05).

Figure 5 illustrates the dose effect of F3 on MA-10 cell progesterone production, which was significantly stimulated with dosages of 4, 8, and 10 mg/mL of F3 compared with the control (P < .05). Unlike F1 without any effect on MA-10 cell steroidogenesis, F3 induced more proges-
Time Course Effects of F1 and F3 on Progesterone Production in MA-10 Cells

To investigate the time effect of F1 and F3 on progesterone production in MA-10 cells, F1 and F3 at 2 mg/mL without or with 50 ng/mL of hCG were added to cells, and media were then collected after 0, 1, 3, 6, 12, and 24 hours to determine progesterone production. Figure 6 illustrates that progesterone production significantly increased with F1 after a 1-hour incubation compared with the control, and the maximal response appeared at 3 hours of incubation (P < .05). Similarly, the addition of F1 at 2 mg/mL on hCG-stimulated progesterone production was significantly increased compared with hCG treatment alone after 1 hour of incubation (P < .05), and the maximal response occurred at 3 hours of incubation (P < .05).

Figure 7 shows that progesterone production significantly increased by 2 mg/mL of F3 treatment after a 1-hour incubation compared with the control, and the maximal response occurred at 3 hours of incubation (P < .05). Similarly, addition of F3 at 2 mg/mL on hCG-stimulated progesterone production was significantly increased compared with hCG treatment alone after a 1-hour incubation (P < .05), and the maximal response was at 1–3 hours of incubation (P < .05).

Effects of CS and F3 on StAR Protein Expression in MA-10 Cells

As mentioned above, StAR protein is essential in steroidogenesis, and the effects of CS and F3 on StAR protein expressions in MA-10 cells were determined. MA-10 cells were treated with hCG, CS (2 and 10 mg/mL), and F3 (2 and 10 mg/mL) for a 3-hour incubation. Cells were then collected and the expression of StAR protein was determined with Western blot analysis. Figure 8 illustrates that StAR protein expression significantly (P < .05) increased upon treatments with hCG, CS, and F3 compared with control treatments. In fact, CS and F3 at 10 mg/mL had a stimulatory effect similar to that of hCG on StAR protein expression in MA-10 mouse Leydig cells.
Discussion

The results show that crude CS, F1, and F3 had stimulatory effects on steroid production in MA-10 cells. The stimulatory effect of F3 on MA-10 cell steroidogenesis was more pronounced than that of crude CS and F1. CS was more effective than F1; however, F2 had no effect on MA-10 cells. These results indicate that F3 and F1 contain factors that could stimulate MA-10 cell steroidogenesis. Furthermore, CS and F3 could induce more progesterone as cells were treated with hCG.

The factors that potentiate LH/hCG action have been observed in many peptides that induce more steroid production as Leydig cells were treated with LH/hCG hormones simultaneously (Huang et al, 1995). Thus it is possible that CS and F3 activate other signal pathways to enhance the LH/hCG-stimulated cAMP signal transduction pathway, and then steroidogenesis. At least 7 different isoforms of adenylate cyclase (Watson et al, 1994) and more isoforms of beta and gamma G-proteins (Tang and Gilman, 1991; Katz et al, 1992; Ueda et al, 1994) have been found. Different combinations of G-proteins, after their interaction with distinct receptors, may activate miscellaneous responses through adenylate cyclase isoforms with different levels of cAMP production (Katsushika et al, 1992; Ueda et al, 1994; Manolopoulos et al, 1995), phospholipid hydrolysis (Katz et al, 1992; Ueda et al, 1994), retinal phosphodiesterase (Tang and Gilman, 1991), or ion channels (Logothetis et al, 1987; Tang and Gilman, 1991). It is possible that in MA-10 cells, hCG and CS or F3 associate with different receptors that couple to independent adenylate cyclase isoforms through different isoform G-proteins. Studies have shown that cAMP production can be synergistically stimulated by two different ligands through different adenylate cyclase isoforms. Examples of this are prostaglandin E-enhanced adenylate cyclase cAMP formation stimulated with forskolin through two EP3 subtype receptors with different efficiencies (Harazono et al, 1994), and phorbol ester-potentiated isoproterenol-stimulated cAMP production on type VII adenylate cyclase (Watson et al, 1994). Therefore, it is not unusual that the additive effect of progesterone and intracellular cAMP production by hCG and CS or F3 occurred in MA-10 cells. It is also possible that CS and F3 induce more expression of LH receptors on cells, which effectively increases LH binding to prompt an increase in steroidogenesis. Therefore, the contents in CS and F3 may have similar factors that can potentiate Leydig cells to produce more steroids. This phenomenon is not unprecedented.

F1 did not have the effect of inducing more steroid release in MA-10 cells with hCG treatment, although F1 alone did stimulate MA-10 cell steroidogenesis in a dose-dependent relationship. It is possible that the factors that induced more steroid release still existed in F3 after CS was extracted. Certainly, this remains to be further clarified.

The time course effects of F1 and F3 on MA-10 cell steroidogenesis indicates that F1 and F3 had an acute effect on cell steroid production. These results suggest that F1 and F3 have similar properties as LH/hCG, which have their maximal stimulatory effects at 3 hours on Leydig cell steroidogenesis (Huang et al, 1995). It is well known that LH and hCG are glycoproteins that contain polysaccharides (Pigny et al, 1992). Recent studies have shown that many polysaccharides extracted from herbs have pharmacological activities (Ukai et al, 1983). It was demonstrated that one of the CS components, polysaccharides, may be the pharmacological part of CS (Yoshida et al, 1989), and which play an important role in living organisms (Teti, 1992). Polysaccharides have been found in interstitial tissues (Ruoslahti, 1989) as structural molecular forms (cell wall, chitin, receptor, etc) with functions in recognizing, connecting, or contacting molecules for cell interaction and communication (Hynes, 1987). Polysaccharides are also part of immunoglobulins, hormones, and enzymes (Lapthorn et al, 1994). It has been shown that the glyco portion of LH/hCG plays an important role in association with the LH receptor to activate the signal pathway for steroidogenesis (Pigny et al, 1992). It is possible that parts of the polysaccharides in CS and F3 may be similar to LH in their structure with the ability to recognize LH receptors on Leydig cells and then stimulate steroid production. However, this remains to be elucidated.

It has been demonstrated that StAR protein has a critical function in the transfer of cholesterol from the outer to the inner mitochondrial membrane during hCG-stimulated steroidogenesis (Stocco and Clark, 1996; Stocco, 2000). Also, the expression of StAR protein is highly associated with the cAMP signal transduction pathway. These results show that the increase in StAR protein expression was enhanced by addition of CS and F3 to MA-10 cells, which was consistent with the stimulatory effect of CS on progesterone production in MA-10 cells. These results strongly suggest that StAR was involved in the action of CS and F3 on MA-10 cell steroidogenesis. Thus, CS probably activated the cAMP signal transduction pathway to stimulate the expression of StAR protein, which resulted in a significant increase in progesterone production.

In summary, our findings provide evidence that CS and F3 increase steroid production and potentiate hCG function in MA-10 cells, which results from an increase in StAR protein expression. We are currently investigating the mechanism of action of CS and F3 on normal mouse
Leydig cells. The effects of the finer extraction of CS on Leydig cell function are also under investigation.

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