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Cordycepin Stimulated Steroidogenesis in MA-10 Mouse Leydig Tumor Cells through the Protein Kinase C Pathway

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ABSTRACT: Cordycepin (3′-deoxyadenosine) is an adenosine analogue isolated from *Cordyceps sinensis*, which is a Chinese herbal medicine known to have many benefits, including adjustment of the physical condition, an anticancer effect, and enhancement of sexual performance. It was previously demonstrated that cordycepin could simultaneously activate steroidogenesis and apoptosis in MA-10 mouse Leydig tumor cells. However, the mechanism remains elusive. Thus, aim of the present study was to investigate the steroidogenic and apoptotic mechanism of cordycepin in MA-10 cells. MA-10 cells were treated with cordycepin at various dosages and time courses plus different protein kinase inhibitors. Steroid production, protein expression, and cell viability were then determined. Results illustrated that cordycepin stimulated MA-10 cell steroidogenesis in dose- and time-dependent relationships. However, cordycepin could not induce steroidogenic acute regulatory (StAR) protein expression. However, cordycepin did activate the phospholipase C/protein kinase C (PLC/PKC), but not PKA and PI3K, pathway to induce MA-10 cell steroidogenesis. Moreover, cordycepin could stimulate the phosphorylation of PKC, extracellular signal-regulated kinase 1/2 (ERK1/2), and c-Jun N-terminal kinase (c-JNK), but not p38, in MA-10 cells. In addition, cordycepin could activate the PKC pathway to induce MA-10 cell death, and this death effect was not caused by cordycepin-stimulated progesterone from MA-10 cells. In conclusion, cordycepin stimulated intracellular PLC/PKC and MAPK signal transduction pathways to induce steroidogenesis and cell death in MA-10 mouse Leydig tumor cells.

KEYWORDS: *cordycepin, steroidogenesis, protein kinase C pathway, Leydig tumor cells, Cordyceps sinensis*

INTRODUCTION

Cordycepin (3′-deoxyadenosine) is an adenosine derivative purified from *Cordyceps sinensis*.[1] It has been illustrated that cordycepin acts as polyadenylation inhibitor to terminate transcriptional elongation by preventing the addition of a poly(A) tail to nuclear RNA in HeLa cells.[2] In fact, studies have also demonstrated its multiple pharmacological actions, such as immune system activation, antioxidation activity, and antitumor effect.[3−5] Previously, we demonstrated that cordycepin could act through adenosine receptors (A1, A2a, and A3) to activate steroidogenic acute regulatory (StAR) protein expression and to stimulate steroidogenesis in normal mouse Leydig cells in vitro and in vivo.[6] Correspondingly, the stimulatory effect of cordycepin in steroidogenesis could also be observed in MA-10 mouse Leydig tumor cells.[7] However, cordycepin could activate adenosine receptor without stimulating StAR protein expression to up-regulate MA-10 cell steroidogenesis.[7]

Steroidogenesis in Leydig cells can be regulated by various signal transduction pathways, including cAMP/protein kinase A (PKA), mitogen-activated protein kinase, and protein kinase C (PKC) signaling pathways.[8−10] These kinases can modulate cellular processes by protein phosphorylation. In fact, it is well-established that StAR protein is essential for steroidogenesis and that extracellular regulating kinase (ERK) 1/2 phosphorylation driven by mitochondrial PKA could promote StAR protein activation and then steroidogenesis.[9] Besides, the family of MAPKs, including ERK, c-Jun NH(2)-terminal kinase (JNK), and p38, has been shown with the stimulatory effects on steroidogenesis.[9,11] However, other investigations have demonstrated the inhibitory effects of MAPKs on steroidogenesis.[12] Studies also show that the PKC signaling pathway can be activated by diacylglyceride (DAG), phospholipase C (PLC), and Ca^{2+} to regulate Leydig cell steroidogenesis.[13]

We have previously demonstrated that cordycepin could not induce *StAR* promoter and StAR protein expressions in MA-10 cells,[7] and this phenomenon was confirmed again in the present study. Thus, it is possible that cordycepin might activate MAPK and/or PKC pathways without activating the PKA/StAR protein cascade to induce steroidogenesis in MA-10 cells. We, therefore, treated MA-10 cells with cordycepin plus various kinase inhibitors and used radioimmunoassay and Western blotting to determine which pathway would be activated. Moreover, we have found that cordycpin could induce MA-10 cell apoptosis through the activation of adenosine receptor and caspase cascade.[7,14] However, the intracellular signal transduction pathway upstream of the caspase cascade mediated by cordycepin in MA-10 cells remains unclear. Thus, an MTT cell viability assay was performed with the treatment of cordycepin

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plus various kinase inhibitors to elucidate the possible apoptotic mechanism.

Some studies have demonstrated that progesterone has the anticancer activity in breast cancer cell lines.[15] Other studies also demonstrate that progesterone could up-regulate p53 gene expression and cause ovarian cancer apoptosis.[16] It is possible that progesterone, produced from cordycepin-activated MA-10 cells, might induce MA-10 cell death. To demonstrate whether tumor cell death effect is actually induced by cordycepin but not progesterone, different concentrations of progesterone were used to treat MA-10 cells to confirm the exact action of cordycepin on MA-10 cell death phenomenon.

MATERIALS AND METHODS

Chemicals. Cordycepin, H89, GF109203X, wortmannin, U73122, U0126, SP600125, SB202190, PD98059, sodium orthovanadate, Waymouth MB 752/1 medium, bovine serum albumin (BSA), chorionic gonadotropin from human (hCG) (potency = 10000 IU/g), and 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma Chemical (St. Louis, MO, USA). Donkey anti-rabbit and anti-mouse IgG conjugated with horseradish peroxidase were purchased from Amersham International (Arlington Heights, IL, USA). Antibody against β-actin, phospho-PKC (pan) $_{(\gamma Thr514)}$, ERK1/2, phospho-ERK1/2$_{(Thr202/Tyr204)}$, JNK/SAPK, phospho-JNK/SAPK$_{(Thr183/Tyr185)}$, p38 MAP kinase, and phospho-p38$_{(Thr180/Tyr182)}$ MAPK were purchased from Cell Signaling (Beverly, MA, USA). Antibody against StAR was a generous gift from Dr. Strauss (University of Pennsylvania Medical Center, Philadelphia, PA, USA). Progesterone [1,2,6,7-^{3} (N)]- (90−115 Ci/mmol or 3.33−4.255 TBq/mmol) used for radioimmunoassay was purchased from PerkinElmer Inc. (Boston, MA, USA). Antiserum to progesterone was a kind gift from Dr. Paulus S. Wang (National Yang Ming University, Taipei, Taiwan, Republic of China).

Cell Culture. MA-10 mouse Leydig tumor cells were a gift from Dr. Mario Ascoli (Department of Pharmacology, University of Iowa, Iowa City, IA, USA). MA-10 cells were grown in modified Waymouth's MB 752/1 medium containing 24 mM Hepes, 1.12 g/L $NaHCO_3$ supplemented with 10% fetal bovine serum, and 0.04 g/L gentamicin sulfate. Cells were maintained at 37 °C in a humidified environment containing 95% air and 5% CO_2 for all of the following experiments. Cells with a starting density of 2×10^4 cells per well were plated onto 96-well plates and grown to 80−90% confluence over a period of approximately 24 h. After the incubation time, media were collected and stored at −20 °C until assayed for progesterone by radioimmunoassay (RIA), and cells were lysed with lysis buffer (50 mM Tris base, 150 mM NaCl, 1% NP40, 0.1% SDS, and 5% deoxychloride acid) containing 1% protease inhibitor cocktail and then saved for protein quantification.

Morphological Study. MA-10 cells were seeded at a concentration of 6×10^5 cells in a 6 cm Petri dish (Techno Plastic Products AG, Trasadingen, Switzerland) supplemented with 2 mL of serum medium. After reaching 70−80% confluence, cells were treated without or with 100 μM cordycepin and different concentrations of progesterone for 12 h, respectively. Cell morphology was then observed and recorded under a light microscope (Olympus CK40, Hamburg, Germany). Apoptosis was characterized by the loss of cellular contact with the matrix and the appearance of plasma membrane blebbing.[7]

RIA. Media from cell cultures with different treatments were harvested. Twenty microliters of sample was loaded into a glass tube, and 100 μL each of progesterone antiserum and ^{3}H progesterone were loaded in at 37 °C for 30 min. The reaction was stopped by putting the tubes in ice for 3 min. Charcoal solution (100 μL at 2.5 g/L PBS) was added into the tubes at 4 °C for 15 min and centrifuged for 10 min to spin down the charcoal−^{3}H progesterone complex.[17] Approximately 250 μL of the supernatant was poured into 2 mL of scintillation fluid, and samples were counted in a β-counter for 2 min.

MTT Cell Viability Assay. Methylthiazoletetrazolium assay was employed to determine cell viability with the treatment of cordycepin. MA-10 cells were seeded in a 96-well plate (Techno Plastic Products AG) with 1×10^4 cells in 100 μL of serum medium in each well. After reaching 70−80% confluence, cells were treated with cordycepin and/or various kinase inhibitors. MTT was added with a final concentration of 0.5 mg/mL and then incubated for 4 h at 37 °C. The medium was removed, and DMSO (100 μL) was added into each well to dissolve the crystals by shaking the plate gently for 20 min in the dark. The absorbance (optical density, OD) values in each treatment were then determined at λ = 570 nm by an ELISA Microplate Reader (VersaMax, Molecular Devices Corp., Sunnyvale, CA, USA). All dosages of protein kinase inhibitors used in the present studies were tested, showing no effect on cell viability of MA-10 cells.[7]

Immunoblot Analysis. MA-10 cells (2.5×10^5) were cultured in a 3.5 cm dish. After reaching 70−80% confluence, cells were treated with cordycepin and/or various kinase inhibitors. After treatments, cells were rinsed with cold PBS and harvested by using 30 μL of lysis buffer (50 mM Tris-base, 150 mM NaCl, 1% w/v NP40, 0.1% w/v SDS, 0.5% v/v deoxychloride acid, and 1 mM PMSF). The cell lysate was subjected to centrifugation at 12000g for 20 min at 4 °C. The supernatant, which contained cell protein, was collected and stored at −20 °C until use. The protein concentration of the crude cell lysate was determined according to the Lowry method. Immunoblot analysis was performed as previously described.[6,17] In brief, total protein was solubilized in 1× SDS sample buffer and loaded on a 12.5% SDS-PAGE. Electrophoresis was performed in SDS-PAGE running buffer (24 mM Tris-HCl, 0.19 M glycine, 0.5% SD, pH 8.3). The proteins were transferred to polyvinylidence difluoride (PVDF) membranes in transfer buffer (20 mM Tris-HCl, 150 mM glycine, 10% methanol, 0.01% SDS). The PVDF membrane with transferred protein was soaked in 100% methanol for 10 s and air-dried for 30 min for blocking and then incubated in freshly prepared 5% skimmed milk containing the primary antibodies, including anti-StAR, anti-p38 MAPK, antiphospho-p38 MAPK, anti-ERK1/2, antiphospho-ERK1/2, anti-JNK/SAPK, and antiphospho-JNK/SAPK, for 16−18 h at 4 °C. After three washings with PBS containing 0.5% Tween-20 for 30 min, the target protein signal in PVDF membrane was detected with 1:4000 dilution of horseradish peroxidase-conjugated secondary antibody and then visualized by enhanced chemiluminescence (ECL) detection kit (PerkinElmer Inc.). Proteins of interest were quantitated by UVP EC3 (UVP, Upland, CA, USA) computer-assisted image analysis system with Labworks software version 4.6 (UVP). The amount of β-actin (43 kDa) in each lane was also detected as a control to normalize the expression of StAR, p38, ERK1/2, and JNK proteins.

Statistical Analysis. All data were expressed as the mean ± SEM of at least three independent experiments. Statistically significant differences between control and treatments were determined by one-way analysis of variance (ANOVA) and then the least significant difference (LSD) analysis. Statistical significance was set at $p < 0.05$.

RESULTS

Time and Dose Effects of Cordycepin on Progesterone Production in MA-10 Cells. To test the hypothesis that cordycepin could induce Leydig cell steroidogenesis, MA-10 mouse Leydig tumor cells were incubated with different dosages of cordycepin (1 nM−100 μM) for 0, 3, 6, 12, and 24 h, respectively. Figure 1A demonstrates that MA-10 cell steroidogenesis was significantly induced by 100 μM cordycepin after 12 and 24 h treatments, respectively ($p < 0.05$). The progesterone production induced by 100 μM cordycepin was 3-fold higher compared to control (46.2 ± 9.8 vs 196.7 ± 39.2 pg/μg protein and 52.1 ± 3.3 vs 167.6 ± 20.6 pg/μg protein for 12 and 24 h treatments, respectively; $p < 0.01$). To determine the optimal concentration of cordycepin inducing Leydig cell steroidogenesis within 12 h, the concentrations were further subdivided from 10 μM to 1 mM. As Figure 1B illustrates, the progesterone production

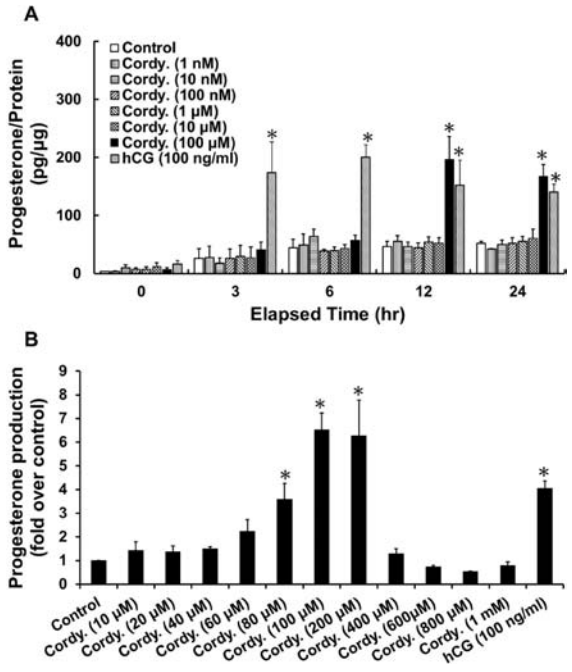

Figure 1. Time and dose effect of cordycepin on progesterone production in MA-10 cells. Cells were treated without (control) or with different dosages of cordycepin (1 nM−100 μM) for 0−24 h (A) or with different dosages of cordycepin (10 μM−1 mM) for 12 h (B). The treatment of hCG (100 ng/mL) was the positive control. Media were collected and assayed for progesterone production by RIA. Each column represents the mean ± SEM of three independent experiments in triplicate of each treatment. ∗ above the bar indicates significant difference compared to control in each specific time treatment (p < 0.05).

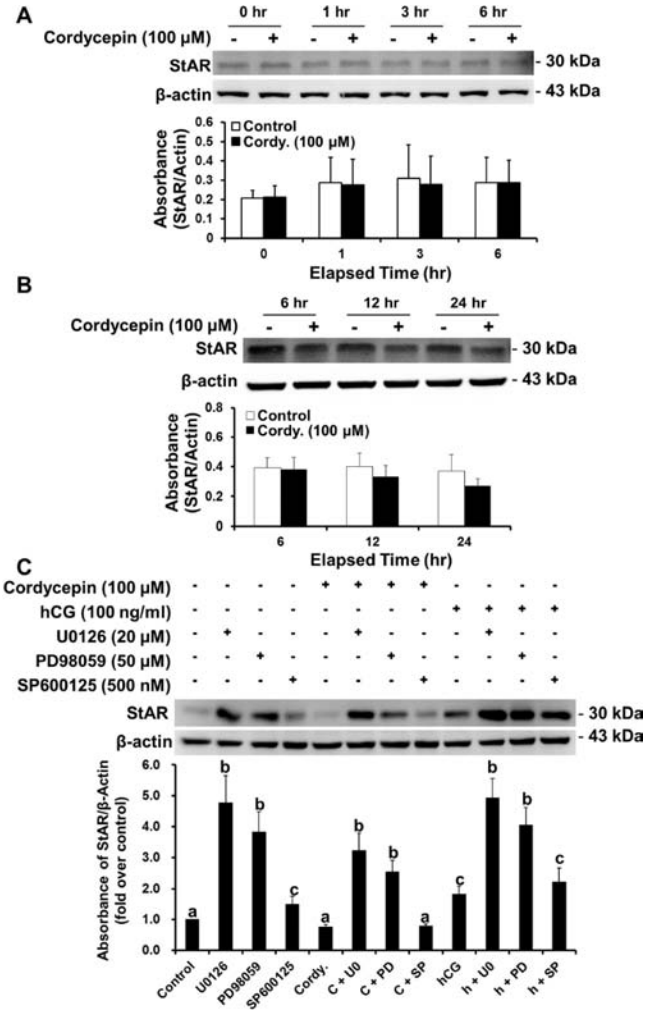

Figure 2. Effect of MEK1/2, ERK1/2, and JNK inhibitors with or without cordycepin and hCG on StAR protein expression in MA-10 cells. MA-10 cells were treated without (control) or with 100 μM cordycepin alone for 0, 1, 3, and 6 h (A) or for 6, 12, and 24 h (B), respectively. In addition, panel C illustrates that MA-10 cells were preincubated with U0126 (MEK1/2 inhibitor, 20 μM; U0), PD98059 (ERK1/2 inhibitor, 50 μM; PD), or SP600125 (JNK inhibitor, 500 nM; SP) for 30 min and then treated without (control) or with 100 μM cordycepin and 100 ng/mL hCG, respectively, in the presence of specific inhibitor for 12 h. StAR protein specific bands were then detected by Western blotting. Each upper panel illustrates a representative Western blot of MA-10 cell StAR protein expression in response to cordycepin treatment, which was repeated at least three times. The integrated optical densities of StAR protein (30 kDa) after normalization with β-actin (43 kDa) in each lane using Labworks imaging software are demonstrated in the lower panels. Each column represents the mean ± SEM of three independent experiments. Different letters (a−c) above the bar indicate the significant differences of StAR protein expression level among each treatment (p < 0.05) (C, Cordy. = cordycepin).

gradually elevated as cordycepin concentration increased (60−200 μM) at 12 h in MA-10 cells. Once the cordycepin concentration increased to 400 μM, the stimulatory effects were reduced to control level. According to the above results, 100 μM cordycepin with a 12 h treatment was then used to investigate the mechanism regarding the cellular signal transduction pathway.

Effects of Cordycepin on StAR Protein Expression in MA-10 Cells. To investigate whether cordycepin would regulate StAR protein expression, MA-10 cells were incubated in the presence or absence of cordycepin (100 μM) for different time durations (0, 1, 3, 6, 12, and 24 h). Cell lysate was collected and StAR protein expression was investigated by immunoblot analysis. Data showed that cordycepin could not increase StAR protein expression under 0−6 h (Figure 2A) or 6−24 h (Figure 2B) treatments (p > 0.05). However, inhibiting MEK1/2 expression by PD98059 and U0126 (Figure 2C) could significantly induce 5-fold higher StAR protein expression compared to control in MA-10 cells (p < 0.05). Furthermore, when cells were preincubated with these MEK inhibitors prior to the addition of cordycepin, there was an elevation in the production of StAR protein compared to cordycepin alone treatment (Figure 2C). Similar results were obtained where PD98059 and U0126 could dramatically increase hCG-induced StAR protein expression (p < 0.05; Figure 2C). Thus, the ERK cascade may negatively regulate steroidogenesis, and this can be explained by the attenuation of StAR protein expression in MA-10 cells.

Effects of PKA, PI3K, PKC, PLC, and IP_3-Ca^{2+} Protein Kinase Inhibitors on Cordycepin-Activated Progesterone Production in MA-10 Cells. Selective protein kinase inhibitors were used to determine the possible signal transduction pathway activating Leydig cell steroidogenesis. Cells were preincubated with H89 (PKA inhibitor), wortmannin (PI3K inhibitor), GF109203X (PKC inhibitor), U73122 (PLC inhibitor), and sodium orthovanadate (IP^3-induced Ca^{2+}

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release inhibitor) for 30 min and then cotreated with cordycepin (100 μM) for another 12 h. Results elucidated that GF109203X (1 nM−1 μM; Figure 3A), U73122 (5−40 μM; Figure 3B), and sodium orthovanadate (10−40 μM; Figure 3C) alone had no effect on cordycepin-activated progesterone production in MA-10 cells ($p > 0.05$). Interestingly, GF109203X (1 μM) significantly inhibited cordycepin-activated progesterone production about 79% in MA-10 cells ($p < 0.05$; Figure 3A). Similar inhibitory effects were observed after U73122 (20 μM; 96.7% decrease) or sodium orthovanadate (40 μM; 67.6% decrease) co-incubation with cordycepin for 12 h of treatment (Figure 3, panels B and C, respectively). H89 and wortmannin did not inhibit cordycepin-activated steroidogenesis after 12 h of treatment ($p > 0.05$; data not shown). Here, we demonstrated that PLC/PKC pathways were involved in the steroidogenic effect of cordycepin in MA-10 cells. The intracellular IP_3−Ca^{2+} channel could also participate in the stimulatory effect induced by cordycepin in MA-10 cells.

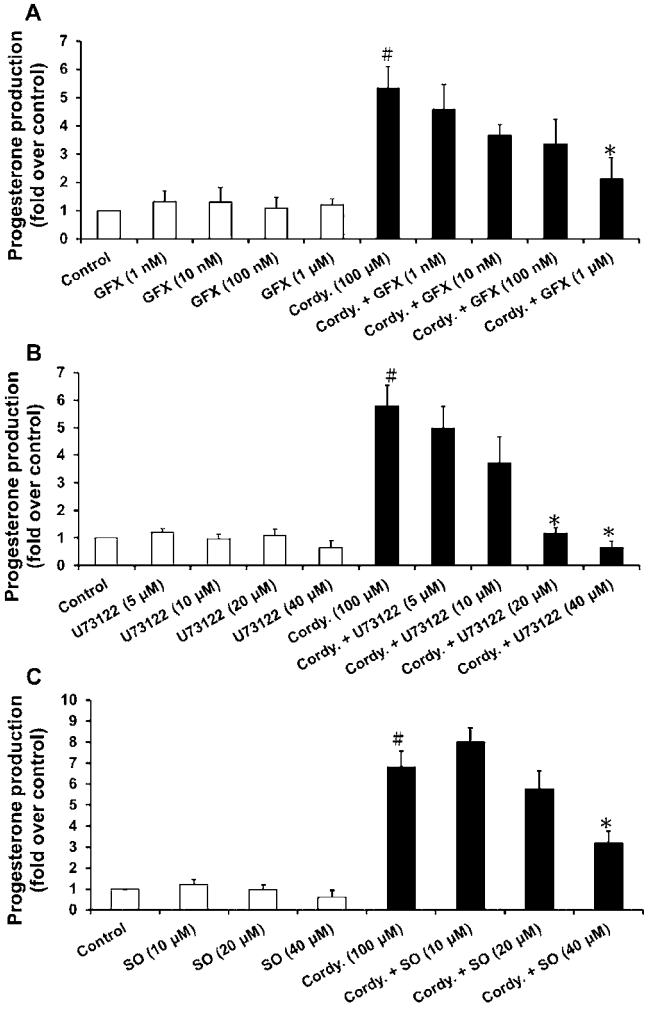

Figure 3. Dose effect of GF109203X, U73122, and sodium orthovanadate on cordycepin-activated progesterone production in MA-10 cells. Cells were preincubated with GF109203X (PKC inhibitor, 1 nM−2 μM; GFX) (A), U73122 (PLC inhibitor, 5 μM∼40 μM) (B), and sodium orthovanadate (IP^3-induced Ca^{2+} release inhibitor, 10−40 μM; SO) (C) for 30 min and then treated without (control) or with 100 μM cordycepin in the presence or absence of inhibitors for 12 h. Media were collected and assayed for progesterone production by RIA. Each column represents the mean ± SEM of three experiments in triplicate of each treatment. # above the bar indicates significant difference between control and cordycepin treatment ($p < 0.05$). ∗ above the bar indicates significant difference between cordycepin and cotreatment groups ($p < 0.05$).

Effects of MAPK Kinase Inhibitors on Cordycepin-Activated Progesterone Production in MA-10 Cells. The mitogen-activated protein kinases (MAPKs) signaling cascade has been implicated in the regulation of steroidogenesis.[18] Therefore, the involvement of MAPKs in cordycepin-activated steroidogenesis was also investigated in the present study. Cells were preincubated with SB202190 (p38 inhibitor), SP600125 (JNK inhibitor), or U0126 (MEK1/2 inhibitor) for 30 min and then co-incubated with cordycepin (100 μM) for another 12 h. As shown in Figure 4A, SB202190 (1−25 μM) did not show

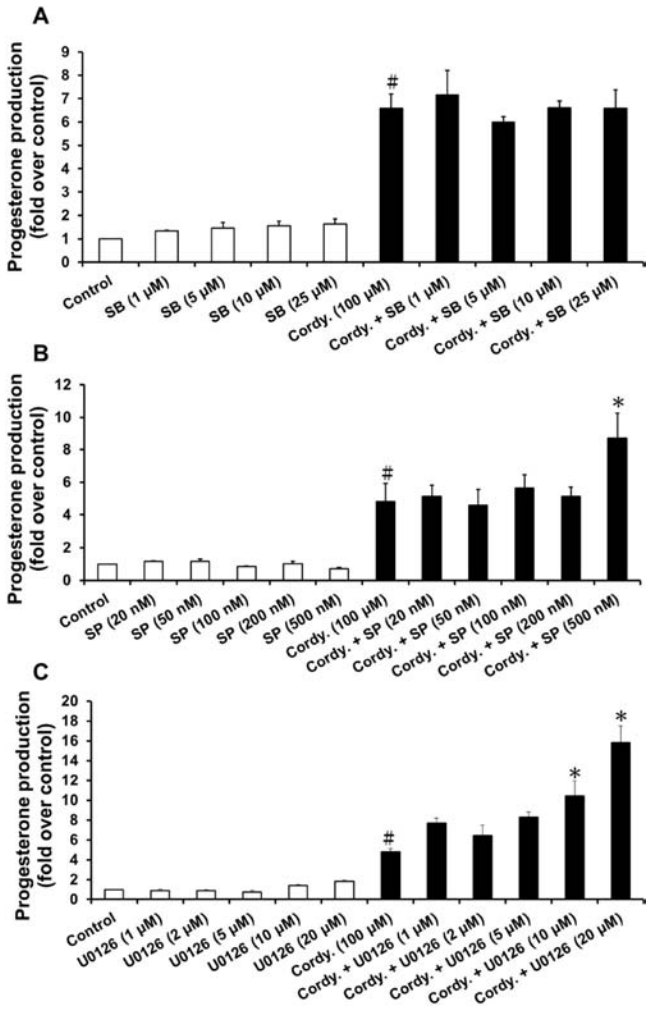

Figure 4. Dose effects of SB202190, SP600125, and U0126 on cordycepin-activated progesterone production in MA-10 cells. Cells were preincubated with SB202190 (p38 inhibitor, 1−25 μM; SB) (A), SP600125 (JNK inhibitor, 20−500 nM; SP) (B), and U0126 (MEK1/2 inhibitor, 1−20 μM) (C) for 30 min and then treated without (control) or with 100 μM cordycepin in the presence or absence of inhibitors for 12 h. Media were collected and assayed for progesterone production by RIA. Each column represents the mean ± SEM of three experiments in triplicate of each treatment. # above the bar indicates significant difference between control and cordycepin treatment ($p < 0.05$). ∗ above the bar indicates significant difference between cordycepin and cotreatment groups ($p < 0.05$).

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any inhibitory effect on cordycepin-induced steroidogenesis after 12 h of incubation ($p > 0.05$). In addition, JNK inhibitor (SP600125; 20−500 nM) had no effect by itself on progesterone production in MA-10 cells (Figure 4B). However, when cells were incubated with SP600125 (500 nM) for 30 min prior to cordycepin induction, there was a 3-fold ($p < 0.05$) increase in cordycepin-activated progesterone production (Figure 4B). Similar to the JNK inhibitor, U0126 (10−20 μM) significantly increased 2−3-fold ($p < 0.05$) cordycepin-activated progesterone production in MA-10 cells (Figure 4C). Thus, in MA-10 cells, cordycepin caused a significant elevation of progesterone production after 12 h, which was dramatically amplified by the addition of SP600125 and U0126. Taken together, we demonstrated that the involvement of MAPKs, especially JNK and ERK1/2, may play inhibitory roles in cordycepin-induced steroidogenesis in MA-10 cells.

Effect of Cordycepin on p38, JNK, and ERK1/2 Protein Expressions and PKC Phosphorylation in MA-10 Cells. The expression of MAPKs and their phosphorylation forms were determined by Western blotting. The changes of p38 protein phosphorylation could not be detected after cordycepin treatment for 5−60 min ($p > 0.05$; Figure 5A). Conversely, the phosphorylation of JNK (Figure 5B), ERK1/2 (Figure 5C), and PKC (Figure 6A) could be significantly detected after cordycepin treatment for 15−30 min ($p < 0.05$). Moreover, the blocking of PKC by GF109203X could further increase the phosphorylation of ERK1/2 about 3-fold compared to cordycepin alone treatment (Figure 6) ($p < 0.05$), suggesting that PKC might decrease ERK1/2 phosphorylation to regulate intracellular protein expression and then regulate Leydig cell steroidogenesis. Taken together, these results showed that StAR protein might not play a major role in regulating the cordycepin-activated steroidogenesis. However, PKC and ERK1/2 phosphorylations may contribute to the stimulatory and inhibitory effects in MA-10 cell steroidogenesis, respectively. Figure 6B also demonstrated that a MEK1/2 inhibitor, U0126, did suppress ERK1/2 phosphorylation with cordycepin and hCG treatments, respectively, as a negative control in MA-10 cells (Figure 6B) ($p < 0.05$).

Effect of Cordycepin and Different Protein Kinase Inhibitors on Cell Viability in MA-10 Cells. We have demonstrated that cordycepin has antitumor activity in OEC-M1 esophageal carcinoma and MA-10 mouse Leydig tumor cell lines.[19,20] In this study, we again observed the effect that cordycepin decreased MA-10 cell viability in dose-dependent (10 nM−1 mM) and time-dependent (0, 1, 3, 6, 12, and 24 h) relationships. Figure 7A illustrates that 100 μM cordycepin significantly decreased MA-10 cell viability to 83, 63, and 41% after 6, 12, and 24 h of treatment, respectively ($p < 0.001$). With higher concentration of cordycepin (1 mM), MA-10 cell death could be observed as early as in 3 h of treatment, and the MA-10 cell viability decreased to 77, 65, 36, and 19% after 3, 6, 12, and 24 h of treatment, respectively ($p < 0.001$). These results confirmed the tumor cell death effect of cordycepin on MA-10 mouse Leydig tumor cells. We illustrated that the PLC/IP_3−Ca^{2+}/PKC pathway was involved in the steroidogenic effect of cordycepin in MA-10 cells. It is possible that PKC and other related signal transduction pathways were involved to induce MA-10 cell apoptosis by cordycepin. Thus, we used different protein kinase inhibitors with cordycepin treatment by MTT assay to examine which specific signal pathway would be activated to affect MA-10 cell viability. Figure 7B showed that cordycepin (100 μM) alone significantly reduced MA-10 cell viability to 68% after 12 h treatment ($p < 0.05$). With the H89, U0126, wortmannin, and U73122 plus cordycepin cotreatment, cell viability were further decreased to 33, 46, 48, and 48%, respectively ($p < 0.05$). However, PKC inhibitor and cordycepin cotreatment significantly rescued 14% MA-10 cell death when compared to cordycepin alone treatment ($p < 0.05$). These results suggested that the PKC pathway activated by cordycepin might be important to the tumor cell death effect, and PKA, PI3K, and MAPK pathways might have protective effects in MA-10 mouse Leydig tumor cells.

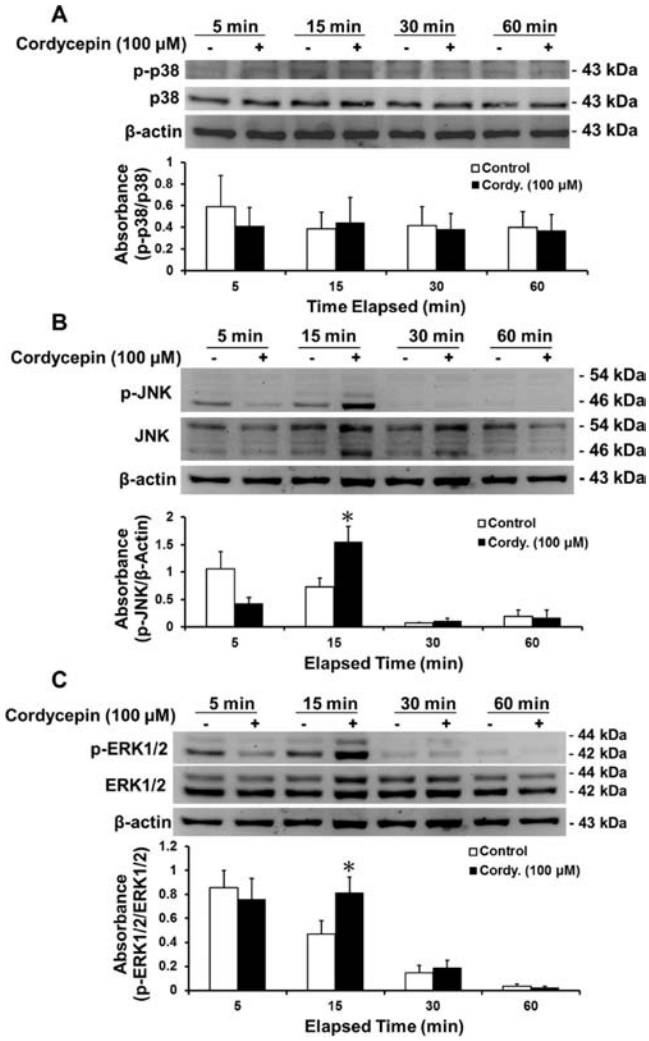

Figure 5. Time course effect of cordycepin on phospho-p38, phosphor-JNK, and phospho-ERK1/2 protein expressions in MA-10 cells. After serum starvation for 12 h, cells were incubated in the presence or absence of 100 μM cordycepin for different times (5, 15, 30, and 60 min). phospho-p38 (A), phospho-JNK (B), and phospho-ERK1/2 (C) protein specific bands were then detected by Western blotting. Each upper panel illustrates a representative Western blot of MA-10 cell protein expression in response to cordycepin treatment. Immunoblot represents the observations from a single experiment repeated three times. The integrated optical densities of phospho-p38, phospho-JNK, and phospho-ERK1/2 after normalization with β-actin (43 kDa) in each lane using Labworks imaging software are demonstrated in the lower panels. Each column represents the mean ± SEM of three independent experiments. ∗ above the bar indicates the significant differences between control and treatment in each specific time point ($p < 0.05$).

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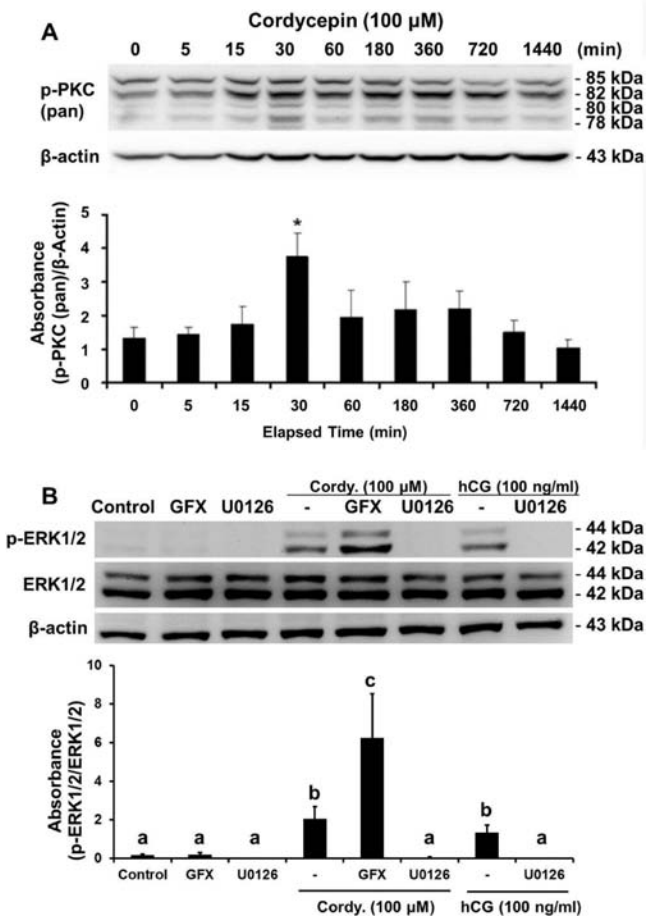

Figure 6. Effect of cordycepin on phosphorylation of PKC, p38, JNK, and ERK1/2 protein expressions in MA-10 cells. After serum starvation for 12 h, cells were incubated in the presence of 100 μM cordycepin for different times (0−1440 min) or preincubated with GF109203X (PKC inhibitor, 1 μM; GFX) or U0126 (MEK1/2 inhibitor, 20 μM) for 30 min and then treated without (control) or with 100 μM cordycepin or 100 ng/mL hCG in the presence or absence of inhibitors for 15 min. Phospho-PKC (A) and phospho-ERK1/2 (B) protein specific bands were then detected by Western blotting. Each upper panel illustrates a representative Western blot of MA-10 cell protein expression in response to cordycepin treatment. Immunoblot represents the observations from a single experiment repeated three times. The integrated optical densities of phospho-PKC and phospho-ERK1/2 after normalization with β-actin (43 kDa) in each lane using Labworks imaging software are demonstrated in the lower panels. Each column represents the mean ± SEM of three independent experiments. ∗ above the bar (A) indicates the significant differences between control and treatment in each specific time point ($p < 0.05$). Different letters (a−c) above the bar (B) indicate the significant differences among each treatment ($p < 0.05$).

Effect of Cordycepin and Progesterone on Cell Viability in MA-10 Cells. Some studies have demonstrated that progesterone may have antitumor activity in specific tumor cell lines.[16,21] It is possible that progesterone, activated by cordycepin, could induce MA-10 cell death. To confirm that the cordycepin, but not progesterone, did directly induce MA-10 cell death, morphological change (Figure 8A−I) and MTT cell viability assay (Figure 8J) were used to examine the cell death effect of cordycepin or progesterone in MA-10 cells. Because cordycepin could maximally induce MA-10 cells to produce 5 ng/mL progesterone in 2×10^4 cells for 12 h of treatment in

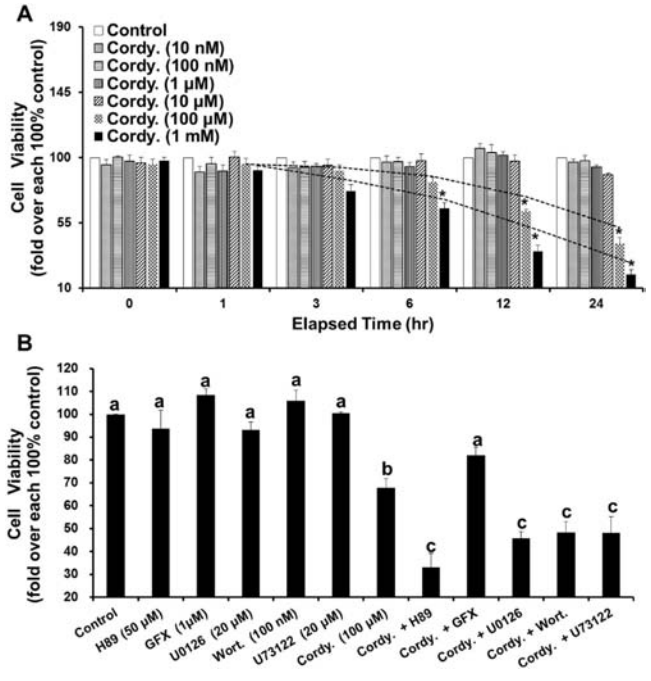

Figure 7. Effect of cordycepin and different protein kinase inhibitors on cell viability in MA-10 cells. Cells were treated without (control) or with different concentrations of cordycepin (10 nM−100 μM) for 0, 1, 3, 6, 12, and 24 h, respectively (A). Or cells were preincubated with H89 (50 μM), GF109203X (1 μM; GFX), U0126 (20 μM), wortmannin (100 nM; Wort.), and U73122 (20 μM) for 30 min and then treated without (control) or with cordycepin (100 μM; Cordy.) in the presence or absence of inhibitors for 12 h (B). Cell viability was quantified by MTT assay. Results are expressed as percentages of cell growth relative to initial number of viable cells in controls (as 100%). Data represent the mean ± SEM of four separate experiments. ∗ above the bar represents significant difference to the control (plain medium) in each specific time treatment ($p < 0.05$). The dot-lines illustrate the decreasing trend of MA-10 cell viability among time course effect of cordycepin. Different letters (a−c) above the bar indicate the significant differences between each treatment ($p < 0.05$).

the present study, we treated MA-10 cells with different concentrations of progesterone (0.5, 1, 5, 10, 50, 100, and 500 ng/mL, respectively) to exclude the progesterone antitumor effect. Results illustrated that lower progesterone concentrations at 1 and 5 ng/mL caused MA-10 cells to round-up, but no cell death effect could be observed (Figure 8D,E). The more congregated cells with membrane blebbing and cell shrinkage could only be found when progesterone concentrations were raised to 10, 50, 100, and 500 ng/mL (Figure 8F−I). MTT cell death assay was further exploited, and the results showed that cell viability was significantly decreased to 62% after 10 ng/mL progesterone treatment for 12 h ($p < 0.05$; Figure 8J). The death effects were significantly increased to about 90% with 50, 100, and 500 ng/mL progesterone for 12 h of treatment ($p < 0.05$; Figure 8J). This experiment illustrated that the tumor cell death effect was actually caused by cordycepin instead of progesterone accumulation, because 5 ng/mL progesterone did not induce MA-10 cell death.

DISCUSSION

In the present study, we demonstrated the effect of cordycepin on mouse Leydig tumor cells, which reveals two important aspects. First, cordycepin could act through the PLC/PKC

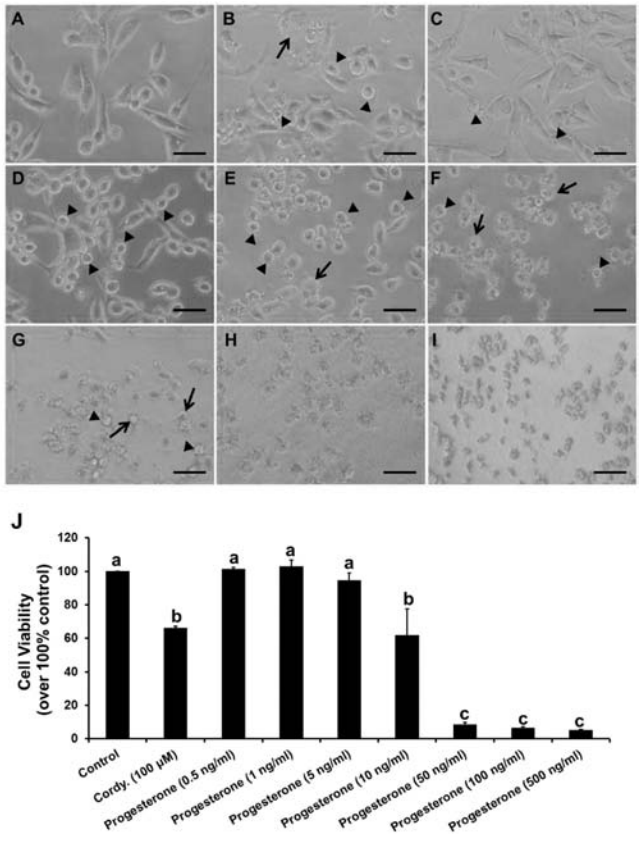

Figure 8. Effect of cordycepin and progesterone on morphological changes and cell viability in MA-10 cells. Cells were treated with plain medium (control) (A), cordycepin (100 μM) (B), 0.5 ng/mL progesterone (C), 1 ng/mL progesterone (D), 5 ng/mL progesterone (E), 10 ng/mL progesterone (F), 50 ng/mL progesterone (G), 100 ng/mL progesterone (H), or 500 ng/mL progesterone (I) for 12 h. Cell morphology was observed and recorded under light microscopy at 500× magnification (bar = 20 μm). Arrowhead (▲) and arrow (→) indicate congregated cells and membrane-blebbed cells, respectively. In addition, cells were treated without (control) or with cordycepin (100 μM; Cordy.) and progesterone (0.5−500 ng/mL) for 12 h, respectively, and cell viability was quantified by MTT assay. Results are expressed as percentages of cell growth relative to initial number of viable cells in controls (as 100%) (J). Data represent the mean ± SEM of four separate experiments. Different letters (a−c) above the bar indicate the significant differences between each treatment ($p < 0.05$).

pathway to increase mouse Leydig tumor cell steroidogenesis. Second, cordycepin could cause MA-10 mouse Leydig tumor cell death, which indicates that it might be an effective source for the future Leydig cell tumor study. We used different protein kinase inhibitors to determine the possible signal transduction pathway activated by cordycepin on steroidogenesis in MA-10 cells. Data illustrated that cordycepin might go through the PLC/PKC pathway to activate Leydig cell steroidogenesis. We also demonstrated that MAPK pathways, especially ERK1/2 and JNK, might negatively regulate cordycepin-activated Leydig cell steroidogenesis, which could not suppress the positive effect of PKC pathway. Furthermore, different protein kinase inhibitors were used to determine the possible signal transduction pathway activated by cordycepin on apoptosis in MA-10 cells. We found that cordycepin would also activate the PKC pathway to induce apoptosis in MA-10 cells. These results highly suggested that cordycepin could activate the PKC pathway to simultaneously induce steroidogenesis and apoptosis in MA-10 cells.

There are four subtypes of adenosine receptors (A1, A2a, A2b, and A3), which all consist of the seven transmembrane domains and interact with G proteins.[22] Adenosine A1 and A3 receptors are Gi protein-coupled cell membrane receptors, and their stimulation would inhibit adenylate cyclase (AC) activity and activate phospholipase C, whereas A2a and A2b receptors are Gs protein-coupled receptors, and their stimulation could induce AC activity with an increase of intracellular cAMP.[23] Because cordycepin possesses a structure similar to that of adenosine, it is highly possible that cordycepin could also stimulate Leydig cells through adenosine receptors. This notion has been confirmed by a binding assay in mouse melanoma cells that cordycepin could bind to adenosine A3 receptors and then act through the Wnt pathway to inhibit the proliferation of B16-BL6 cells.[24]

In fact, we have previously demonstrated that cordycepin could induce steroidogenesis through A1, A2a, and A3 adenosine subtype receptors in primary mouse Leydig cells and through all four adenosine subtype receptors in MA-10 cells.[6,7] Here, we further demonstrated the stimulatory effect of cordycepin in MA-10 cells, which was mediated by PLC/PKC signal transduction pathway without increasing StAR protein expression. In fact, some evidence has shown that steroidogenic effect could be triggered by cAMP- and StAR-independent pathways in Leydig cells.[25,26] Other studies have also shown that activation of the PLC/PKC signal transduction pathway would further induce steroidogenesis.[27] Thus, our finding is not unprecedented, which indicates that the PKC signal pathway is also important in regulating Leydig cell functions. Our results showed the importance of PLC/PKC pathways in Leydig cell steroidogenesis activated by cordycepin.

It is well-known that MAP kinases are involved in the regulation of many important biological functions, including cell proliferation, differentiation, and apoptosis, as well as in carcinogenesis.[28] Interestingly, some papers in the literature have demonstrated that MAP kinases could also regulate Leydig cell steroidogenesis.[29,30] However, that the ERK cascade participates in the regulation of steroidogenesis appears to be contradictory with stimulatory and inhibitory effects.[31,32] In the present study, we did find that JNK and ERK1/2, but not p38, might play inhibitory roles on cordycepin-induced steroidogenesis. We found that the blocking of PKC by GF109203X could further increase the phosphorylation of ERK1/2, suggesting that PKC might decrease ERK1/2 expression to up-regulate intracellular protein expression and then to stimulate MA-10 cell steroidogenesis. In this regard, it seemed that PKC was upstream to ERK1/2 under cordycepin regulation in MA-10 cells. It is also possible that both pathways were simultaneously activated by cordycepin. Thus, it will be worthwhile to uncover the relationships between both pathways regulated by cordycepin in MA-10 mouse Leydig tumor cells.

We have illustrated that CS induced MA-10 cell apoptosis through caspase-8 protein activation.[14] Also, the apoptotic effect could be observed with cordycepin treatment in MA-10 cells through caspase-9, -3, and -7 protein activation.[20] Moreover, cordycepin could activate adenosine subtype receptors to induce apoptosis in MA-10 cells.[7] In this study, we again confirmed the apoptotic phenomenon mediated by cordycepin in MA-10 cell death. Furthermore, the signal transduction pathway activated by cordycepin to induce MA-10

cell death was investigated with different protein kinase inhibitors, and we found that the PKC pathway could be stimulated to induce MA-10 cell apoptosis. Studies have shown that the activation of PKC signal transduction pathway could induce the cell differentiation in hepatocellular carcinoma cells and ovarian cancer cells and the cell apoptosis in human promyelocytic leukemia cells and colorectal cancer cells.[33−36] It is highly possible that cordycepin could also activate the PKC signal pathway to induce MA-10 cell apoptosis and/or differentiation, and we found that the activation of PKC by cordycepin only induced MA-10 cell apoptosis. Thus, our finding is comparable to those studies. However, our results showed that PKC inhibitor could only prevent 14% cell death, suggesting that other pathways, besides PKA and PI3K, might be also involved in this event. Further studies will be needed to clarify the detail mechanism.

It has been shown that a high concentration of progesterone could induce apoptosis in breast, ovarian, and endometrial cancer cells.[37,38] In the present study, progesterone production was induced by cordycepin, and it is possible that cordycepin-induced progesterone could provoke apoptosis in MA-10 cells. We did demonstrate that progesterone in high concentrations (10−500 ng/mL) caused MA-10 cell death. However, such a high concentration of progesterone could not be produced by MA-10 cells activated by cordycepin (the maximal production is 5 ng/mL). Thus, our results highly suggest that cordycepin has direct antitumor activity in MA-10 cells.

Collectively, in this study, we demonstrate that cordycepin has the ability to induce Leydig cell steroidogenesis through PLC/PKC and MAPK signal pathways. Furthermore, the stimulatory effect of PKC and the inhibitory effect of ERK1/2 pathways in cordycepin-activated Leydig cell steroidogenesis might interact for the homeostasis status in regulating steroidogenesis. Besides, cordycepin has a direct antitumor effect in MA-10 cells through the activation of the PKC pathway.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Cunningham, K. G.; Manson, W.; Spring, F. S.; Hutchinson, S. A. Cordycepin, a metabolic product isolated from cultures of *Cordyceps militaris* (Linn.) Link. *Nature* **1950**, *166*, 949.

(2) Zeevi, M.; Nevins, J. R.; Darnell, J. E., Jr. Newly formed mRNA lacking polyadenylic acid enters the cytoplasm and the polyribosomes but has a shorter half-life in the absence of polyadenylic acid. *Mol. Cell. Biol.* **1982**, *2*, 517−525.

(3) Liu, C.; Lu, S.; Ji, M. R. Effects of *Cordyceps sinensis* (CS) on in vitro natural killer cells. *Zhongguo Zhong Xi Yi Jie He Za Zhi* **1992**, *12*, 267−269.

(4) Yamaguchi, Y.; Kagota, S.; Nakamura, K.; Shinozuka, K.; Kunitomo, M. Antioxidant activity of the extracts from fruiting bodies of cultured *Cordyceps sinensis*. *Phytother. Res.* **2000**, *14*, 647−649.

(5) Chen, Y. J.; Shiao, M. S.; Lee, S. S.; Wang, S. Y. Effect of *Cordyceps sinensis* on the proliferation and differentiation of human leukemic U937 cells. *Life Sci.* **1997**, *60*, 2349−2359.

(6) Leu, S. F.; Poon, S. L.; Pao, H. Y.; Huang, B. M. The in vivo and in vitro stimulatory effects of cordycepin on mouse Leydig cell steroidogenesis. *Biosci., Biotechnol., Biochem.* **2011**, *75*, 723−731.

(7) Pan, B. S.; Lin, C. Y.; Huang, B. M. The effect of cordycepin on steroidogenesis and apoptosis in MA-10 mouse Leydig tumor cells. *Evidence Based Complement. Alternat. Med.* **2011**, on-line.

(8) Dyson, M. T.; Kowalewski, M. P.; Manna, P. R.; Stocco, D. M. The differential regulation of steroidogenic acute regulatory protein-mediated steroidogenesis by type I and type II PKA in MA-10 cells. *Mol. Cell. Endocrinol.* **2009**, *300*, 94−103.

(9) Poderoso, C.; Maloberti, P.; Duarte, A.; Neuman, I.; Paz, C.; Maciel, F. C.; Podesta, E. J. Hormonal activation of a kinase cascade localized at the mitochondria is required for StAR protein activity. *Mol. Cell. Endocrinol.* **2009**, *300*, 37−42.

(10) Manna, P. R.; Chandrala, S. P.; King, S. R.; Jo, Y.; Counis, R.; Huhtaniemi, I. T.; Stocco, D. M. Molecular mechanisms of insulin-like growth factor-I mediated regulation of the steroidogenic acute regulatory protein in mouse Leydig cells. *Mol. Endocrinol.* **2006**, *20*, 362−378.

(11) Gyles, S. L.; Burns, C. J.; Whitehouse, B. J.; Sugden, D.; Marsh, P. J.; Persaud, S. J.; Jones, P. M. ERKs regulate cyclic AMP-induced steroid synthesis through transcription of the steroidogenic acute regulatory (StAR) gene. *J. Biol. Chem.* **2001**, *276*, 34888−34895.

(12) Seger, R.; Hanoch, T.; Rosenberg, R.; Dantes, A.; Merz, W. E.; Strauss, J. F., 3rd; Amsterdam, A. The ERK signaling cascade inhibits gonadotropin-stimulated steroidogenesis. *J. Biol. Chem.* **2001**, *276*, 13957−13964.

(13) Newton, A. C. Protein kinase C: poised to signal. *Am. J. Physiol. Endocrinol. Metab.* **2010**, *298*, E395−402.

(14) Yang, H. Y.; Leu, S. F.; Wang, Y. K.; Wu, C. S.; Huang, B. M. *Cordyceps sinensis* mycelium induces MA-10 mouse Leydig tumor cell apoptosis by activating the caspase-8 pathway and suppressing the NF-κB pathway. *Arch. Androl.* **2006**, *52*, 103−110.

(15) Wiebe, J. P. Progesterone metabolites in breast cancer. *Endocr. Relat. Cancer* **2006**, *13*, 717−738.

(16) Bu, S. Z.; Yin, D. L.; Ren, X. H.; Jiang, L. Z.; Wu, Z. J.; Gao, Q. R.; Pei, G. Progesterone induces apoptosis and up-regulation of p53 expression in human ovarian carcinoma cell lines. *Cancer* **1997**, *79*, 1944−1950.

(17) Chen, Y. H.; Leu, S. F.; Jen, C. Y.; Huang, B. M. Effects of sesamol on apoptosis and steroidogenesis in MA-10 mouse Leydig tumor cells. *J. Agric. Food Chem.* **2011**, *59*, 9885−9891.

(18) Tamura, M.; Nakagawa, Y.; Shimizu, H.; Yamada, N.; Miyano, T.; Miyazaki, H. Cellular functions of mitogen-activated protein kinases and protein tyrosine phosphatases in ovarian granulosa cells. *J. Reprod. Dev.* **2004**, *50*, 47−55.

(19) Wu, W. C.; Hsiao, J. R.; Lian, Y. Y.; Lin, C. Y.; Huang, B. M. The apoptotic effect of cordycepin on human OEC-M1 oral cancer cell line. *Cancer Chemother. Pharmacol.* **2007**, *60*, 103−111.

(20) Jen, C. Y.; Lin, C. Y.; Leu, S. F.; Huang, B. M. Cordycepin induced MA-10 mouse Leydig tumor cell apoptosis through caspase-9 pathway. *Evidence Based Complement. Alternat. Med.* **2009**, on-line.

(21) Rocha, A.; Azevedo, I.; Soares, R. Progesterone sensitizes breast cancer MCF7 cells to imatinib inhibitory effects. *J. Cell Biochem.* **2008**, *103*, 607−614.

(22) Ralevic, V.; Burnstock, G. Receptors for purines and pyrimidines. *Pharmacol. Rev.* **1998**, *50*, 413−492.

(23) Zhao, Z.; Ravid, S.; Ravid, K. Chromosomal mapping of the mouse A3 adenosine receptor gene, Adora3. *Genomics* **1995**, *30*, 118−119.
(24) Yoshikawa, N.; Yamada, S.; Takeuchi, C.; Kagota, S.; Shinozuka, K.; Kunitomo, M.; Yoshikawa, N.; Yamada, S.; Takeuchi, C.; Kagota, S.; Shinozuka, K.; Kunitomo, M.; Nakamura, K. Cordycepin (3′-deoxyadenosine) inhibits the growth of B16-BL6 mouse melanoma cells through the stimulation of adenosine A3 receptor followed by glycogen synthase kinase-3β activation and cyclin D1 suppression. *Naunyn Schmiedebergs Arch. Pharmacol.* **2008**, *377*, 591−595.
(25) Lukyanenko, Y. O.; Carpenter, A. M.; Brigham, D. E.; Stocco, D. M.; Hutson, J. C. Regulation of Leydig cells through a steroidogenic acute regulatory protein-independent pathway by a lipophilic factor from macrophages. *J. Endocrinol.* **1998**, *158*, 267−275.
(26) King, S. R.; Matassa, A. A.; White, E. K.; Walsh, L. P.; Jo, Y.; Rao, R. M.; Stocco, D. M.; Reyland, M. E. Oxysterols regulate expression of the steroidogenic acute regulatory protein. *J. Mol. Endocrinol.* **2004**, *32*, 507−517.
(27) Morris, J. K.; Richards, J. S. Luteinizing hormone induces prostaglandin endoperoxide synthase-2 and luteinization in vitro by A-kinase and C-kinase pathways. *Endocrinology* **1995**, *136*, 1549−1558.
(28) Lopez-Ilasaca, M. Signaling from G-protein-coupled receptors to mitogen-activated protein (MAP)-kinase cascades. *Biochem. Pharmacol.* **1998**, *56*, 269−277.
(29) Hirakawa, T.; Ascoli, M. The lutropin/choriogonadotropin receptor-induced phosphorylation of the extracellular signal-regulated kinases in Leydig cells is mediated by a protein kinase a-dependent activation of ras. *Mol. Endocrinol.* **2003**, *17*, 2189−2200.
(30) Manna, P. R.; Chandrala, S. P.; King, S. R.; Jo, Y.; Counis, R.; Huhtaniemi, I. T.; Stocco, D. M. Molecular mechanisms of insulin-like growth factor-I mediated regulation of the steroidogenic acute regulatory protein in mouse Leydig cells. *Mol. Endocrinol.* **2006**, *20*, 362−378.
(31) Gyles, S. L.; Burns, C. J.; Whitehouse, B. J.; Sugden, D.; Marsh, P. J.; Persaud, S. J.; Jones, P. M. ERKs regulate cyclic AMP-induced steroid synthesis through transcription of the steroidogenic acute regulatory (StAR) gene. *J. Biol. Chem.* **2001**, *276*, 34888−34895.
(32) Seger, R.; Hanoch, T.; Rosenberg, R.; Dantes, A.; Merz, W. E.; Strauss, J. F., 3rd; Amsterdam, A. The ERK signaling cascade inhibits gonadotropin-stimulated steroidogenesis. *J. Biol. Chem.* **2001**, *276*, 13957−13964.
(33) Hung, J. H.; Lu, Y. S.; Wang, Y. C.; Ma, Y. H.; Wang, D. S.; Kulp, S. K.; Muthusamy, N.; Byrd, J. C.; Cheng, A. L.; Chen, C. S. FTY720 induces apoptosis in hepatocellular carcinoma cells through activation of protein kinase C δ signaling. *Cancer Res.* **2008**, *68*, 1204−1212.
(34) Nazarenko, I.; Jenny, M.; Keil, J.; Gieseler, C.; Weisshaupt, K.; Sehouli, J.; Legewie, S.; Herbst, L.; Weichert, W.; Darb-Esfahani, S.; Dietel, M.; Schafer, R.; Ueberall, F.; Sers, C. Atypical protein kinase C ζ exhibits a proapoptotic function in ovarian cancer. *Mol. Cancer Res.* **2010**, *8*, 919−934.
(35) Savickiene, J.; Treigyte, G.; Gineitis, A.; Navakauskiene, R. A critical role of redox state in determining HL-60 cell granulocytic differentiation and apoptosis via involvement of PKC and NF-κB. *In Vitro Cell Dev. Biol. Anim.* **2010**, *46*, 547−559.
(36) Gobbi, G.; Di Marcantonio, D.; Micheloni, C.; Carubbi, C.; Galli, D.; Vaccarezza, M.; Bucci, G.; Vitale, M.; Mirandola, P. TRAIL up-regulation must be accompanied by a reciprocal PKCε down-regulation during differentiation of colonic epithelial cell: implications for colorectal cancer cell differentiation. *J. Cell Physiol.* **2012**, *227*, 630−638.
(37) Moe, B. G.; Vereide, A. B.; Orbo, A.; Sager, G. High concentrations of progesterone and mifepristone mutually reinforce cell cycle retardation and induction of apoptosis. *Anticancer Res.* **2009**, *29*, 1053−1058.
(38) Syed, V.; Ho, S. M. Progesterone-induced apoptosis in immortalized normal and malignant human ovarian surface epithelial cells involves enhanced expression of FasL. *Oncogene* **2003**, *22*, 6883−6890.