Effects of Extracted *Cordyceps sinensis* on Steroidogenesis in MA-10 Mouse Leydig Tumor Cells

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Extracts from the mycelium of *Cordyceps sinensis* (CS) were tested to determine the *in vitro* effect on Leydig cell function. MA-10 mouse Leydig tumor cells were used to conduct the experiments. Results showed that progesterone production gradually increased as the dosage of combined water and ethanol extracted CS increased, and there was a statistically significant difference in progesterone production stimulated by 20 mg/ml of CS extracts compared to the control. The combined water and ethanol extracted CS significantly stimulated MA-10 cell steroid production at 12 and 24 h of incubation. In addition, a protein synthesis inhibitor, cycloheximide, did not block the stimulatory effects of CS extracts on MA-10 cell steroid production or total protein expression. Moreover, the expression of steroidogenic acute regulatory (StAR) protein, which is a critical protein for steroidogenesis, stimulated by CS extracts, could not be detected by Western blot analysis. These data indicate that CS extracts might not induce StAR protein and/or other protein expressions to stimulate steroidogenesis in MA-10 mouse Leydig tumor cells.

**Key words** *Cordyceps sinensis*; steroidogenesis; stimulation; Leydig cell

*Cordyceps sinensis* (CS), a fungus parasite on the larva of Lepidoptera, has been used as an effective nutritious medicine in Chinese society for a long time, with such claimed pharmacological properties as antitumor activity, enhancement of immune response, protection of kidney from toxicants, relaxation of persistent contractions of the trachea and aorta, erythropoiesis, antiarrhythmic effects, hypoglycemic activity and vasorelaxant activity. The supply of CS is unsatisfactory for the demand. However, the mycelium of the fungus has recently been cultured, and the dried powder of the mycelium is commercially available. Chinese people also believe that CS can enhance sexual performance and can restore impaired reproductive functions such as impotency or infertility, in both sexes. One report showed that CS increases the weight of the prostate, seminal vesicle and Cowper's gland in castrated rats. This report indicates that CS might directly affect reproductive accessory organs which are androgen-dependent tissues. However, it is also possible that CS acts on the adrenal gland to release androgen to affect the growth of reproductive accessory glands in castrated rat. In fact, it has been illustrated that CS can induce the adrenal gland to release glucocorticoid. Therefore, it is still unclear whether CS has a direct effect on Leydig cell steroidogenesis.

It is well known that testosterone, produced by Leydig cells, is essential in male reproductive functions. In the present study we would like to investigate whether CS directly influences Leydig cell steroidogenesis. A mouse Leydig tumor MA-10 cell line was used for this study. This pure Leydig tumor cell line produces progesterone as the major steroid, instead of testosterone, in response to trophic hormones (LH and hCG) and cAMP analogs. Progesterone production by MA-10 cells following treatment with cAMP analog and/or the extracts of CS was measured to determine the effect of CS on steroidogenesis. Total protein and StAR protein expressions were also measured to determine the mechanism.

**MATERIALS AND METHODS**

**Materials** Mycelium of *Cordyceps sinensis* was kindly supplied by Dr. Peter Pang, Herbal Tech, Edmonton, Canada. Preparation of the extracted CS was as follows: 30 g of cultured *Cordyceps sinensis* mycelium powder was extracted with 240 ml of water in a water bath of 100°C for 3 h with reflux. Eighty ml 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. Thus 50% alcoholic fraction was then 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 were reconstituted by adding 4 times more of the suscrose as a desiccant; the resulting mixture was then used in the present study. The concentration indicated in this study is the net weight of extracted CS.

Dibutyryl-adenosine-3’,5’-cyclic monophosphate (dbcAMP) and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Weymouth medium, fetal bovine serum, horse serum and cell culture related materials were obtained from Gibco-BRL (Grand Island, NY, U.S.A.). Materials related to radioimmunoassay were purchased from DuPont NEN (Boston, MA, U.S.A.). Antiserum to progesterone was obtained from Holly Hills Biologicals, Inc. (Hillsboro, Oregon, U.S.A.), and from Dr. Paulus S. Wang (National Yang-Ming University, Taipei, Taiwan).

**Cell Culture and Radioimmunoassay** The MA-10 cell line is a generous gift from Dr. Mario Ascoli (University of Iowa, Iowa City, IA) and is maintained by standard techniques. This well-studied mouse Leydig tumor cell line produces progesterone as the major steroid in response to both trophic hormone (LH and hCG) and cAMP analogs. 1×10⁶ cells/ml medium or 5×10⁶ cells/100 µl medium were plated in 100 mm diameter tissue culture dishes or 96-well plates, respectively. Cells were grown for 24 h in Weymouth medium containing 15% horse serum. After this time, e-mail: lisaleu@sun5.nlacr.gov.tw

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the medium was removed and the cells were washed with PBS. Chemicals and/or CS extracts were then added to the Weymouth medium without serum at various concentrations and times. Cells were maintained in a humidified atmosphere containing 95% air and 5% CO₂ at 37°C. After the incubation, media were recovered and progesterone levels in each treatment were determined by radioimmunoassay (RIA).22,23

**Total Protein Determination** After the experiment in which progesterone was measured, cells in culture dishes or each well of the 96-well plates were solubilized by 0.5 N HCl. Protein concentration in the cell lysate in each dish or well was determined by the Lowry method.24

**Isolation of Mitochondria** After stimulation by the CS extracts or cAMP analog, MA-10 cells in 100 mm diameter tissue culture dishes were collected by scraping with a plastic cell scraper. The cells were then homogenized with a homogenizer. The homogenates were centrifuged at 600×g for 30 min, and the resultant supernatants were centrifuged at 12000×g for 30 min to pellet the mitochondria.25,26

**Immunoblot Analysis** The mitochondrial pellets were solubilized in sample buffer and loaded onto a 12.5% SDS-PAGE minigel. Electrophoresis was performed at 200 V for 45 min using a standard SDS-PAGE running buffer, and the proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (PVDF) at 100 V for 2 h at 4°C. For immuno-detection of the 30 kDa protein, primary antibody was generated in rabbits against amino acids 88–98 of the 30 kDa protein. The PVDF membrane with transferred protein was incubated in a blocking buffer at room temperature for 1 h. The membrane was then incubated in fresh blocking buffer containing the primary antibody for an additional hour at room temperature. Next, the membrane was washed with PBS containing 0.5% Tween 20, and then incubated for 30 min at room temperature with fresh blocking buffer containing the secondary antibody, donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amersham). The membrane was washed as before and the specific signal was detected by chemiluminescence using a Renaissance kit.25,26

**Statistics** All experiments, with triplicates of each treatment, were repeated at least three times. Data of all experiments were analyzed by analysis of variance (ANOVA), and then the Least Significant Difference (LSD) was used to determine the difference between treatments.

**RESULTS**

**Dose Effect of the Combination of Water and Ethanol Extracted CS on Progesterone Production in MA-10 Cells**

Figure 1 demonstrates the effect of various concentrations of CS extracts on the progesterone production in MA-10 cells. Progesterone production increased with increasing dosages of CS extracts. When incubated with 20 mg/ml of CS extracts, the production of progesterone increased 9-fold compared with the basal level.

**Time Course Effect of the Combination of Water and Ethanol Extracted CS on Progesterone Production in MA-10 Cells**

The effect of different incubation times with CS extracts (20 mg/ml) on progesterone release in MA-10 cells is shown in Fig. 2. Progesterone production gradually increased from 6 to 24 h. In fact, there is a significant difference in the progesterone production stimulated by CS extracts compared with the basal level after 12 h incubation. The maximal response is within 24 h of incubation, similarly to the cells treated with 20 mg/ml of CS extracts.

**Effects of Cycloheximide on Extracted CS or cAMP Analog Stimulated Progesterone Production in MA-10 Cells**

It has been shown that de novo protein synthesis is essential for steroid production in steroidogenic cells. In the present experiments, MA-10 cells, with CS extract (20 mg/ml) or cAMP analog (1 mM) stimulation, were treated with a protein synthesis inhibitor, cycloheximide, for 24 h. Progesterone production stimulated by cAMP analog in MA-10 cells was reduced in the presence of cycloheximide (Fig. 3A). However, cycloheximide didn't have any significantly inhibitory effect on extracted CS-stimulated progesterone production in MA-10 cells (Fig. 3B). Here, we have to point out that the scales of progesterone production in MA-10 cells stimulated by CS extracts or cAMP analog are different.

**Effects of Cycloheximide on Extracted CS or cAMP Analog Induced Total Protein Expression in MA-10 Cells**
heximide alone or cycloheximide with cAMP analog groups, as compared to the control and the cAMP analog alone groups. Interestingly, the amount of total protein was not affected between the control and CS extracts groups without or with increasing doses of cycloheximide.

**Effect of the Extracted CS on the Expression of Steroidogenic Acute Regulatory (STAR) Protein**

To determine whether CS extracts can stimulate the synthesis of a 30 kDa mitochondrial steroidogenic protein, MA-10 cells were treated with CS extracts and/or cAMP analog, and the expression of STAR protein was examined. Results illustrate that cAMP analog (1 mM for 24 h) stimulated the significant expression of STAR protein. In contrast, treatment with 20 mg/ml of CS extracts for 24 h could not induce the expression of STAR protein (data not shown).

**DISCUSSION**

The fruit body of CS with the larva of Lepidoptera has long been used as an effective medicine in traditional Chinese society. Various in vitro or in vivo pharmacological effects of this substance have been reported. In the present studies, we demonstrate that CS extracts can significantly stimulate steroid production in the MA-10 mouse Leydig cell line.

In steroidogenic tissue, the production of a steroid hormone is regulated by trophic hormone stimulation involving new protein synthesis. In this study, a protein synthesis inhibitor, cycloheximide, did reduce cAMP analog stimulated progesterone production or total protein expression in MA-10 cells. However, cycloheximide did not block the stimulatory effect of CS extracts on MA-10 cell progesterone production or total protein expression. These results indicate that CS extracts might stimulate MA-10 cell steroid production without new protein synthesis.

To further confirm whether CS extracts do not induce new protein synthesis, Western blotting was used to detect STAR protein expression. It has been shown that STAR is a steroidogenic tissue specific, hormone induced, rapidly synthesized protein previously shown to be involved in the acute regulation of steroidogenesis, probably by promoting the transfer of cholesterol to the inner mitochondrial membrane and the cytochrome P450 side-chain cleavage (P450scc) enzyme.25,37–39 According to the data, CS extracts did not induce any expression of the STAR protein. These results suggest that CS extracts stimulate MA-10 cell steroidogenesis through a pathway without STAR or other protein expressions.

As we described in the results, the effect of CS extracts in stimulating MA-10 cell progesterone is far less than cAMP analog. A 100-fold increase in progesterone was induced by cAMP analog in MA-10 cells. CS extracts only induced about a 4- to 9-fold increase in steroid production compared to the basal level. It is possible that CS extracts might only enhance the basal activities of steroidogenic enzymes, including STAR protein. Therefore, the induction of any new protein synthesis by CS extracts is not necessary. However, it is still possible that CS extracts may induce a low amount of STAR protein expression, which is not easy to detect by protein assay or Western blot. Therefore, a more sensitive method of detecting STAR protein or gene expression under
the CS extracts will be needed to determine the mechanism. Certainly, we still cannot exclude the possibility that CS extracts might use another pathway to stimulate Leydig cell function.

It is known that long-term treatment with Chinese medicine is required for disease therapy or nutritious remedy. For long-term consumption, it is logical that CS, with its low stimulatory effect on Leydig cells, can improve reproductive functions. Likewise, CS is a complex compound. It will be interesting to pinpoint which component of CS has the effect on Leydig cell steroidogenesis.

In summary, CS extracts stimulate MA-10 mouse Leydig tumor cell steroidogenesis. The stimulatory effect of CS extracts might not involve the new synthesis of proteins.

REFERENCES