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Cordycepin Induces Apoptosis of CGTH W-2 Thyroid Carcinoma Cells through the Calcium−Calpain−Caspase 7-PARP Pathway

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Cordycepin, a nucleoside isolated from *Cordyceps sinensis*, is an inhibitor of polyadenylation and has an antitumor effect. We used CGTH W-2, a follicular thyroid carcinoma cell line, to study the mechanism of the anticancer effect of cordycepin. Cordycepin decreased cell viability and resulted in apoptosis but not necrosis. Cordycepin increased intracellular calcium levels triggering calpain activation, which led to apoptosis. BAPTA/AM and calpeptin inhibited the cordycepin-induced cleavage of caspase 7 and poly (ADP-ribose) polymerase (PARP), implying an upstream role of calcium and calpain. CGTH W-2 cells expressed four subtypes of adenosine receptors (AR), A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR. Specific antagonists to AR subtypes all blocked cordycepin-induced apoptosis to different degrees. Small interfering RNA for A_1AR and A_3AR abrogated cordycepin-induced apoptosis. In conclusion, the cordycepin-induced apoptosis of CGTH W-2 cells is mediated by the calcium−calpain−caspase 7-PARP pathway, and ARs are involved in the apoptotic effect of cordycepin.

KEYWORDS: Cordycepin; thyroid carcinoma cells; apoptosis; signaling mechanism

INTRODUCTION

Cordyceps sinensis has been used as a traditional medicine or healthy food in China for years. Cordycepin or 3′-deoxyadenosine, a compound purified from *Cordyceps sinensis*, is an inhibitor of polyadenylation, a process associated with cell survival and the stability of mRNA (mRNA) (*1*, *2*). It has an anti-inflammatory effect (*3*), inhibits platelet aggregation (*4*), and has an anticancer effect (*5*, *6*). Its anticancer effects have been studied in several types of cancer cells (*7*−*11*). The action of cordycepin is mainly mediated by cell death through apoptosis or cell cycle arrest. Several signaling pathways have been proposed for the apoptotic effects of cordycepin. The caspase-dependent pathway includes activation of caspases 2, 8, and 10 in HL-60 cells (*2*), and activation of caspases 9, 3, and 7 in mouse Leydig tumor cells (*12*). Other caspase-independent pathways are reduction in the mitochondrial transmembrane potential in K562 cells (*13*), direct inhibition of RNA synthesis in multiple myeloma cells (*1*), and calcium-μ-calpain activation in erythocytes (*14*). However, the downstream signaling distal to calpain triggered by cordycepin remains elusive.

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Adenosine receptors (ARs) are subdivided into A_1 adenosine receptor (A_1AR), A_{2A}AR, A_{2B}AR, and A_3AR (*15*) and are G protein-coupled receptors (GPCRs), with A_1AR and A_3AR interacting with Gi/o proteins to inhibit adenylyl cyclase and activate phospholipase C (PLC), whereas A_{2A}AR and A_{2B}AR interact with Gs (*15*). In addition to polyadenylation inhibition, cordycepin inhibits mouse melanoma and lung carcinoma cancer cell growth by activating A_3AR in vivo (*16*). Though cell growth inhibition by cordycepin is mediated by A_3AR, it is not known whether its apoptotic effect is also mediated by A_3AR. The involvement of other subtypes of ARs in the anticancer effect of cordycepin cannot be ruled out.

The general treatment for thyroid cancer is total thyroidectomy using radioiodine. However, the metastatic rate of thyroid carcinomas (mostly lung and bone metastases) is relatively high (*17*). In view of the fact that thyroid cancer is the common endocrine carcinoma in young women in Taiwan, we are involved in searching for compounds that induce apoptosis of the well-differentiated follicular thyroid carcinoma cell line, CGTH W-2. We have previously reported that the peroxisome proliferator-activated receptor γ agonists, ciglitazone and 15-deoxy-$\Delta^{12,14}$ prostaglandin J2, and magnaolol induce apoptosis of CGTH W-2 cells via the cytochrome *c*-caspase 3 pathway (*18*, *19*). The aims of this study were to elucidate the signaling pathways involved in cordycepin-induced apoptosis and to investigate whether the effect of cordycepin was mediated through ARs in thyroid carcinoma.

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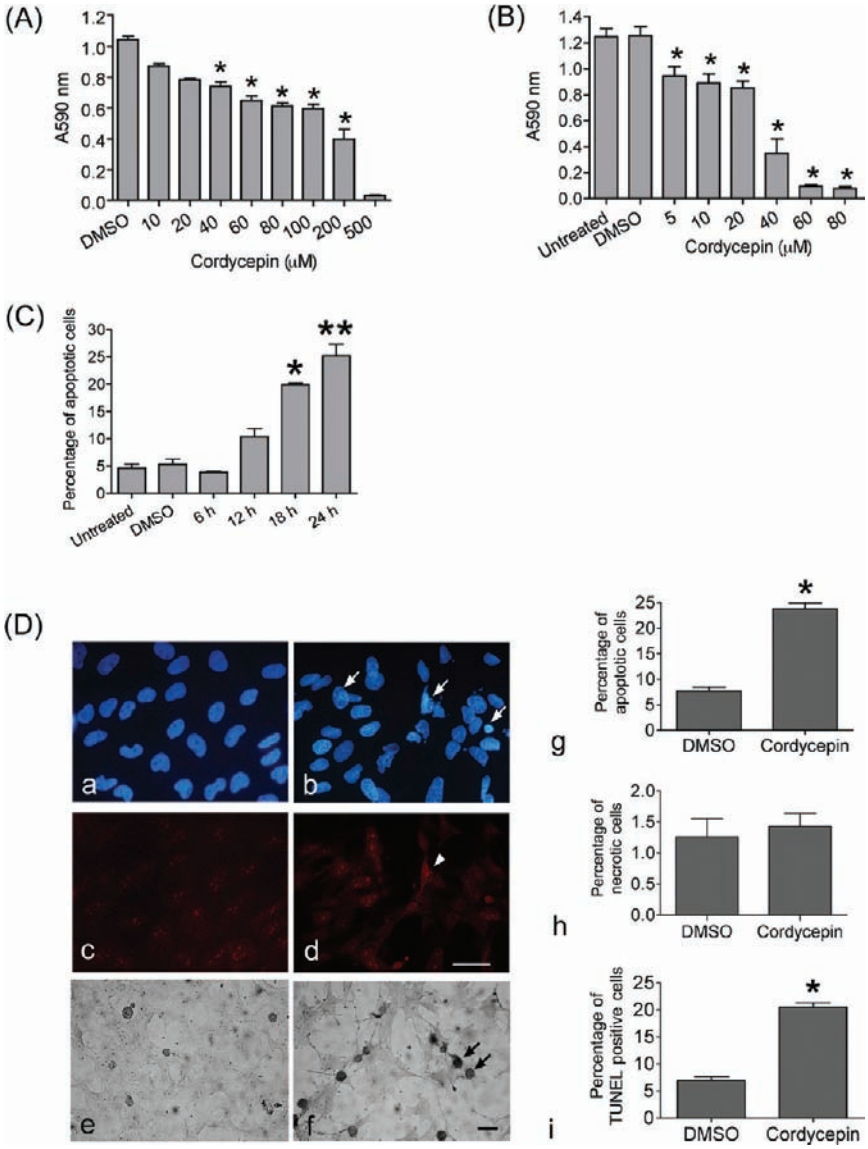

Figure 1. Cordycepin induces the apoptosis of CGTH W-2 cells. (**A**,**B**) Cordycepin reduces cell viability. CGTH W-2 cells were untreated or treated for 24 h with 0.2% DMSO or different concentrations of cordycepin in serum-containing medium (**A**) or serum-free medium (**B**), then were assayed for cell viability using the MTT test. The results are expressed as the absorbance at 590 nm (A_{590}). $n = 3$. *, $p < 0.05$ compared to the DMSO group. (**C**) Cells were treated for 6, 12, 18, or 24 h with 40 μM cordycepin or 0.2% DMSO, then DAPI staining was used to identify the apoptotic cells and quantify the results. *, $p < 0.05$ compared to the DMSO-group. (**D**) Cells were treated for 24 h with 0.2% DMSO (a, c, and e) or 40 μM cordycepin (b, d, and f), and apoptotic nuclei were identified by DAPI staining (arrow in b; a, control) or TUNEL staining (arrow in f; e, control). PI-positive cells are necrotic cells (arrowhead in d; c, control). Bars = 40 μm. (g−i) Quantitative analyses of apoptotic and necrotic cells in the cordycepin- and DMSO-treated groups. $n = 6$. *, $p < 0.05$ or **, $p < 0.01$ compared to the DMSO group.

MATERIALS AND METHODS

Cell Culture. The CGTH W-2 cell line, derived from a metastatic thyroid follicular carcinoma from a Chinese patient in Taiwan (*20*), was a generous gift from Dr. Jen-Der Lin (Chang Gung Memorial Hospital). The cells were grown in RPMI 1640 medium containing 10% fetal bovine serum (both from Gibco BRL, Grand Island, NY), 1 mM sodium pyruvate (Sigma, St. Louis, MO), and 100 IU/mL penicillin and streptomycin (pH 7.2) (Gibco BRL, Grand Island, NY) in a humidified atmosphere of 5% CO_2−95% air at 37 °C.

Drugs. Cordycepin, propidium iodide (PI), 4′,6-diamidino-2-phenylindole dilactate (DAPI), BAPTA/AM, and MRS1523 were purchased from Sigma (St. Louis, MO). DPCPX, ZM241385, and MRS1706 were from TOCRIS (TOCRIS Cookson Inc., Bristol, UK). Calpeptin and staurosporine were obtained from Biomol (Biomol International, Plymouth Meeting, PA).

Cell Survival Assay. CGTH W-2 cells were plated at 2×10^4 cells per well of a 24-well plate and incubated for 24 h for cell adhesion. Then, different concentrations of cordycepin or 0.2% dimethyl sulfoxide (DMSO, Sigma) were added in culture medium for 24 h. After two washes with phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, and 8 mM Na_2HPO_4, pH 7.4), 500 μL of RPMI medium containing 0.5 mg/mL of 2.3. 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (Sigma) was added to each well and incubation continued for another 4 h. The reaction solution was then removed, and the cells were lysed with 500 μL of DMSO and the absorbance at 590 nm measured with a spectrophotometer (Beckman Coulter Inc., Fullerton, CA).

Apoptosis and Necrosis Assays. PI vital stain was used to detect plasma membrane disruption in necrotic cells. After various treatments, cells were washed with serum-free medium, and incubated for 30 min with PI (50 μg/mL in serum-free medium) in a CO_2 incubator. After a brief wash with PBS, the cells were fixed in 5% formalin and 0.5% Triton X-100 for 10 min at room temperature, then, after three washes with PBS, were stained for 15 min with 1 μg/mL of DAPI in 0.9% NaCl and mounted in fluorescence mounting medium (70% glycerol and 2% propyl gallate in

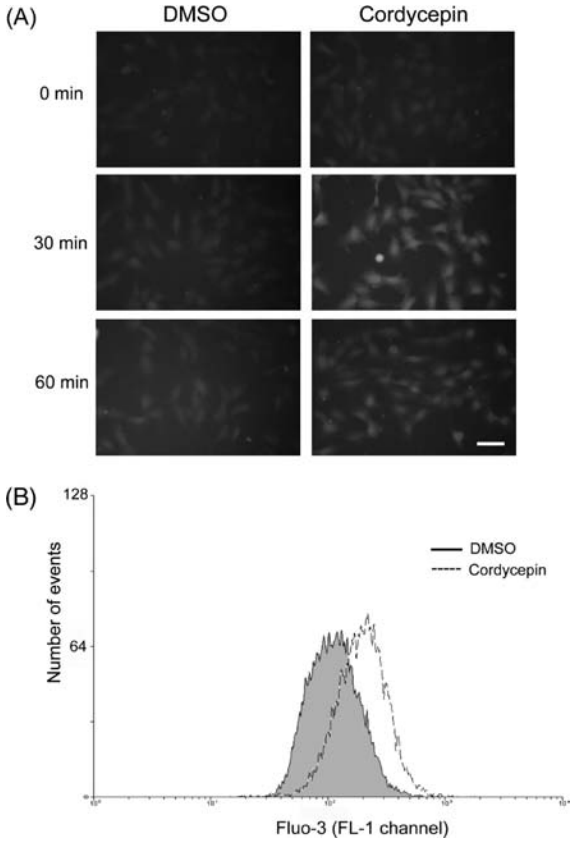

Figure 2. Cordycepin increases intracellular calcium levels. CGTH W-2 cells were loaded with fluo-3/AM, then treated for 0, 30, or 60 min with 0.2% DMSO or 40 μM cordycepin. (**A**) Fluorescent images were recorded with the same exposure time at the different time intervals. Bar = 40 μm. (**B**) Cells were trypsinized and washed, then analyzed by flow cytometry. The black line is the DMSO group, and the gray dotted line indicates the cordycepin treated group for 60 min.

PBS). TUNEL (Calbiochem, La Jolla, CA) staining was also used to detect apoptotic cells. Briefly, the cells were fixed with 4% paraformaldehyde in PBS, rinsed with PBS, permeabilized with methanol, and incubated with TUNEL reaction mixture according to the manufacturer's instructions. All experiments were performed on triplicate dishes and more than one hundred cells were examined per dish.

Reverse Transcription−Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from CGTH W-2 cells using TRI reagent (Ambion, Austin, TX). Reverse transcription was performed on 2 μg of RNA using M-MLV reverse transcriptase (Mbiotech Inc., Seoul, Korea). The generated cDNA was used for PCR amplification using primers for human ARs, β-actin, and GAPDH, designed using MacVector7.2. The sequences were as follows: A_1AR, forward, GCCACAGACCTACTTC-CACA; backward, CC-TTCTCGAACTCACACTTG; A_{2A}AR, forward, CTTCAACTTCTTTGCCTGTGTGC; backward, ATTGGTGTGGG-AGAGGACGATG; A_{2B}AR, forward, GGGATGGAAC-CACGAAT-GAAAG; backward, GAAAAGTGTAGCGGAAGTCTCGG; A_3AR, forward, GGCTGGAACATGAAACTGAC; backward, GAGGTAC-TACTTGGGATA-GC; β-actin, forward, GCCATTGTTACCAACTG-GGACG; backward, TTGATGTCA-CGCACGATTTCC; and GAPDH, forward, TGGTATCGTGGAAGGACTCA; backward, AGTGGGTG-TCGCTGTTGAAG. PCR reactions were performed at 94 °C for 5 min, 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, followed by a final extension step of 72 °C for 5 min. Because the mRNA (mRNA) expressions of ARs are weak to detect, we used 40 cycles for the PCR reaction. The amplified products were electrophoresed on 1.2% agarose gels and stained with ethidium bromide.

Small Interfering RNA (siRNA) Transfection. SiRNA for A_1AR or A_3AR targeting the mRNA coding sequence was, respectively, designed by Dharmacon (ON-TARGET plus SMART pool, Dharmacon Corporation, Lafayette, CO) or Ambion (*Silencer* Select Predesigned siRNA, Ambion, Austin, TX). Negative control and GAPDH siRNA were purchased from Ambion. Electroporation was used to transfect siRNAs as advised in the instruction manual (Amaxa, Germany) for 48 h to detect mRNA expressions. Briefly, 10^6 cells were trypsinized and resuspended in 100 μL of Nucleofector solution (Amaxa), and 100 μM of siRNA duplexes were electroporated. GAPDH siRNA was used as a positive control to confirm the transfection efficiency.

Intracellular Calcium Levels Measured Using Fluo-3/AM. CGTH W-2 cells were washed once with PBS and loaded for 1 h at room temperature with 10 μM fluo-3/AM (Invitrogen) in PBS. After a PBS wash, 0.2% DMSO or cordycepin was added to the culture dish and calcium images recorded immediately on a fluorescence microscope. We also used flow cytometry to further confirm the increase of calcium. After loading with fluo-3/AM, CGTH W-2 cells were treated with cordycepin for 1 h, then cells were washed and trypsinized. The change of calcium was measured by flow cytometry using excitation at 488 nm.

Western Blotting. After the various treatments, CGTH W-2 cells were washed once with ice-cold PBS, homogenized in lysis buffer (10 mM EGTA, 2 mM $MgCl_2$, 60 mM PIPES, 25 mM HEPES, 0.15% Triton X-100, 1 μg/mL of pepstatin A, 1 μg/mL of leupeptin, 1 mM NaF, and 1 mM phenylmethylsulfonyl fluoride), and sonicated for 10 s twice. The concentrations of proteins were determined using a Bio-Rad protein assay kit (Bio-Rad Life Science, Hercules, CA) and samples of proteins (50 μg per lane) electrophoresed on a 10% SDS polyacrylamide gel and transferred to a nitrocellulose membrane (Schleicher & Schuell Inc., Keene, NH). Strips from the membrane were then blocked by incubation for 30 min at room temperature with 5% nonfat milk in Tris-buffered saline, pH 8.2, containing 0.1% Tween (TBS-Tween) and incubated overnight at 4 °C with a 1:1000 dilution of monoclonal mouse antibody against GAPDH (BioVision Research Products, Mountain View, CA), α-spectrin (Calbiochem, San Diego, CA), or rabbit antibodies against cleaved caspase 3, cleaved caspase 7, or cleaved poly (ADP-ribose) polymerase (PARP) (Cell Signaling Technology Inc., Beverly, MA), all diluted in TBS-Tween. After washes with TBS-Tween, the strips were incubated for 2 h at room temperature with a 1:7500 dilution of alkaline phosphatase-conjugated antimouse or antirabbit IgG antibodies (Promega Corp., Madison, WI), and the bound antibody was visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma) as chromogen. The density of the bands on the nitrocellulose membrane was quantified by densitometry using Gel Pro 3.1 (Media Cybernetics, Silver Spring, MD), taking the density of the band in the control sample as 100% and expressing the density of the band in the test sample as a percentage of this.

Statistical Analysis. All experiments were performed at least three times, and the results are expressed as the mean ± SD for the total number of experiments. Statistical differences between means were assessed using the Kruskal−Wallis test and post tested using the Mann−Whitney test, a p value less than 0.05 being considered statistically significant.

RESULTS

Cordycepin Reduces the Viability of CGTH W-2 Cells through Apoptosis, but Not Necrosis. Treatment with cordycepin for 24 h reduced CGTH W-2 cell viability in the presence or absence of serum as shown by the MTT assay (**Figure 1A**,**B**). The concentration-effect of cordycepin on cell death was more obvious in the absence of serum, while only a slight concentration effect (between 40 μM-100 μM) was observed in the presence of serum. Compared to DMSO, 40 μM cordycepin decreased cell viability by about 25% and 60−100 μM cordycepin reduced cell viability by around 30% (IC_{50} = 170 μM). In order to study the protective effects of different AR antagonists against cordycepin cytotoxicity, we used 40 μM cordycepin throughout the rest of this study. Double-staining with DAPI and PI was performed to identify apoptotic and necrotic cells, respectively. **Figure 1C** shows that the percentages of apoptotic cells increased in a time-dependent manner, which peaked between 18 and 24 h. In the DMSO group, fewer cells had apoptotic nuclei and showed PI staining

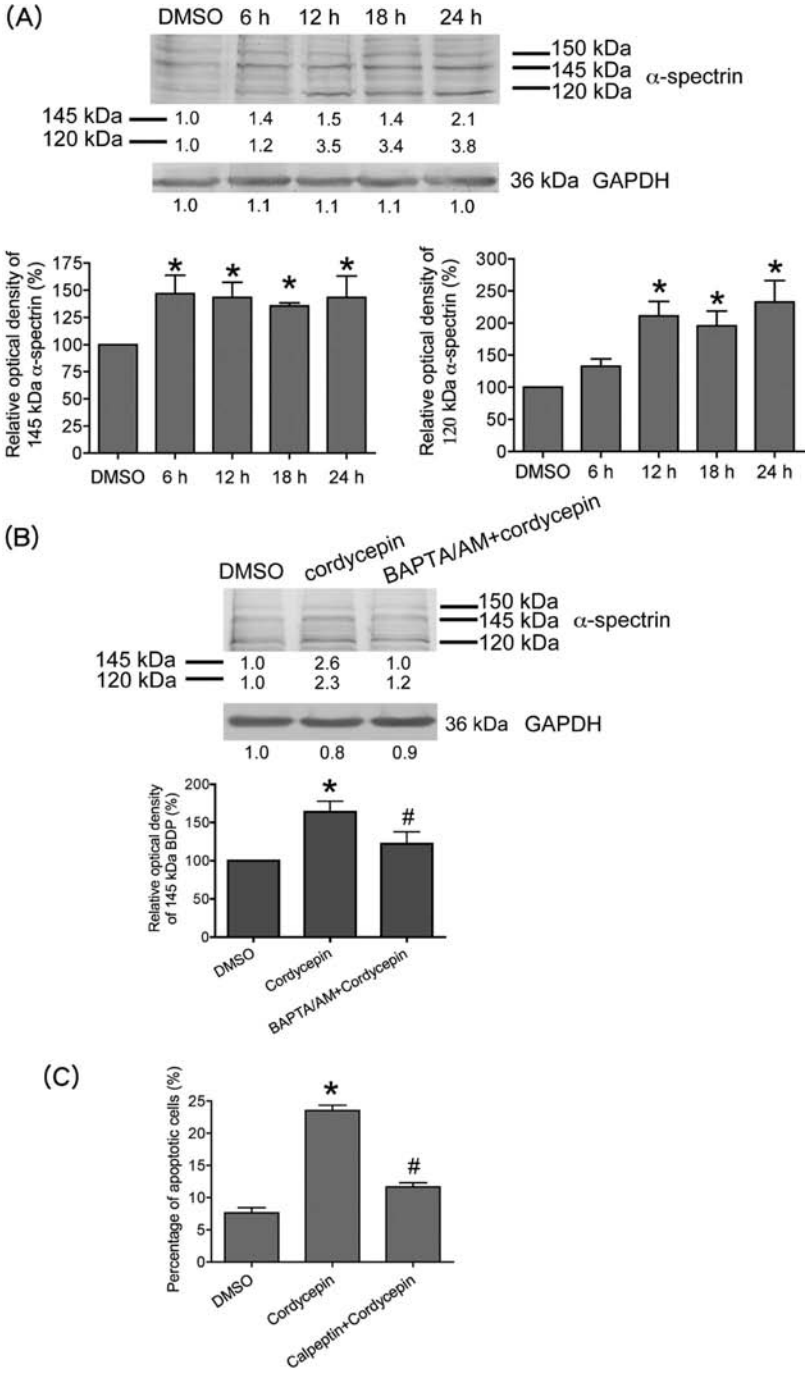

Figure 3. Calpain is involved in the cordycepin-induced cell apoptosis. (**A**) Calpain is activated by cordycepin. CGTH W-2 cells were treated for different times with 40 μM cordycepin, then cell lysates were examined for the 145 kDa and 120 kDa breakdown product (BDP) of α-spectrin. A typical blot and the summarized densitometric scans are shown ($n = 5$). *, $p < 0.05$ compared to the DMSO group. GAPDH is the loading control. (**B**) The activation of calpain by cordycepin is dependent on intracellular calcium. CGTH W-2 cells were treated for 24 h with 0.2% DMSO or 40 μM cordycepin or for 30 min with 10 μM BAPTA/AM, then for 24 h with cordycepin in the continued presence of BAPTA/AM and the cell lysates analyzed for the 145 kDa BDP of α-spectrin. GAPDH is the loading control. $n = 3$. (**C**) Calpeptin inhibits cordycepin-induced apoptosis. CGTH W-2 cells were treated for 24 h with 0.2% DMSO or 40 μM cordycepin or for 30 min with 10 μM calpeptin, then for 24 h with cordycepin in the continued presence of calpeptin. Apoptotic cells were identified by DAPI staining. $n = 6$. *, $p < 0.05$ compared to the DMSO group. #, $p < 0.05$ compared to the cordycepin-treated group.

(**Figures 1D**, a and c). After 24 h of treatment with cordycepin, DAPI-stained cells with condensed or fragmented nuclei were increased by approximately 25% (**Figures 1D**, b and g). We further used the TUNEL assay to confirm the cordycepin-induced apoptosis (**Figure 1D**, f, control **e**). Forty micromolar cordycepin raised the apoptosis ratio to 18% (**Figure 1D**, i; 6% in the DMSO-group) without any difference in the percentage of PI-positive necrotic cells compared to the DMSO-treated group (**Figure 1D**, c, d and h). These data demonstrate that cordycepin reduces the cell viability of CGTH W-2 by apoptosis but not necrosis.

Cordycepin Increases Intracellular Calcium Levels and Triggers Calpain Activation. Recently, cordycepin was reported to increase intracellular calcium levels and to activate μ-calpain in mouse erythrocytes (*14*). We therefore used fluo-3/AM to detect the changes in intracellular calcium levels after different times of

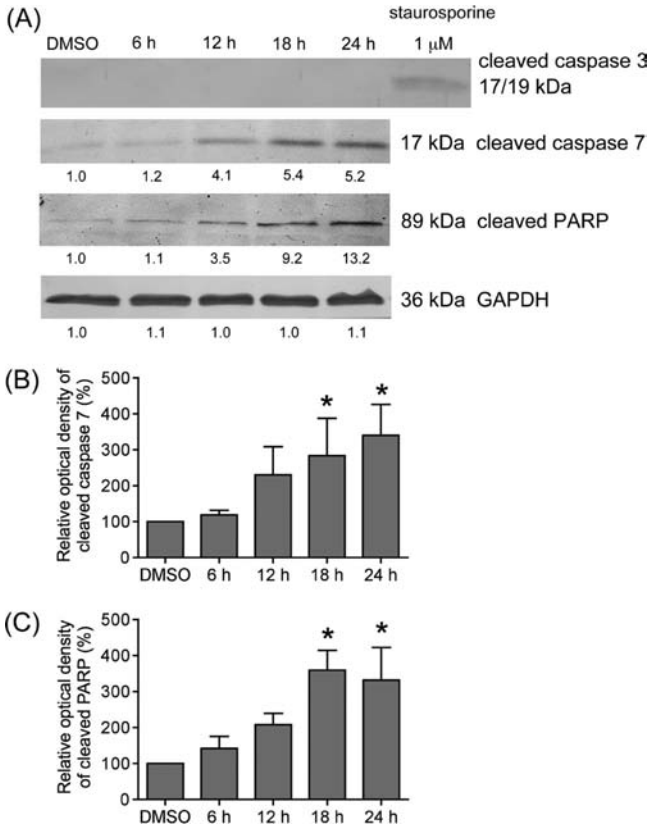

Figure 4. Cordycepin induces a time-dependent cleavage of caspase 7 and PARP. (**A**) CGTH W-2 cells were treated for the indicated time with 0.2% DMSO or 40 μM cordycepin, then cell lysates were analyzed for cleaved caspase 3 (17/19 kDa), cleaved caspase 7 (17 kDa), or cleaved PARP (89 kDa) by Western blotting. Staurosporine (1 μM) treatment for 24 h was used as a positive control (right lane). The results shown are representative of those obtained in 6 experiments. GAPDH is the loading control. Quantitative analyses of the levels of cleaved caspase 7 (**B**) or cleaved PARP (**C**) ($n = 6$) are shown. *, $p < 0.05$, compared to the DMSO group.

cordycepin treatment (**Figure 2**). Cordycepin increased intracellular calcium levels after 30 min, and the effect lasted for at least 60 min (**Figure 2A**). By flow cytometry, we further confirmed that cordycepin elevated intracellular calcium levels after 60 min of treatment (**Figure 2B**).

We next focused on the role of calpain, a calcium-dependent cysteine protease, in cordycepin-induced apoptosis. The activation of calpain leads to the activation of caspases 3 and 7, followed by downstream cleavage of α-spectrin (*21*). Calpain activation results in the cleavage of 280 kDa α-spectrin to breakdown products (BDPs) of 150 and 145 kDa, while caspase activation results in the cleavage of α-spectrin to BDPs of 150 and 120 kDa (*22*). The 150 kDa BDP is therefore not specific for calpain, but the presence of the 145 kDa BDP can be used as a selective assay for calpain activity (*20*). Western blot analyses showed that levels of the 145 kDa BDP of α-spectrin was increased at 6 h and kept constant up to 24 h after cordycepin treatment (**Figure 3A**), indicating the activation of calpain. Besides, the levels of 120 kDa BDP were increased from 12 to 24 h after cordycepin treatment, suggesting caspase activation (**Figure 3A**).

A calcium chelator, BAPTA/AM (10 μM), blocked the cordycepin-induced increased levels of the α-spectrin 145 kDa BDP (**Figure 3B**), demonstrating that cordycepin-induced calpain activation was calcium-dependent. Moreover, DAPI staining showed that 10 μM calpeptin, a calpain inhibitor, inhibited

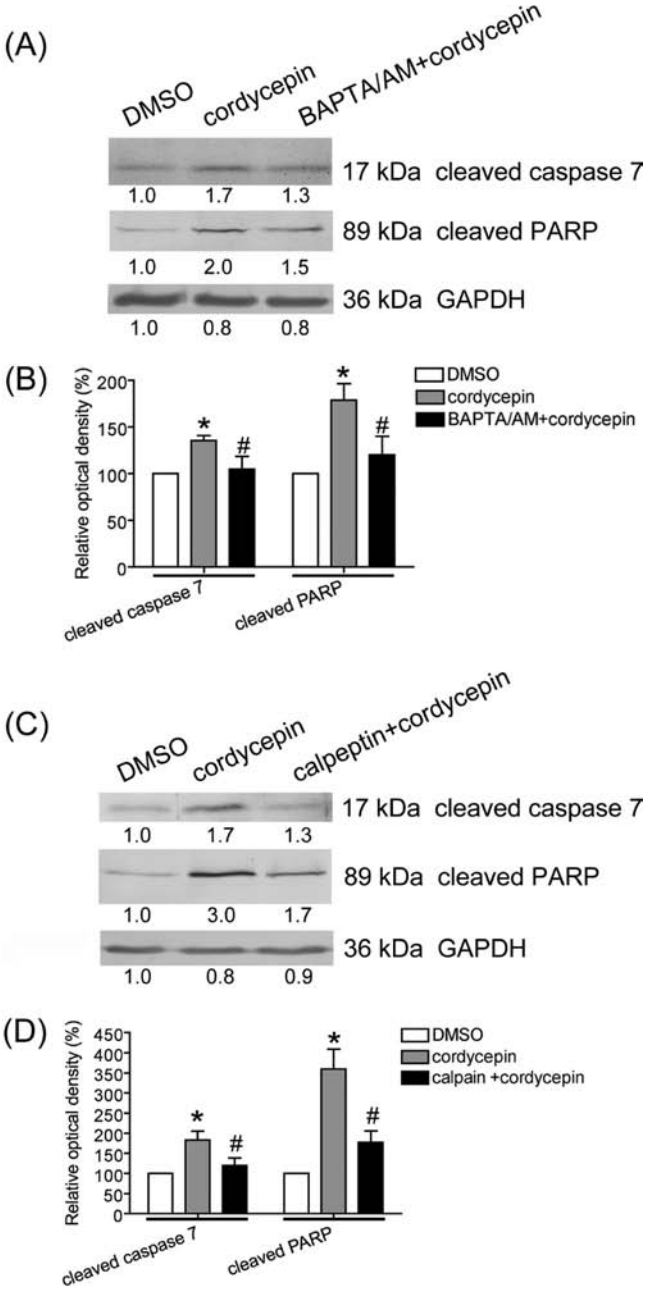

Figure 5. Cordycepin-induced cleavage of caspase 7 and PARP is mediated by calpain. CGTH W-2 cells were treated for 24 h with 0.2% DMSO or 40 μM cordycepin or for 30 min with 10 μM BAPTA/AM, then for 24 h with cordycepin in the continued presence of BAPTA/AM (**A**) or with 10 μM calpeptin instead of BAPTA/AM (**C**), then the cell lysates were analyzed for cleaved caspase 7 and PARP by Western blotting (**B** and **D**). The results shown in **A** and **C** are representative of those obtained in 3 or 6 experiments, respectively. GAPDH is the control. *, $p < 0.05$ compared to the DMSO group. #, $p < 0.05$ compared to the cordycepin-treated group.

cordycepin-induced apoptosis (**Figure 3C**), confirming that cordycepin-induced apoptosis is calpian dependent. These results show that cordycepin induced the increase in intracellular calcium levels, which leads to calpain activation and subsequent cell apoptosis.

Apoptotic Effect of Cordycepin Is Mediated by the Calpain−Caspase 7-PARP Pathway. Activation of caspase 3 or 7 results in cleavage of the downstream protein, PARP (*23*). We failed to detect the cleavage of caspase 9 (data not shown) or

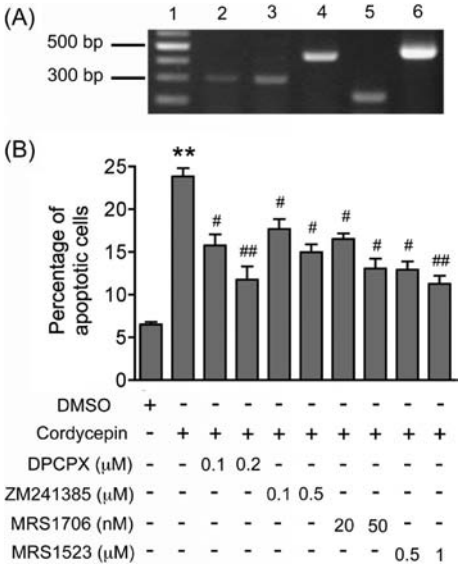

Figure 6. A_1AR, A_{2A}AR, A_{2B}AR, and A_3AR are involved in cordycepin-induced apoptosis. (**A**) RT-PCR of AR mRNAs in untreated CGTH W-2 cells. Lane 1: DNA markers. Lanes 2−6: amplification fragments corresponding to A_1AR mRNA (309 bp) (lane 2), A_{2A}AR mRNA (304 bp) (lane 3), A_{2B}AR mRNA (432 bp) (lane 4), A_3AR mRNA (220 bp) (lane 5), or β-actin (lane 6, loading control) in an agarose gel stained with ethidium bromide. (**B**) Effects of AR antagonists on cordycepin-induced apoptosis. CGTH W-2 cells were treated (i) for 24 h with 0.2% DMSO or 40 μM cordycepin or (ii) for 30 min with 100 nM or 200 nM DPCPX, 100 nM or 500 nM ZM241385, 20 nM or 50 nM MRS1706, and 0.5 μM or 1 μM MRS152340, then for 24 h with cordycepin in the continued presence of the AR antagonists. Apoptotic cells were identified by DAPI staining. $n = 6$. **, $p < 0.01$ compared to the DMSO group. #, $p < 0.05$; ##, $p < 0.01$ compared to the cordycepin-treated group.

caspase 3 (**Figure 4A**) after cordycepin treatment, whereas 1 μM staurosporine, which caused 96% cell apoptosis used as a positive control, induced the cleavage of caspase 3 to the 17/19 kDa fragment (**Figure 4A**). It is known that calpain activation results in caspase 7 cleavage into 18/17 kDa fragments (*24*). Here, we noted that a 17 kDa fragment of caspase 7 was generated by cordycepin treatment (**Figure 4A**). Cells treated with cordycepin showed a significant time-dependent increase in levels of cleaved caspase 7 and PARP (**Figure 4A**). Quantitative analyses of cleaved caspase 7 (**Figure 4B**) and PARP levels (**Figure 4C**) showed an over 3-fold increase in cordycepin-treated cells compared to that in the DMSO controls. These results suggest that cordycepin causes caspase 7 activation and PARP cleavage.

To determine whether calcium and calpain were upstream of caspase 7-PARP, we found that pretreatment with BAPTA/AM or calpeptin blocked both effects (**Figures 5A** and **C**). Quantitative analyses of the levels of cleaved caspase 7 and cleaved PARP are presented in **Figures 5B** and **D**. These results indicate that the caspase 7-PARP cascade was downstream of calcium-calpain cascade. Taken together, our data show that cordycepin-induced CGTH W-2 cell apoptosis is mainly mediated through the calcium−calpain−caspase 7-PARP pathway.

Induction of Apoptosis by Cordycepin through ARs. Because it has been reported that the growth inhibitory effect of cordycepin on mouse melanoma and lung carcinoma is mediated through A_3ARs (*6*, *16*), we next investigated the roles of ARs in cordycepin-induced apoptosis. Untreated CGTH W-2 cells expressed mRNAs for all four AR subtypes by RT-PCR analysis

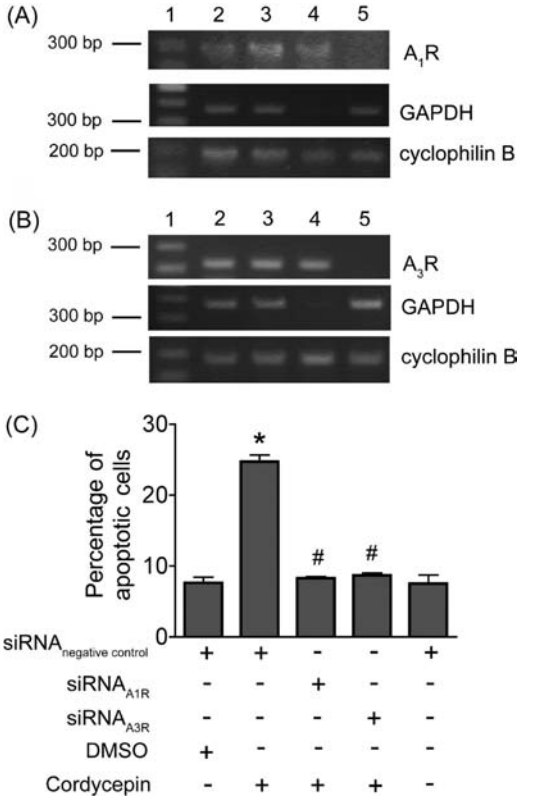

Figure 7. A_1AR or A_3AR siRNA prevents cordycepin-induced apoptosis. (**A**) RT-PCR of A_1AR mRNA. The amplification fragment corresponds to A_1AR mRNA (309 bp). Lane 1: DNA marker. Lane 2: untreated cells. Lane 3: negative control siRNA. Lane 4: GAPDH siRNA, positive control. Lane 5: A_1AR siRNA. CyclophilinB is the internal control. (**B**) RT-PCR of A_3AR mRNA. The amplification fragment corresponds to A_3AR mRNA (220 bp). Lane 1: DNA marker. Lane 2: untreated cells. Lane 3: negative control siRNA. Lane 4: GAPDH siRNA, positive control. Lane 5: A_3AR siRNA. CyclophilinB was used as the internal control. (**C**) A_1AR or A_3AR siRNA abolishes the apoptosis induced by cordycepin. CGTH W-2 cells were left untreated or were transfected with $siRNA_{negative\ control\ (ctrl)}$ for 72 h, then DMSO or cordycepin was added for 24 h. Experimental groups were transfected with $siRNA_{A1R}$ or $siRNA_{A3R}$ for 72 h, then cordycepin was added for 24 h. Apoptotic cells were identified by DAPI staining. $n = 3$. *, $p < 0.05$, compared to the $siRNA_{negative\ ctrl}$-DMSO group. #, $p < 0.05$, compared to the $siRNA_{negative\ ctrl}$-cordycepin group.

(**Figure 6A**). To elucidate the relationship between ARs and cordycepin-induced apoptosis, we used specific antagonists of each AR. DPCPX (500 nM, an A_1AR antagonist) or MRS1523 (1 μM, an A_3AR antagonist) blocked cordycepin-induced apoptosis more effectively than ZM241385 (100 or 500 nM, an A_{2A}AR antagonist) or MRS1706 (20 or 50 nM, an A_{2B}AR antagonist) (**Figure 6B**). Furthermore, we used the siRNA approach to further confirm the roles of A_1AR and A_3AR in cordycepin-induced apoptosis. After transfection with A_1AR or A_3AR siRNAs, expression of A_1AR and A_3AR mRNAs in CGTH W-2 cells were effectively silenced (**Figures 7A** and **B**). Transfection with A_1AR or A_3AR siRNA significantly abrogated the cordycepin-induced apoptosis of CGTH W-2 cells (**Figure 7C**). These results verified that cordycepin-induced apoptosis is mediated through ARs.

DISCUSSION

In this study, we found that cordycepin induced the death of CGTH W-2 cells by apoptosis but not necrosis. Cordycepin

induced an increase in internal calcium and activation of calpain, which triggered caspase 7 activation and PARP cleavage. All four types of ARs contributed to this apoptotic effect of cordycepin.

A_1AR, $A_{2A}AR$, $A_{2B}AR$, and A_3AR have been shown to regulate cell cycle and cell survival (*15*). In RCR-1 astrocytoma cells and CW2 human colonic cancer cells, activation of A_1AR by adenosine leads to apoptosis accompanied by activation of caspases 3, 8, and 9 (*25*, *26*). Nakamura et al. (*16*) showed that the A_3AR antagonist prevents the cordycepin-induced growth inhibition of mouse melanoma or lung carcinoma cells with an IC_{50} of 39 μM or 48 μM, respectively. A_3AR antagonist abrogates cordycepin-induced growth inhibition of B16-BL6 mouse melanoma cells via decreased expression of A_3R-dependent GSK-3β and cyclin D1 in mouse melanoma (*8*). Besides, A_3AR agonists cause caspase 9- and caspase 3-dependent apoptosis in human lung cancer cells and colon carcinoma (*27*). In this study, A_1AR, $A_{2A}AR$, $A_{2B}AR$, and A_3AR are responsible for the cordycepin-induced apoptosis in CGTH W-2 cells. A molecular approach by siRNA to knockdown A_1AR and A_3AR was used to confirm the role of A_1AR and A_3AR in the cordycepin anticancer effect. Interestingly, siRNA-mediated knock down of A_1AR or A_3AR alone led to almost complete inhibition of the apoptosis-inducing effect of cordycepin. In the rat hippocampus, the activation of A_3AR leads to the desensitization of A_1AR, showing an interaction between A_1AR and A_3AR (*28*). The possible collaborative actions between A_1AR and A_3AR can explain why the knock down of A_1AR or A_3AR alone resulted in almost complete abolishment of the apoptosis-inducing effect of cordycepin.

There are two possible sources of the additional intracellular calcium, the intracellular calcium pool, and the extracellular medium. In A_1AR or A_3AR-transfected CHO cells, the specific A_1AR or A_3AR agonist caused an increase in inositol phosphatase-dependent calcium levels, and this effect was blocked by pertussis toxin (*29*, *30*). In human neutrophils and promyelocytic leukemia cells, the A_3AR agonist-induced increase in intracellular calcium levels is not reversed by 0.5 mM EGTA but by the PLC inhibitor (*31*), demonstrating that the A_3AR agonist-induced release of calcium from the intracellular pool is mediated by PLC activation. In this study, the elevated intracellular calcium might originate from the extracellular pool via the A_1AR/A_3AR-GPCR-PLC pathway. However, the additional intracellular calcium may come from intracellular pools. Various stimuli cause endoplasmic reticulum stress and trigger calcium release, leading to apoptosis (*21*, *32*).

Caspase activation has been previously reported in cordycepin-induced apoptosis in other cell types. Immunostaining and membrane fractionation studies provided no support for the cytosolic release of cytochrome *c* (data not shown), and no cleavage of caspase 3, 8, or 9 was detected by Western blotting. These results showed that neither the intrinsic nor the extrinsic pathway were involved in the cordycepin-induced apoptosis of CGTH W-2 cells. Instead, another effector caspase, caspase 7, was activated by cordycepin treatment in this study. The increase in intracellular calcium levels leads to downstream calpain activation, and subsequent activation of caspase 7 and 12 has been reported in human prostate and melanoma cells (*33*, *34*). Thus, the calcium−calpain−caspase 7 pathway is also present in other cell types. This difference in caspase activation in different studies may be due to the different cell models used. Besides caspase 3 and 7, the activation of caspase 2 or 12 can also lead to PARP cleavage (*35*, *36*). Therefore, caspase 7 may not be the only one upstream of cleaved PARP in cordycepin-induced apoptosis in CGTH W-2 cells.

In CGTH W-2 cells, cordecepin-induced calpain stimulation led to caspase 7 activation. The evidence for calpain activation is the presence of 145 kDa BDP of α-spectrin. In this study, cordycepin-induced increase in calpain activity was not dose-dependent, as previously reported by Martinez et al. (*37*). Thapsigargin-increased calpain activity assayed by α-spectrin cleavage was not dose-dependent in rat PC12 cells (*37*). In addition, the appearance of 120 kDa BDP of α-spectrin was noted. The 120 kDa BDP of α-spectrin can be produced by enzyme cleavage of caspase 3 or 7 (*22*). Since caspase 3 is not activated by cordycepin treatment, it is possible that caspase 7 activated by calpain is responsible for the production of 120 kDa BDP of α-spectrin.

Cordycepin is also a well-known polyadenylation inhibitor. Polyadenylation determines the synthesis of functional mRNA and its transport from the nucleus to the cytoplasm. After cordycepin injection, nuclear precursor mRNA polyadenylation in rat brain cells is suppressed, and some translatable mRNAs are degraded (*38*). Levels of both total RNA and poly(A)$^+$ RNA are decreased by cordycepin treatment in multiple myeloma cells, and this leads to apoptosis (*1*). Recently, cordycepin is shown to inhibit protein translation via adenosine 5′-monophosphate-activated protein-activated kinase in NIH 3T3 fibroblasts (*39*). It is possible that cordycepin-induced apoptosis in CGTH W-2 cells can be mediated either by the suppression of polyadenylation or by the inhibition of protein synthesis.

In conclusion, in addition to its known function of polyadenylation inhibition, cordycepin triggers the apoptosis of CGTH W-2 cells through all four subtypes of ARs. Drugs or ligands that target ARs might be useful in thyroid cancer therapy in addition to total thyroidectomy and radioiodine administration.

ABBREVIATIONS USED

ARs, adenosine receptors; BDP, breakdown product; DAPI, 4′,6-diamidino-2-phenylindole dilactate; DMSO, dimethyl sulfoxide; GPCR, G protein-coupled receptor; mRNA, mRNA; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PARP, poly (ADP-ribose) polymerase; PBS, phosphate-buffered saline; PI, propidium iodide; PLC, phospholipase C; RT-PCR, reverse transcription−polymerase chain reaction; small interfering RNA, siRNA; TBS, Tris-buffered saline.

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