Original Article

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Cordycepin induces apoptosis in human bladder cancer T24 cells through ROS-dependent inhibition of the PI3K/Akt signaling pathway

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Summary **Cordycepin, a derivative of nucleoside adenosine, is one of the active ingredients extracted from the fungi of genus *Cordyceps*, which have been used for traditional herbal remedies. In this study, we examined the effect of cordycepin on the proliferation and apoptosis of human bladder cancer T24 cells and its mechanism of action. Cordycepin treatment significantly reduced the cell survival rate of T24 cells in a concentration-dependent manner, which was associated with the induction of apoptosis. Cordycepin activated caspase-8 and -9, which are involved in the initiation of extrinsic and intrinsic apoptosis pathways, respectively, and also increased caspase-3 activity, a typical effect caspase, subsequently leading to poly (ADP-ribose) polymerase cleavage. Additionally, cordycepin increased the Bax/Bcl-2 ratio and truncation of Bid, and destroyed the integrity of mitochondria, which contributed to the cytosolic release of cytochrome c. Moreover, cordycepin effectively inactivated the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway, while LY294002, a PI3K/Akt inhibitor, increased the apoptosis-inducing effect of cordycepin. Cordycepin further enhanced the intracellular levels of reactive oxygen species (ROS), while the addition of N-acetyl cysteine (NAC), a ROS inhibitor, significantly diminished cordycepin-induced mitochondrial dysfunction and growth inhibition, and also blocked the inactivation of PI3K/Akt signaling pathway. Furthermore, the presence of NAC significantly attenuated the enhanced apoptotic cell death and reduction of cell viability by treatment with cordycepin and LY294002. Collectively, the data indicate that cordycepin induces apoptosis through the activation of extrinsic and intrinsic apoptosis pathways and the ROS-dependent inactivation of PI3K/Akt signaling in human bladder cancer T24 cells.**

Keywords: Cordycepin, bladder cancer, T24 cells, apoptosis, ROS, PI3K/Akt

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1. Introduction

Apoptosis, a programmed cell death, is one of the most actively studied areas of cancer treatment, and

is largely divided into death receptor (DR)-mediated extrinsic and mitochondria-mediated intrinsic pathways (*1,2*). The extrinsic pathway begins with the activation of caspase-8 by the formation of the death-inducing signaling complex through the binding of death ligand to the cell surface DR. On the other hand, the intrinsic pathway is characterized by the release of pro-apoptotic proteins, such as cytochrome c from the mitochondria to the cytoplasm, with increased mitochondrial permeability and the activation of caspase-9 (*1,3*). Caspase-8 and -9 as initiator caspases ultimately activate downstream effector caspases, including caspase-3, which induce apoptosis through the cleavage of cellular substrates. In addition, these pathways are strictly regulated by a group of proteins that are composed of pro- and anti-apoptotic proteins, such as Bcl-2 protein family proteins (*4,5*). Meanwhile, apoptosis is precisely regulated by a wide variety of cellular signaling pathways. Among them, the phosphoinositide 3-kinase (PI3K)/Akt signaling pathway is involved in the inhibition of apoptosis and the promotion of cell growth, thus playing a key role in the pathogenesis of various tumors (*6-8*). Moreover, there is growing interest in reactive oxygen species (ROS), which induce apoptosis of cancer cells through dysregulation of the PI3K/Akt signaling pathway (*9-11*). Therefore, inhibiting the PI3K/Akt signaling pathway, while promoting the generation of ROS, can be an attractive approach to cancer treatment.

Bladder cancer is the sixth most prevalent malignancy in the United States and causes more than 16,000 deaths annually that has a considerable morbidity and mortality impact with particularly poor prognosis (*12,13*). About 90% of affected patients are older than 55 years, and three to four times greater in men than in women to develop the disease (*12*). Established risk factors included male sex, older ages, personal or family history, cigarette smoking and underlying disease such as diabetes mellitus and obesity (*12,13*). The common treatment for bladder cancer focuses on radical cystectomy, but most patients experience relapse after excision (*14*). Because of this, adjuvant chemotherapy is usually performed in an effort to delay recurrence and prolong survival (*14,15*). However, there are reported that adjuvant chemotherapy responded to 50% of muscle-invasive bladder cancer patients (*15,16*). Therefore, there is a need to develop a novel treatment strategies for the overcome these challenges of bladder cancer. In this respect, numerous medical plants and herbal pharmacologically compounds are coming into the spotlight, due to their low cost, low toxicity, and low hostility as dietary supplements (*17*). Recently, many studies into the natural compounds that have been traditionally used in the treatment of various diseases have shown great interest in their use as potential resources for cancer chemoprevention and chemotherapy (*18-20*). Among them, cordycepin is a type of nucleoside analogue that is isolated from the fungi belonging to the Cordyceps genus, such as *Cordyceps militaris* and *C. sinensis* (*21-23*). Although various pharmacological actions of cordycepin have been known, research on the anticancer activity, including the induction of apoptosis of cancer cells, has been conducted most extensively (*21,23-25*). For example, the intrinsic and extrinsic apoptosis pathways may be involved in the induction of apoptosis of human hepatocarcinoma and prostate cancer cells, and mouse Leydig tumor cells by cordycepin (*26-28*). It has also been found that the increase of ROS production in leukemia, gastric and prostate cancer cells plays an important role in the induction of intrinsic apoptosis pathway (*28-30*). In addition, the anticancer effects of cordycepin involve the disturbance of various cell signaling pathways, and in particular, cordycepin-induced apoptosis in human gastric and ovarian cancer cells and leukemia and glioma cells was accompanied by inactivation of the PI3K/Akt signaling pathway (*31-36*). Although the induction of apoptosis by cordycepin in a certain gastric cancer cell line was accompanied by the production of ROS and inactivation of the PI3K/Akt signaling pathway (*24*), the underlying mechanism of ROS involved in the inactivation of PI3K/Akt signaling pathway by cordycepin is still not well known. Therefore, in this study, we investigated the effect of cordycepin on the induction of apoptosis, and evaluated whether its effect was associated with the ROS generation and PI3K/Akt signaling pathway in human urinary bladder transitional cell carcinoma T24 cells.

2. Materials and Methods

2.1. *Cell culture and cordycepin treatment*

T24 cells were purchased from the American Type Culture Collection (Manassas, MD, USA). Cells were cultured at 37°C in 5% CO_2 humidified incubator in complete media consisting of RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin (WelGENE Inc., Daegu, Republic of Korea). Cordycepin obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) was dissolved in dimethylsulfoxide (DMSO, Sigma-Aldrich Chemical Co.) to a final concentration of 100 μg/mL, and prior to use, the stock solution was diluted with cell culture medium to the desired concentration.

2.2. *Cell viability*

The viability of the cells was assessed by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetra-zolium bromide (MTT) assay, as previously described (37). Briefly, T24 cells were seeded onto 96-well plates at a

density of 1×10^4 cells/well, and incubated overnight. Thereafter, the cells were treated with the desired concentrations of cordycepin for 48 h, and the cells were then incubated with 50 μg/mL MTT solution (Invitrogen, Waltham, MA, USA) for 2 h. Formazan crystals were dissolved in DMSO, and then the absorbance was measured by microplate reader (VERSA Max, Molecular Device Co., Sunnyvale, CA, USA) at 540 nm. The morphological changes of cells were visualized by phase-contrast microscopy (Carl Zeiss, Oberkochen, Germany).

2.3. *Detection of apoptotic morphological changes*

Apoptotic cells containing chromatin condensation and apoptotic body formation in the nuclei were detected by 4′,6′-diamidino-2-phenylindole (DAPI, Sigma-Aldrich Chemical Co.) staining. After treatment with cordycepin for 48 h, the cells were harvested, washed with phosphate-buffered saline (PBS), and then fixed with 3.7% paraformaldehyde (Sigma-Aldrich Chemical Co.) in PBS for 10 min at room temperature (RT). The cells were washed with PBS, and stained with 1 μg/mL DAPI solution for 10 min under light-shielded conditions. The cells were washed with PBS, and the fluorescence intensity was observed using fluorescence microscopy (Carl Zeiss).

2.4. *Determination of apoptosis by flow cytometer*

The magnitude of apoptosis was measured by flow cytometer using propidium iodide (PI) staining. In brief, the cells treated with cordycepin were washed with cold PBS, fixed in ice-cold 70% ethanol, and stored at 4°C. The cells were suspended in cold PBS containing 50 μg/mL PI, 100 μg/mL ribonuclease A, 0.1% (w/v) sodium citrate and 0.1% (v/v) Nonidet-P40 (Sigma-Aldrich Chemical Co.), then incubated on ice for 30 min in the dark at RT. Flow cytometric analysis was carried out using a flow cytometer (BD Biosciences, San Jose, CA, USA), and Cell Quest software was used to determine the relative DNA content. The sub-G1 population was calculated to estimate the apoptotic cell population.

2.5. *Agarose gel electrophoresis for DNA fragmentation assay*

After treatment with cordycepin for 48 h, the cells were lysed in lysis buffer containing 10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM ethylenediaminetetraacetic acid, and 0.5% Triton X-100 for 1 h at RT. The lysates were vortexed, and cleared by centrifugation at 10,000× g for 30 min. After extraction of fragmented DNA in the supernatant using phenol:chloroform:isoamyl alcohol (25:24:1, v/v/v, Sigma-Aldrich Chemical Co.), electrophoretic analysis was performed on 1.0% agarose gels containing 0.1 μg/mL ethidium bromide (EtBr, Sigma-Aldrich Chemical Co.).

2.6. *Protein extraction and Western blot analysis*

Total protein was extracted from the cells using the Bradford Protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), according to the manufacturer's protocol. NE-PER nuclear and cytoplasmic extraction reagents (Thermo Fisher Scientific Inc., Waltham, Utah, USA) were applied for the preparation of mitochondrial and cytosolic extracts of cells, according to the manufacturer's instructions. After quantification of protein concentration using the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA), an equal amount of protein from the samples was separated by denaturing sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, and transferred onto polyvinylidene difluoride (PVDF) membranes (Schleicher & Schuell, Keene, NH, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Triton X-100 (TBST) for 1 h, and probed with specific primary antibodies at 4°C overnight. After washing three times with TBST, the membranes were incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies for 2 h at RT. The expression of protein was detected by enhanced chemiluminescence (ECL) kit (GE Healthcare Life Sciences, Little Chalfont, UK), and visualized by Fusion FX Image system (Vilber Lourmat, Torcy, France).

2.7. *Caspase activity*

The activity of caspases was measured using caspase activity assay kits (R&D Systems, Minneapolis, MN, USA), according to the manufacturer's protocol. In brief, cells were harvested, and lysed in the lysis buffer provided in the kit. The supernatants were collected, incubated with the supplied reaction buffer containing dithiothreitol, with or without substrates [Asp-Glu-Val-Asp (DEAD) for caspase-3; Ile-Glu- Thr-Asp (IETD) for caspase-8; and Leu-Glu-His-Asp (LEHD) for caspase-9] labeled with p-nitroaniline (pNA) at 37°C for 2 h in the dark. The optical density of the reaction mixture was determined by absorbance at 405 nm using a microplate reader.

2.8. *Measurement of mitochondrial membrane potential (MMP, Δψm)*

To observe the changes of MMP, 5,5‘,6,6'-tetrachloro-1,1',3,3'-tetraethyl-imidacarbocyanine iodide (JC-1; Sigma-Aldrich Chemical Co.) staining was performed. After treatment with various concentrations of cordycepin, 10 μM JC-1 was added to the cells for 30 min at 37°C. Subsequently, the cells were washed with

PBS to remove unbound dye, and at least 10,000 cells were collected for each sample. The amounts of MMP were detected at 488/575 nm using a flow cytometer (BD Biosciences), by following the manufacturer's protocol.

2.9. *Statistical analysis*

The results of quantitative studies are reported as mean ± standard deviation (SD) using GraphPad Prism software (version 5.03; GraphPad Software, Inc., La Jolla, CA, USA). All experiments were repeated at least three times. To compare data, One-way analysis of variance (ANOVA) with Dunnett's *post-hoc* test was used, and $p < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. *Cordycepin inhibits T24 cell viability*

In evaluate the cytotoxicity of cordycepin in T24 cells, the cells were incubated with different concentrations of cordycepin for 48 h, and cell viability was assessed by MTT assay. Figure 1A shows that cordycepin significantly reduced T24 cells viability in a concentration-dependent manner, and fifty percent inhibitory concentration (IC_{50}) values of cordycepin was 41.62 μM. Therfore, we decided that 40 μM (IC_{50}) appropriated as the maximum concentration for investigation of apoptotic effects of cordycepin. Under phase-contrast microscope, the phenotypic characteristics of cordycepin-treated cells showed irregular cell outlines, decrease of cell density, and increase of detached cell (Figure 1B).

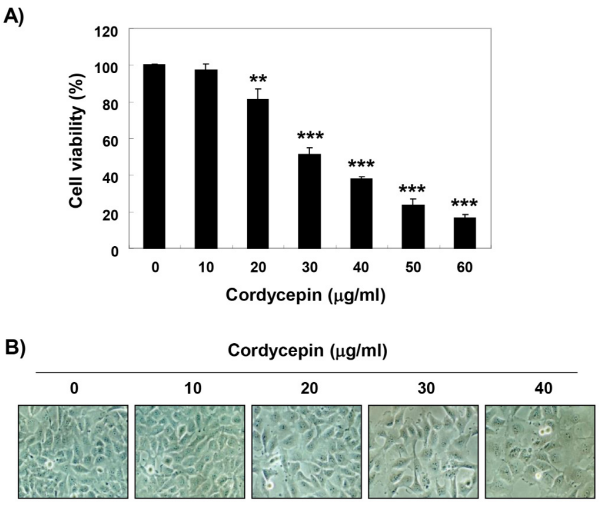

Figure 1. Cordycepin decreases the survival of human bladder cancer T24 cells. (A) T24 cells were treated with the indicated concentrations of cordycepin. The cell viability was assessed after 48 h by MTT assay, as described in the Materials and Methods. Each bar represents the mean ± SD of three independent experiments (**$p < 0.001$ and ***$p < 0.0001$ when compared to control). **(B)** Morphological changes of T24 cells were observed by phase-contrast microscopy. Representative photographs of the morphological changes are presented.

3.2. *Cordycepin induces apoptotic cell death in T24 cells*

We determined whether the growth inhibition of T24 cells by cordycepin was associated with apoptosis induction. Figure 2A shows the results of DAPI staining, which reveal that the morphological changes of nuclei observed in apoptosis-inducing cells, such as nuclear fragmentation, and chromatin condensation, were increased, depending on cordycepin treatment concentration. In addition, the results of flow cytometry analysis and agarose electrophoresis showed that the percentage of sub-G1 cells and fragmentation of DNA were increased in cells treated with cordycepin in a concentration-dependent manner (Figure 2B-D).

3.3. *Cordycepin activates caspases in T24 cells*

We next assessed whether cordycepin activated the caspase signaling pathway in T24 cells, and found that cordycepin reduced the expression of pro-caspase-3, -8, and -9 (Figure 3A) and increased their enzymatic activity in a concentration-dependent manner (Figure 3B). Cordycepin also induced cleavage of poly (ADP-ribose) polymerase (PARP), one of the major substrate

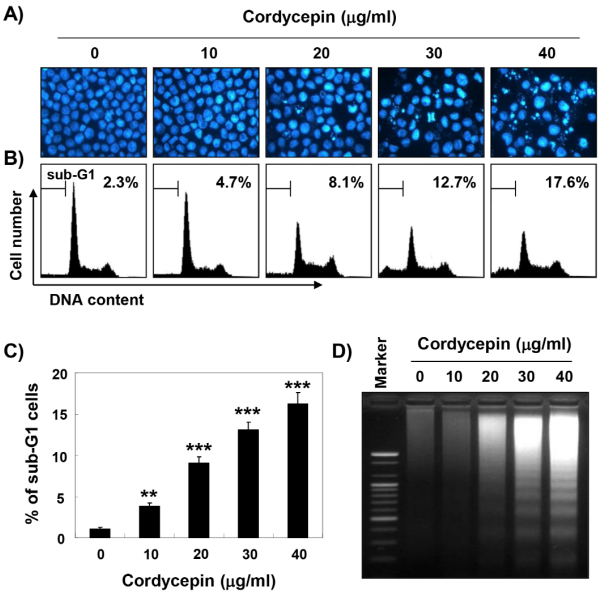

Figure 2. Cordycepin induces apoptosis in T24 cells. (A) After treatment with different concentrations of cordycepin for 48 h, the cells were collected, fixed, and stained with DAPI solution. The stained nuclei were pictured under a fluorescence microscope. **(B** and **C)** The cells cultured under the same conditions were collected, and stained with PI solution for flow cytometry analysis. **(B)** The percentages of apoptotic sub-G1 cells were determined. **(C)** Data were expressed as the mean ± SD of three independent experiments (**$p < 0.001$ and ***$p < 0.0001$ when compared to control). **(D)** DNA fragmentation was analyzed by the extraction of genomic DNA, electrophoresis in agarose gel, and then visualization by EtBr staining.

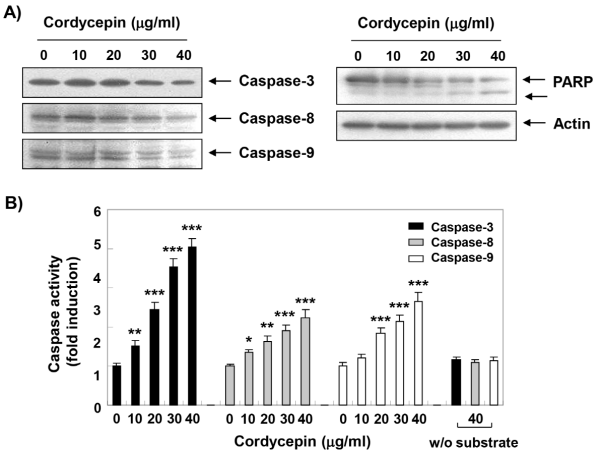

Figure 3. Cordycepin induces the activation of caspases and degradation of PARP in T24 cells. T24 cells were treated with the indicated concentrations of cordycepin for 48 h. **(A)** The cell lysates were prepared, and equal amounts of cellular proteins were separated on SDS-polyacrylamide gels, and transferred to PVDF membranes. The membranes were probed with the indicated antibodies, and the proteins were visualized using an ECL detection system. The equivalent loading of proteins in each well was confirmed by actin. **(B)** The activities of caspases were evaluated using caspases colorimetric assay kits. The data were expressed as the mean ± SD of three independent experiments (*$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$, when compared to control).

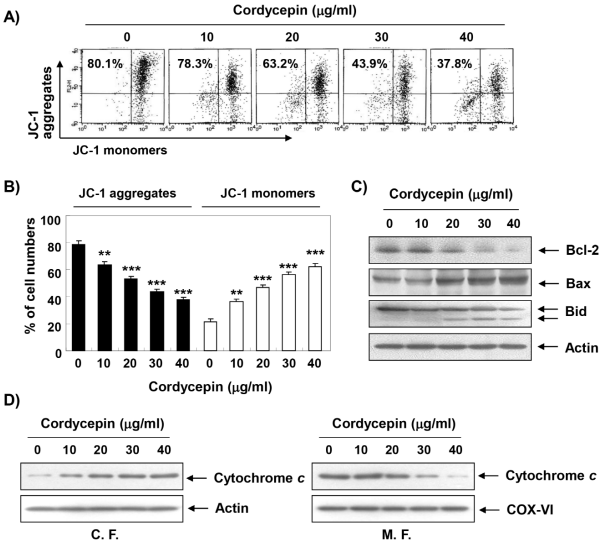

Figure 4. Cordycepin reduces the values of MMP (Δψm), modulates the levels of Bcl-2 family proteins, and induces the cytosolic release of cytochrome c in T24 cells. (A) After 48 h incubation with the indicated concentrations of cordycepin, the cells were stained with JC-1 dye, and were then analyzed by flow cytometry, in order to evaluate the changes in MMP. **(B)** Each bar represents the percentage of cells with JC-1 aggregates and monomers (mean ± SD of triplicate determinations, **$p < 0.001$ and ***p 0.0001, when compared to control). **(C)** The cell lysates were prepared, and the expression of Bcl-2 family proteins (Bc-2, Bax and Bid) was evaluated by Western blot analysis with whole cell lysates. Equal protein loading was confirmed by an analysis of actin. **(D)** Cytosolic and mitochondrial proteins were prepared, and analyzed for cytochrome c expression by Western blot analysis. Equal protein loading was confirmed by the analysis of actin and cytochrome oxidase subunit VI (COX VI) in each protein extract. The results shown are representative of three independent experiments.

proteins of activated caspase-3 (Figure 3A).

3.4. *Cordycepin induces mitochondrial dysfunction in T24 Cells*

We further assessed whether mitochondrial dysfunction was involved in the induction of cordycepin-induced apoptosis, in order to study additional mechanisms involved in inducing apoptosis by cordycepin. As can be seen from the results of JC-1 staining, the MMP-dependent formation of JC-1 aggregates in mitochondria was maintained at a relatively high rate in T24 cells not treated with cordycepin (Figure 4A and B). However, JC-1 aggregates were reduced after treatment with cordycepin in a concentration-dependent manner, indicating a significant depletion of MMP after cordycepin treatment. As indicated in Figure 4C, we also found that cordycepin increased the expression of pro-apoptotic Bax, and decreased the expression of anti-apoptotic Bcl-2. Additionally, the expression of truncated BH3 interacting-domain death agonist (tBid) was increased, and the release of cytochrome c from the mitochondria to the cytoplasm was promoted in cordycepin-treated T24 cells (Figure 4C and D).

3.5. *Cordycepin inactivates PI3K/Akt signaling pathway in T24 cells*

To determine the effect of cordycepin on the PI3K/Akt signaling pathway, we measured the phosphorylation level of PI3K protein and its downstream component Akt. Figure 5A shows that when cells were exposed to cordycepin, the expressions of phosphorylated (p)-PI3K and p-Akt were gradually decreased with increasing time of cordycepin treatment, while total PI3K and Akt protein levels remained constant during cordycepin treatment, which suggests that cordycepin was able to block the activation of the PI3K/Akt pathway in T24 cells. To further confirm the role of the PI3K/Akt pathway in cordycepin-mediated apoptosis, cells were co-treated with LY294002, a specific PI3K inhibitor and cordycepin. The results obtained from DAPI staining, agarose gel electrophoresis, and flow cytometric analysis showed that apoptosis was significantly increased in cells treated with PI3K inhibitor and cordycepin, compared to with cordycepin alone (Figure 5B-E). In addition, after co-treatment with LY294002 and cordycepin, the reduction of cell viability by cordycepin was further reduced (Figure 5F).

3.6. *Cordycepin induces ROS-dependent mitochondrial dysfunction and growth inhibition in T24 cells*

We next investigated whether cordycepin induced the production of ROS, and the effects of increased ROS on cordycepin-induced apoptosis and inhibition of the PI3K/Akt signaling pathway. Examining the

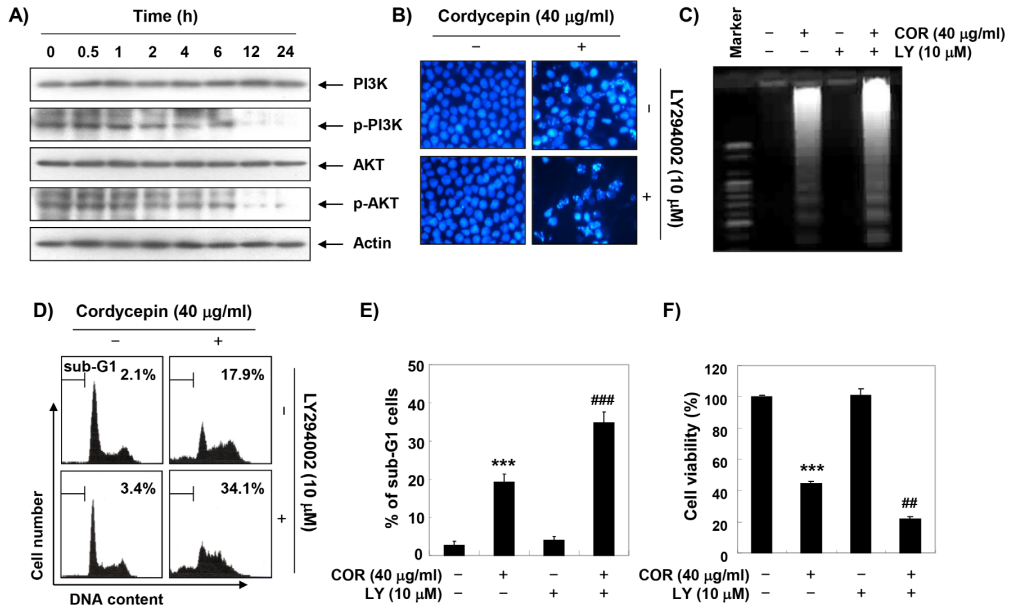

Figure 5. Cordycepin induces the inactivation of PI3K/Akt signaling pathway in T24 cells. The cells were treated with 40 μg/mL cordycepin for the indicated times **(A)**, or pre-treated with 10 μM LY294002 for 1 h, and then treated with 40 μg/mL cordycepin for a further 48 h **(B-F)**. **(A)** The cell lysates were prepared, and the expression of PI3K and Akt proteins was evaluated by Western blot analysis with whole cell lysates. Actin was used as an internal control. **(B)** The DAPI-stained nuclei were then observed by fluorescence microscopy (original magnification, ×400). **(C)** DNA fragmentation was analyzed by the extraction of genomic DNA, electrophoresis in agarose gel, and then visualization by EtBr staining. **(D)** The percentages of apoptotic sub-G1 cells were determined. **(E)** Data were expressed as the mean ± SD of three independent experiments (***$p < 0.0001$, when compared to control; ###$p < 0.001$, when compared to cordycepin-treated cells). **(F)** The cell viability was measured by MTT assay. Data were expressed as the mean ± SD of three independent experiments (***$p < 0.0001$, when compared to control; ##$p < 0.001$, when compared to cordycepin-treated cells). COR, cordycepin; LY, LY294002.

generation of ROS using 2',7'-dichlorofluorescin diacetate (DCF-DA) showed that the accumulation of ROS was highest after 1 h of cordycepin treatment, and then gradually decreased thereafter (data not shown). However, cells co-treated with N-acetyl cysteine (NAC), a potent ROS scavenger, showed significantly reduced ROS levels, compared to those of cordycepin alone treated cells (Figure 6A and B). Consistent with these flow cytometric results, fluorescence microscopy observations confirmed that NAC treatment significantly inhibited cordycepin-induced ROS generation (Figure 6C). In addition, NAC significantly prevented cordycepin-induced loss of MMP (Figure 6D and E), and reduced viability in T24 cells (Figure 6F).

3.7. *Cordycepin-mediated inactivation of PI3K/Akt signaling is ROS-dependent in T24 cells*

We further investigated the role of PI3K/Akt signaling pathway on ROS generation-mediated apoptosis by cordycepin. Figure 7A shows that when the production of ROS was artificially blocked, the reduced phosphorylation levels of PI3K and Akt by cordycepin were maintained at the control level. In addition, NAC treatment significantly protected the apoptosis induced by the co-treatment of cordycepin and LY294002, as observed by the nuclear morphological changes, DNA fragmentation assay, and flow cytometric analysis (Figure 7B-E). Consistent with these results, the reduced cell viability by co-treatment with cordycepin and LY 294002 was also significantly restored by blocking ROS production (Figure 7F).

4. Discussion

Many previous studies have shown that cordycepin induces apoptosis in a variety of cancer cells under conditions that are not toxic to normal cells (*21,23,38*). It has also been reported that several cellular signaling pathways are involved in the induction of apoptosis of cancer cells by cordycepin (*27,32,35,38-40*), and that the accumulation of ROS associated with mitochondrial dysfunction acts as a major signal in this process (*27,29-30,32,39,41*). Although the possibility of involvement of PI3K/Akt signaling pathway was suggested in several previous studies (*32-36,42*), the link between this pathway and the production of ROS is largely unknown.

Our results indicated that cordycepin activated both caspase-8 and -9, initiator caspases for the activation of extrinsic and intrinsic pathways, respectively, and increased the truncation of Bid in T24 cells, consistent with previous studies in breast and prostate cancer cells (*28,42,43*). In addition, mitochondrial dysfunction was induced as confirmed by the loss of MMP in cordycepin-treated T24 cells. The loss of MMP was accompanied by a down-regulation in the Bcl-2/Bax ratio and promotion of cytochrome c release into the cytoplasm from mitochondria, which are typically

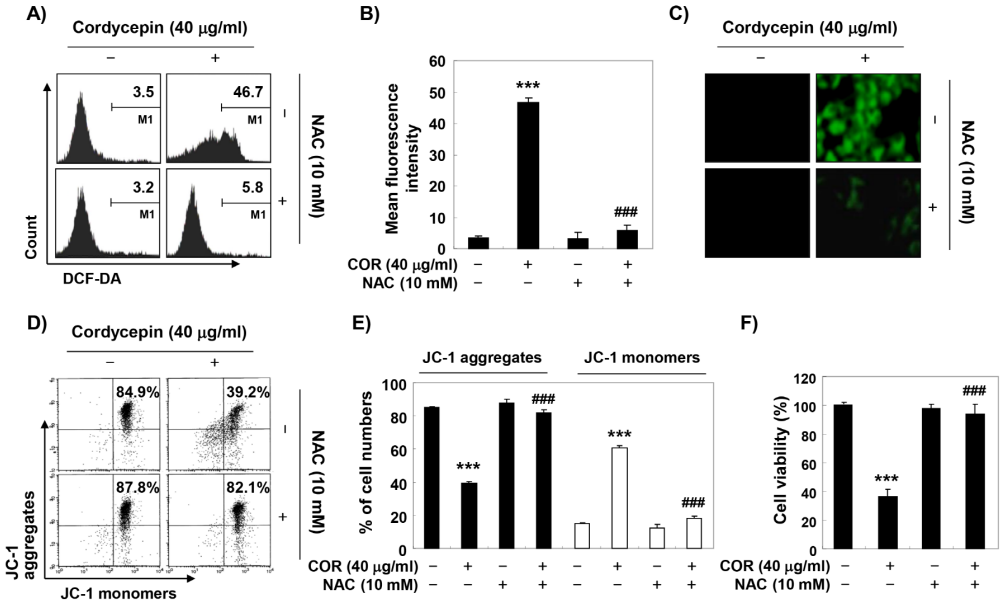

Figure 6. Cordycepin induces ROS generation in T24 cells. (A-C) The cells were either treated with 40 μg/mL cordycepin for 1 h, or pre-treated with 10 mM NAC for 1 h, before cordycepin treatment. **(A)** The medium was discarded, and the cells were incubated at 37°C in the dark for 20 min with new culture medium containing DCF-DA. ROS generation was measured by flow cytometry. **(B)** Each bar represents the mean ± SD of three independent experiments (***$p < 0.0001$, when compared to control; ###$p < 0.0001$, when compared to cordycepin-treated cells). **(C)** Images were obtained by fluorescence microscopy (original magnification: ×200). The images presented here are captured from one experiment, and are representative of at least three independent experiments. **(D-F)** The cells were either treated with 40 μg/mL cordycepin for 48 h, or pre-treated with 10 mM NAC for 1 h, before cordycepin treatment. **(D)** The cells were stained with JC-1 dye, and were then analyzed by flow cytometry, in order to evaluate the changes in MMP. **(E)** Each bar represents the percentage of cells with JC-1 aggregates and monomers (mean ± SD of triplicate determinations, ***$p < 0.0001$, when compared to control; ###$p < 0.0001$, when compared to cordycepin-treated cells). (F) The cell viability was measured by MTT assay. Data were expressed as the mean ± SD of three independent experiments (***$p < 0.0001$, when compared to control; ###$p < 0.0001$, when compared to cordycepin-treated cells). COR, cordycepin.

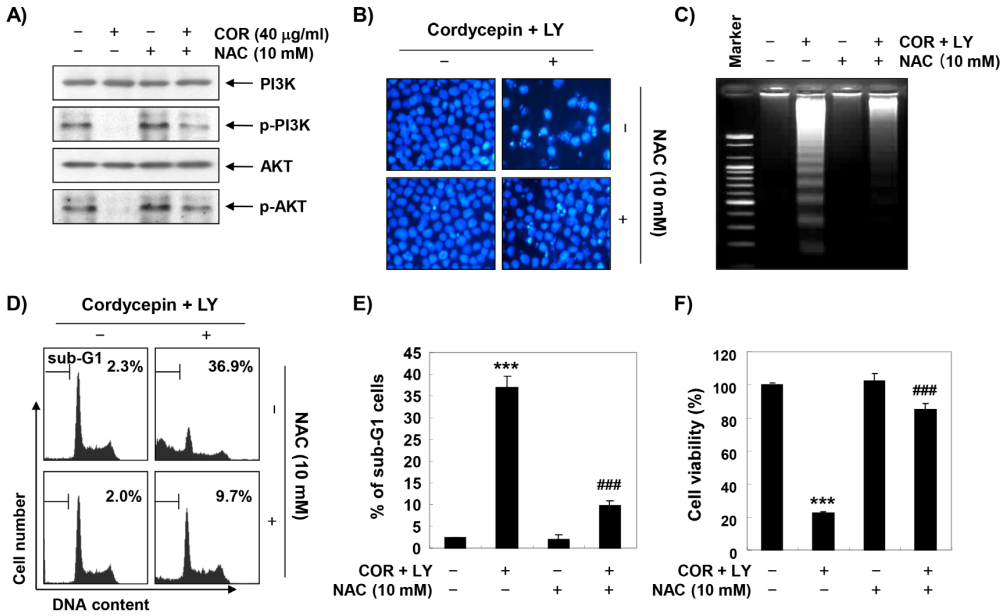

Figure 7. Cordycepin induces the ROS-dependent inactivation of PI3K/Akt pathway in T24 cells. (A) The cells were either treated with 40 μg/mL cordycepin for 48 h, or pre-treated with 10 mM NAC for 1 h before 40 μg/mL cordycepin treatment, and then collected. The cellular proteins were prepared, and the expression of PI3K and Akt proteins was evaluated by Western blot analysis. **(B-F)** The cells were pre-treated with 10 μM LY294002 for 1 h, and then treated with 40 μg/mL cordycepin for a further 48 h, in the presence or absence of 10 mM NAC. **(B)** The DAPI-stained nuclei were then observed by fluorescence microscopy (original magnification, ×400). **(C)** DNA fragmentation was analyzed by the extraction of genomic DNA, electrophoresis in agarose gel, and then visualization by EtBr staining. (D-F) The percentages of apoptotic sub-G1 cells and cell viability were determined by flow cytometry and MTT assay, respectively. (E and F) Data were expressed as the mean ± SD of three independent experiments (***$p <$ 0.0001, when compared to control; ###$p < 0.001$, when compared to cordycepin-treated cells).

observed in the activated intrinsic pathway (*4,5*). Cordycepin treatment also significantly increased the activity of caspase-3, and induced the cleavage of PARP. On the other hand, as is well known in previous studies, caspase-8 activated by the initiation of the extrinsic pathway cleaved and converted Bid, a pro-apoptotic protein belonging to the Bcl-2 family proteins, to tBid (*4,44,45*). tBid in turn translocates to the mitochondria to promote the permeability of the mitochondrial outer membrane, leading to the accumulation of cytochrome c, counteracting the cytoprotective activity of Bcl-2 protein, and amplifying the intrinsic pathway (*45,46*). Therefore, the results indicate that cordycepin induced apoptosis in T24 cells by simultaneously activating the extrinsic and intrinsic pathways through tBid-mediated crosstalk.

Abnormal activation of the PI3K/Akt signaling pathway has recently been shown to be involved in the pathogenesis of multiple human tumors, including bladder cancer (*9-11,47,48*). Activated PI3K initiates the activation of Akt, a downstream kinase of PI3K, which can inhibit apoptosis by protecting caspase cascade through phosphorylation of caspase-9, and promotes the expression of anti-apoptotic proteins of the Bcl-2 family proteins, thereby enhancing cell survival and the proliferation of cancer cells (*49,50*). Because these ultimately contribute to resistance to chemotherapy for inducing apoptosis in cancer cells, PI3K and its regulatory factors are attractive targets for cancer treatment. Therefore, we analyzed whether this signaling pathway was involved in the induction of T24 cell apoptosis by cordycepin, and found cordycepin suppressed the phosphorylated levels of Akt, as well as PI3K. This means that the PI3K/Akt signaling pathway is inactivated by cordycepin treatment, and the results are in good agreement with previous studies performed on several other cancer cell lines (*31-36,42,51*). Furthermore, in line with our previous study using leukemia cells (36), LY294002, a pharmacological inhibitor of PI3K, significantly enhanced the apoptotic effect of cordycepin and further reduced cell viability, supposing that cordycepin-induced apoptosis is mediated by blocking the PI3K/Akt signaling pathway.

Accumulated evidence has shown that the low levels of ROS in the cell act as a secondary messenger in the intracellular signaling pathways, while excessively high levels of ROS induce oxidation of cellular macromolecules, and promote apoptosis through the activation of extrinsic and/or intrinsic pathways (*52-54*). Recent previous studies have reported that several bioactive compounds generated ROS to activate apoptosis signaling in cancer cells, while ROS-dependent suppressing the activity of the PI3K/Akt signaling pathway (*9-11*). These observations suggest that inducing the production of ROS in cancer cells can be used in therapeutic strategies, such as the induction of apoptosis through the inhibition of cell survival signals, such as PI3K/Akt. Therefore, we further assessed whether cordycepin-induced apoptosis in T24 cells was correlated with the production of ROS, and the relationship between ROS production and the PI3K/Akt signaling pathway. Consistent with previous studies (*27-28,30,32,41,43*), our current results showed that cordycepin treatment markedly increased the levels of ROS production; however, as can be predicted, the ROS scavenger, NAC greatly blocked the accumulation of ROS by cordycepin. The quenching of ROS generation also significantly diminished cordycepin-induced disruption of MMP to the control level, followed by significant survival restoration, indicating that ROS act as upstream signaling molecules to enhance cordycepin-induced apoptosis in T24 cells. These results are consistent with our previous findings using prostate cancer cells (*43*), and signified that the cordycepin-induced apoptosis of T24 cells was ROS-dependent. Furthermore, the presence of NAC markedly attenuated cordycepin-induced dephosphorylation of PI3K and Akt proteins, and NAC treatment also significantly blocked the enhanced apoptosis and viability reduction induced by co-treatment of cordycepin and LY294002. Taken together, these results lead us to suggest that the production of ROS by cordycepin plays a critical role in the induction of apoptosis through simultaneous initiation of both extrinsic and intrinsic pathways in T24 cells, and acts as an upstream signal related to the effect of cordycepin on the inactivation of the PI3K/Akt signaling pathway. However, further studies are warranted to determine the direct relationship between cordycepin-mediated inactivation of PI3K/Akt signaling pathway and other cellular signaling pathways, and the identification and role of intracellular organelles involved in ROS generation by cordycepin.

In conclusion, our findings demonstrate that cordycepin exerts an anti-proliferative effect on human bladder cancer T24 cells, through the activation of extrinsic and intrinsic apoptosis pathways. As evidence for this, cordycepin activated caspase-8 and -9, which belong to the initiator caspases of the extrinsic and intrinsic pathways, respectively, followed by the activation of effector caspase 3, resulting in the degradation of PARP. Cordycepin also induced the truncation of Bid and mitochondrial dysfunction, which was associated with an increase in Bax/Bcl-2 expression ratio and cytochrome c release into the cytoplasm. Moreover, the induction of apoptosis by cordycepin was accompanied by inhibition of the PI3K/Akt signaling pathway, and excessive production of ROS. In addition, artificial interception of the PI3K/Akt signal pathway further increased cordycepin-induced apoptosis, and the interruption of ROS generation led T24 cells to escape from apoptosis, while maintaining the activity of PI3K/Akt signaling pathway. Based on these finding, we suggest that cordycepin has chemopreventive potential by inducing apoptosis through ROS-dependent inactivation of the PI3K / Akt signaling pathway in T24 cells.

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