Full Paper

Cordycepin Prevents Hyperlipidemia in Hamsters Fed a High-Fat Diet via Activation of AMP-Activated Protein Kinase

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Abstract. Hyperlipidemia is a major risk factor for cardiovascular diseases. In this study, we investigated the potential effects of cordycepin (3'-deoxyadenosine), a bioactive component of the fungus *Cordyceps militaris*, on hyperlipidemia. We found that in male Syrian golden hamsters fed a high-fat diet (HFD), daily administration of cordycepin effectively reduced the accumulation of serum total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-c) and suppressed HFD-associated increases in relative retroperitoneal fat. It also increased the levels of phospho-AMP-activated protein kinase (AMPK) and phospho-acetyl-CoA carboxylase (phospho-ACC) in liver and retroperitoneal adipose tissues. In HepG2 cells, cordycepin stimulated robust concentration- and time-dependent AMPK activation that correlated with the activation of ACC and the suppression of lipid biosynthesis. However, pretreatment with compound C, a specific inhibitor of AMPK, substantially abolished the effects of cordycepin on AMPK activation and lipid biosynthesis inhibition. These results indicate that cordycepin prevents hyperlipidemia via activation of AMPK. Experiments on abnormal metabolic mice indicated that cordycepin can also improve insulin sensitivity effectively.

Keywords: cordycepin, hyperlipidemia, AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), *Cordyceps militaris*

Introduction

Cardiovascular diseases are among the leading causes of death throughout the world, and their occurrence is related to a variety of factors. Among these many risk factors, elevated levels of blood lipids are considered to be of particular concern and are extremely widespread (1). Common manifestations of hyperlipidemia include elevated levels of total cholesterol (TC), triglycerides (TG), and low-density lipoprotein cholesterol (LDL-c); all three of these are known to be important susceptibility

factors for developing heart diseases, including atherosclerosis and coronary heart disease (CHD) (2 – 4). Although diet control and exercise can prevent hyperlipidemia and are always recommended, individuals with a higher risk of developing hyperlipidemia may also require effective lipid-lowering therapy in order to re-attain healthy levels of blood lipids.

AMP-activated protein kinase (AMPK), a key cellular energy sensor, plays a critical role in the regulation of fat metabolism (5). When activated, it decreases fatty acid levels by phosphorylating and thus inhibiting acetyl-CoA carboxylase (ACC), a critical enzyme for controlling fatty acid biosynthesis and oxidation. The activation of AMPK also decreases TC and TG levels by inhibiting the activity of glycerol-3-phosphate acyltransferase (GPAT) and HMG-CoA reductase, the two rate-limiting

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enzymes in TC and TG synthesis, respectively (6). AMPK has therefore been proposed as a major therapeutic target for obesity and obesity-linked metabolic disorders such as hyperlipidemia (7).

Cordycepin, also known as 3'-deoxyadenosine, is a bioactive compound present in species of fungi belonging to the genus *Cordyceps* (8). It was initially identified as a selective inhibitor of poly (A) synthesis (9), a property that has been intensively investigated in cancer treatment (10, 11). In recent years, many other pharmacological properties of cordycepin have been uncovered, including anti-viral (12), anti-fungal (13), anti-inflammatory (14), anti-hyperglycemia (15), and anti-atherosclerosis actions (16). Cordycepin is structurally highly analogous to adenosine, an activator of AMPK (17); however, the effects of cordycepin on AMPK activation and lipid metabolism have not been studied to date.

In the present study, we investigated the antihyperlipidemic and lipid-lowering capacities of cordycepin in Syrian golden hamsters fed a high-fat diet (HFD). Furthermore, we tested the effects of cordycepin on TC and TG biosynthesis, as well as on the phosphorylation and activation of AMPK in HepG2 cells. We also studied the effect of compound C, a specific inhibitor of AMPK, on the AMPK-activating and lipid biosynthesis inhibiting role of cordycepin in HepG2 cells. Our results, presented here, suggest that cordycepin is an effective AMPK activator capable of suppressing HFD-induced hyperlipidemia.

Materials and Methods

HFD hamsters

All experiments were performed according to the guidelines of the Chinese Academy of Medical Science for Institutional Animal Care. Fifty-eight male Syrian golden hamsters (Vital River Laboratory Animal Technology Co., Ltd., Beijing, China) were kept in a humidity-controlled room on a 12-h light–dark cycle with food and water available ad libitum. After 1 week of acclimation, body weights were measured. Hamsters weighing 90 ± 10 g were divided into six diet regimen groups. These regimens were as follows: a control diet consisting of normal food ($n = 8$); a HFD ($n = 10$); a HFD in combination with one of three cordycepin dosages (12.5, 25, or 50 mg/kg per day, i.g.; $n = 10$ per group); and a HFD in combination with fenofibrate (40 mg/kg per day; $n = 10$) (Laboratoires Fournier S.A., Chenove, France). The food for the HFD was prepared by mixing pellets from the normal diet with groundnut oil (200 g/kg HFD) and cholesterol (2 g/kg HFD). At the end of the 2-week period, the weight of each animal was measured and blood samples were collected for the estimation of serum TC, TG, LDL-

c, and high-density lipoprotein cholesterol (HDL-c) (Jian Cheng Biotechnology Company, Nanjing, China) after they were fasted overnight. Hamsters were then euthanized and the weight of the liver and retroperitoneal adipose tissue was determined.

Abnormal metabolic mice

Four-week-old male C57BL/6 mice were obtained from the Animal Center of the Institute of Laboratory Animal Sciences, Chinese Academy of Medical Sciences & Peking Union Medical College. Mice were housed 4 per cage and kept in a humidity-controlled room on a 12-h light–dark cycle with food and water available ad libitum. One group of mice used for the control was allowed to eat normal rodent chow. The rest of the mice were fed with a modified high-fat diet (mHFD) ad libitum for 20 weeks. The normal rodent chow contained (in energy %) 12% fat, 62% carbohydrate, and 26% protein, with a total energy content of 12.6 kJ/g. The mHFD was formulated to balance micronutrient content on a per calorie basis and contained 60% fat, 14% protein, and 26% carbohydrate, with total energy content of 21.0 kJ/g. The mHFD contained much less choline bicitrate (0.6 g/kg) and DL-methionine (1.5 g/kg). Fatty acid composition of the fats (mainly from lard) in mHFD was 36% saturated fatty acids, 45% monounsaturated fatty acids, and 19% polyunsaturated fatty acids (PUFA).

After 20-week feeding, the mice fed with mHFD were randomly divided into three groups, the model group, cordycepin group, and fenofibrate group, with 10 mice per group. Cordycepin (50 mg/kg body weight per day) and rosiglitazone (30 mg/kg body weight per day) (Laboratoires Fournier S.A.) were administered by gavage for the following 6 weeks. The model group and the control group received distilled water. All mice were sacrificed by decapitation after 4-h food deprivation. Blood samples were collected for the assays of glucose and insulin.

Serum was separated by centrifugation at 4°C and analyzed immediately or stored at –20°C. Blood glucose levels were determined with a glucose analyzer (EKF Diagnostic Company, Barleben, Germany). Insulin levels were measured by radioimmunoassay (Northern Bioengineering Institute, Beijing, China).

Oil-Red O stain

Liver tissues of hamsters were removed and each liver lobe was cut into small pieces. To detect fat deposition in the liver, frozen sections were rinsed with distilled water, stained with 0.2% Oil-Red O (Sigma-Aldrich, Shanghai, China) and 60% 2-propanol (Sigma-Aldrich) for 10 min at 37°C, and then rinsed with distilled water. We observed the liver tissues under a microscope (Leica DM4000B, Bensheim, Germany) and photographed the samples with

a digital camera.

Cell culture

HepG2 cells, which originated from the American Type Culture Collection (ATCC) (Manassas, VA, USA), were obtained from the China Union Medical University. Cells were grown to 70%–80% confluence and then incubated in 0.02% BSA (Sigma-Aldrich) / DMEM (Gibco-BRL, Grand Island, NY, USA) for 24 h. Cells were then washed and incubated with the indicated concentration of either cordycepin or the AMPK activator 5-aminoimidazole-4-carboxamide 1-beta-ribofuranoside (AICAR; Sigma-Aldrich, China) in 0.02% BSA/DMEM or 0.02% BSA/DMEM alone for the indicated periods of time. Cordycepin (3'-deoxyadenosine), with a purity of 99% as determined by HPLC, was provided by the Key Laboratory of Natural Drugs Biosynthesis (Chinese Academy of Medical Sciences & Peking Union Medical College).

Compound C pretreatment

To verify the specificity of cordycepin in AMPK activation, we tested the effect of the AMPK inhibitor Compound C (Sigma-Aldrich) on the AMPK-activating roles of cordycepin in HepG2 cells. Cells were grown to 70%–80% confluence and incubated in 0.02% BSA/DMEM for 24 h. Compound C (40 μ M) was then added to the culture. After another 30 min, cells were treated with cordycepin (10^{-6} M) or solvent only for 6 h.

MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assays were performed as follows: First, the cytotoxicity of cordycepin on HepG2 cells (IC_{50}) was determined for each period of exposure to determine an appropriate test concentration. Cells (10^6 M) were then treated with cordycepin for the indicated time period prior to the addition of MTT (Sigma-Aldrich) and then incubated for an additional 4 h at 37°C. The absorbance of the cell lysate solution was measured at 490 nm. Each experiment was performed in triplicate.

Western blotting

Livers, retroperitoneal adipose tissues and HepG2 cells were lysed in lysing buffer containing 10% glycerol, 1% Triton X-100, 135 mM NaCl, 20 mM Tris (pH 8.0), 2.7 mM KCl, 1 mM $MgCl_2$, and protease and phosphatase inhibitors (0.5 mM PMSF, 2 μ M pepstatin, and 2 μ M okadaic acid). Protein concentrations were assessed using the Bradford method, and aliquots of samples containing 40 μ g protein were subjected to SDS-PAGE (12% and 7.5% for P-AMPK and P-ACC, respectively) and then transferred to PVDF membranes (Amersham Phar-

macia, Uppsala, Sweden). Immunoblotting was performed using phospho-AMPK α (Thr-172, 1:3000) and phospho-ACC (1:2000) antibodies (Cell Signaling Technology, Beverly, MA, USA), as well as anti-beta-actin (included as a loading control; Abcam, Inc., Cambridge, MA, USA). Following incubation with horseradish peroxidase-conjugated secondary antibodies (Sigma-Aldrich, China), proteins were detected with ECL plus kits (Amersham, Piscataway, NJ, USA). Immunoreactive bands were quantified using NIH image analysis software.

Lipid synthesis assay

HepG2 cells cultured as described above were washed with fresh medium after being treated with cordycepin or AICAR or cordycepin and compound C and then incubated for 2.5 h with [$1-^{14}C$]acetate acid (0.2 μ Ci/ml) (Amersham Biosciences, Uppsala, Sweden). Lipids were extracted with *n*-hexane : 2-propanol (3:2, v/v) and dried under a nitrogen stream. Aliquots of lipid samples were loaded onto thin-layer chromatography plates and chromatographed in a solvent mixture containing hexane, diethyl ether, and acetic acid (85:30:1, v/v/v). Bands corresponding to TG and cholesteryl esters were scraped off, immersed in nonaqueous scintillation fluid, and counted by measuring radioactivity.

Evaluation of insulin sensitivity

The insulin sensitivity was estimated by the insulin tolerance test (ITT), oral glucose tolerance test (OGTT) in vivo, and the homeostasis model assessment of insulin resistance (HOMA-IR) index. Food was removed for 2 h before the tests. Insulin (0.4 IU/kg body weight) (Lilly, Fegersheim, France) was administered by subcutaneous injection, and blood samples were taken from tail vein droplets at time points of 0, 30, 60, and 120 min after insulin loading. The blood glucose concentration was immediately determined. In the OGTT, a glucose load (2 g/kg body weight) was given by gavage, and the blood samples were collected and determined as above. HOMA-IR index was calculated by the formula (HOMA-IR = FINS $\times$ FBG / 22.5) as reported previously (FBG: fasting blood glucose, FINS: fasting insulin).

Statistical analyses

Data are presented as the mean $\pm$ S.E.M. One-way ANOVA was used to determine significant differences among groups, after which the modified Student's *t*-test with the Bonferroni correction was used for comparison between individual groups. $P < 0.05$ was considered statistically significant.

Results

Effects of cordycepin treatment on HFD-induced hyperlipidemia in hamsters

To determine the effect of cordycepin on regulating lipid metabolism, adult hamsters were fed a normal diet, a HFD, a HFD together with cordycepin treatment (12.5, 25, or 50 mg/kg per day), or a HFD with fenofibrate treatment (40 mg/kg per day). The food intake and body weight of hamsters were measured every other day during the experiment. The results showed that the food intake of animals was not affected by cordycepin or fenofibrate treatment. However, the body weights of hamsters treated with cordycepin (50 mg/kg) or fenofibrate (40 mg/kg) was significantly decreased as compared with the HFD control at the end of the two weeks (Table 1). In all HFD groups, marked increases were also observed in relative retroperitoneal fat levels (Fig. 1A). Among the HFD-treated groups, however, these values were significantly lower in the groups treated with the highest concentrations of cordycepin (50 mg/kg per day) compared to HFD alone (Fig. 1A).

HFD feeding also led to significant increases in serum TC, TG, and LDL-c (Table 1). Treating hamsters fed the HFD with any of the three cordycepin dosages significantly decreased the levels of serum TC, TG, and LDL-c compared to untreated HFD-fed hamsters. Interestingly, the effect of 50 mg/kg cordycepin on preventing lipid accumulation was comparable to that of 40 mg/kg fenofibrate, an established hypolipidemic agent. The lipid accumulation in liver was visualized by Oil-Red O stain. Cordycepin and fenofibrate both decreased the proportion of lipid droplets in the liver markedly (Fig. 1B), suggesting a preventive role of cordycepin in liver lipid

accumulation.

Cordycepin increases AMPK phosphorylation

Activation of AMPK has been proposed to be a central event in maintaining cellular energy homeostasis and is known to play a key role in fat metabolism (5). Phosphorylation of threonine 172 in its α -subunit activates the kinase domain of AMPK, which in turn phosphorylates other target proteins. ACC is a major substrate for AMPK and, as such, the phosphorylation status of ACC is also often assayed as a proxy for AMPK activation. Thus, to determine whether cordycepin influences fat metabolism in hamsters by regulating AMPK activity, we measured levels of phospho-AMPK (Thr-172) and phospho-ACC in HepG2 cells, a human hepatocellular liver carcinoma cell line (18).

As shown in Fig. 2A, treatment with cordycepin (50 mg/kg) significantly increased the levels of phospho-AMPK and phospho-ACC in liver tissues of hamsters fed a HFD. These data suggested that the antihyperlipidemia effect of cordycepin was probably due to its AMPK-activating activity. To further validate this issue, HepG2 cells were used to test the concentration- and time-dependent effects of cordycepin on AMPK activation. As illustrated in Fig. 3, treating HepG2 cells with cordycepin led to concentration- and time-dependent increases in phospho-AMPK levels. Stimulating HepG2 cells for 3 h with 1 μ M cordycepin led to a significant increase in phospho-AMPK levels compared to unstimulated cultures, and the fold-difference reached a maximum at the highest dosage (10 μ M vs. control, 2.9 ± 0.3 -fold higher, $P < 0.05$) (Fig. 3A). Phospho-AMPK levels increased rapidly when HepG2 cells were treated with 1 μ M cordycepin, with significant increases observable

Table 1. Effects of cordycepin on body weight, food intake, TC, TG, HDL, and LDL in high-fat-diet (HFD) hamsters

	Control (n = 8)	HFD (n = 10)	HFD + cordycepin (12.5 mg/kg, n = 10)	HFD + cordycepin (25 mg/kg, n = 10)	HFD + cordycepin (50 mg/kg, n = 10)	HFD + fenofibrate (40 mg/kg, n = 10)
Body weight (g)	130.8 $\pm$ 3.9	151.0 $\pm$ 3.7***	141.4 $\pm$ 4.9	142.1 $\pm$ 5.7	137.3 $\pm$ 4.9††	136.7 $\pm$ 3.1††
Food intake (g/24 h)	11.58 $\pm$ 1.17	11.79 $\pm$ 1.37***	11.84 $\pm$ 1.64	11.63 $\pm$ 1.17	10.79 $\pm$ 2.79	10.57 $\pm$ 1.21
Serum						
TC (mM)	2.61 $\pm$ 0.23	8.07 $\pm$ 2.85***	6.30 $\pm$ 1.54	6.01 $\pm$ 1.72	5.35 $\pm$ 1.46†	5.24 $\pm$ 1.30†
TG (mM)	1.81 $\pm$ 0.38	7.00 $\pm$ 2.07***	5.17 $\pm$ 1.54	4.63 $\pm$ 1.17††	3.99 $\pm$ 1.80††	3.97 $\pm$ 1.70††
HDL (mM)	0.69 $\pm$ 0.17	0.84 $\pm$ 0.33	0.93 $\pm$ 0.28	0.78 $\pm$ 0.10	0.78 $\pm$ 0.13	0.85 $\pm$ 0.14
LDL (mM)	1.55 $\pm$ 0.20	4.52 $\pm$ 1.04***	3.27 $\pm$ 0.81	2.80 $\pm$ 0.77††	2.30 $\pm$ 0.48†††	2.51 $\pm$ 0.45†††
Hepatic						
TC (mmol/g Pr)	1.45 $\pm$ 0.45	2.69 $\pm$ 0.55***	2.32 $\pm$ 0.35	2.02 $\pm$ 0.17	1.92 $\pm$ 0.35††	2.28 $\pm$ 0.34
TG (mmol/g Pr)	0.58 $\pm$ 0.21	1.47 $\pm$ 0.43***	1.11 $\pm$ 0.29	0.99 $\pm$ 0.29	0.87 $\pm$ 0.31††	1.34 $\pm$ 0.21

Values are means $\pm$ S.D. *** $P < 0.001$ vs. control groups. † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$: HFD groups vs. HFD + cordycepin group.

Fig. 1. Effects of cordycepin on lipid accumulation in hamsters fed a high-fat diet (HFD). A) HFD-fed rats treated with 50 mg/kg per day cordycepin show significant decreases in retroperitoneal fat weights relative to untreated rats (right panel). * $P < 0.05$ vs. control, † $P < 0.05$: HFD group vs. HFD + cordycepin groups and HFD + fenofibrate group; $n = 10$ per group. B) Representative images of the Oil-Red O staining of liver tissues. Lipid accumulation was indicated by arrows. Bar = 25 μm .

after 1 h; levels then peaked after 3 h and decreased gradually over the next 9 h (Fig. 3B). Moreover, cordycepin (1 μM) treatment also caused a substantial increase in the levels of phosphorylated ACC after 3 and 6 h (Fig. 3C), providing further evidence that cordycepin is capable of activating AMPK.

We also found that treatment with cordycepin significantly increased the levels of phospho-AMPK and phospho-ACC in retroperitoneal adipose tissues of hamsters fed a HFD, as shown in Fig. 2B. Thus, cordycepin is also capable of activating AMPK in the visceral fat tissue of the hamsters.

Cordycepin inhibits lipogenesis in HepG2 cells

TC and TG concentrations in the blood are strongly associated with cardiovascular diseases. We therefore tested the effects of cordycepin on lipid synthesis in HepG2 cells using a [$1\text{-}^{14}\text{C}$]acetate acid absorption assay. Cordycepin significantly inhibited the biosynthesis of TC and TG ($P < 0.01$) in a wide range (0.1 – 10 μM , Fig.

4A). Treatment with 1 μM cordycepin was able to strongly inhibit the biosynthesis of TC and TG ($P < 0.01$) for at least 3 h (Fig. 4B). Together, these findings suggest that cordycepin promotes AMPK activity, thereby reducing lipid deposition.

Specificity of cordycepin in AMPK activation in HepG2 cells

To verify the specificity of cordycepin in AMPK activation, we tested the effect of the AMPK-inhibitor Compound C on the phosphorylation of AMPK and its downstream target ACC. In HepG2 cells, pretreatment with Compound C (40 μM) almost completely suppressed cordycepin-induced phosphorylation of AMPK and ACC without changing the expression levels of total AMPK, ACC, and β -actin (Fig. 5A). Similarly, the ability of cordycepin to reduce the biosynthesis of TC and TG contents in HepG2 cells was almost abolished by pretreatment with Compound C. These results indicate that reduced TG and cholesterol levels mediated by cordyce-

Fig. 2. Cordycepin enhances AMPK activation in liver and adipose tissues of hamsters fed a HFD. Treatment with 50 mg/kg cordycepin for two weeks significantly increased the levels of phospho-AMPK and phospho-ACC in liver tissues (A) and adipose tissues (B). Protein levels were standardized against β -actin levels, and the data are expressed as means $\pm$ S.D. of four to five individual samples. * $P < 0.05$ vs. control.

pin treatment in HepG2 cells is mainly achieved through AMPK activation (Fig. 5B).

Cordycepin does not affect cellular proliferation

Cordycepin can inhibit RNA synthesis and also possesses anti-tumor properties that are attributable to its cytotoxicity (19). We therefore used an MTT cytotoxicity assay to discern whether the hypolipidemic effects of cordycepin on HepG2 cells are attributable to cytotoxic effects. Incubating HepG2 cells with cordycepin (1 or 10 μ M) for 1, 3, 6, or 12 h had no adverse effect on cell proliferation (data not shown), suggesting that the inhibition of lipogenesis by cordycepin is likely due to its hypolipidemic and AMPK-activating properties, not to its cytotoxicity.

Cordycepin improves insulin resistance

To test the effect of cordycepin on insulin resistance, the abnormal metabolic mice induced by modified HFD were used. As shown in Table 2, abnormal metabolic mice showed significantly enhanced serum insulin levels as compared with the normal control. Treating with cordycepin greatly decreased the levels of serum insulin. HOMA-IR index was also higher in abnormal metabolic

mice and decreased after the treatment with cordycepin (Table 2).

After injection with insulin, the abnormal metabolic mice displayed reduced response to exogenous insulin in the ITT. Treatment with cordycepin significantly improved the insulin sensitivity (Fig. 6A). In OGTT, the abnormal metabolic mice displayed impaired oral glucose tolerance. Treatment with cordycepin significantly improved the glucose tolerability (Fig. 6B).

Discussion

Cordycepin is a bioactive component found in *Cordyceps militaris*, a fungus that is widely used in traditional Chinese medicine to treat a variety of diseases (20–24). Even though many pharmacological properties of cordycepin have been explored, previous studies have mainly focused on its anti-tumor and immune-regulating effects; and to our knowledge, no report on activity related to lipid metabolism has been published.

In this study, we evaluated the antihyperlipidemic effect of cordycepin in HFD-induced hyperlipidemic hamsters. Daily administration of cordycepin (50 mg/kg per day) significantly suppressed the increase of fat ac-

Fig. 3. Cordycepin activates AMPK in HepG2 cells. A) Top panel: Representative Western blot showing phospho-AMPK levels in HepG2 cell lysates. Cells were treated with 1 mM 5-aminoimidazole-4-carboxamide 1- β -D-ribofuranoside (AICAR) or 0 (control), 10, 1, 0.1, 0.01, or 0.001 μ M cordycepin for 3 h prior to harvesting. Compared to untreated control cells (left-most lane), cordycepin induces AMPK phosphorylation in a dose-dependent manner. Cordycepin 10 μ M induces AMPK phosphorylation to the same extent as the specific AMPK activator AICAR. Bottom panel: Quantification of phospho-AMPK band intensities normalized to the intensity of in-group total AMPK, represented as a fold change from the control (mean $\pm$ S.E.M., averaged over four independent experiments; * P < 0.05 vs. control). β -Actin was included in all Western blots as a loading control. B) Top panel: Representative Western blot showing phospho-AMPK levels in HepG2 cell lysates. Cells were treated with control solution or 1 μ M cordycepin for the indicated period of time before harvesting (control, no treatment). Bottom panel: Quantification of phospho-AMPK band intensities calculated as described in A. C) Representative western blot showing phospho-ACC levels in HepG2 cell lysates. Cells were treated with 1 μ M cordycepin or control solution for 3 or 6 h before harvesting.

Fig. 4. Inhibition of lipid synthesis by cordycepin. HepG2 cells were incubated with 0.01–10 μ M cordycepin for 6 h (A) or with 1 μ M cordycepin for 3 and 6 h (B) and then radiolabeled with [14 C]acetate acid. Intracellular triglycerides (TG) and cholesterol were extracted and analyzed as described in the Materials and Methods section. Values are expressed as percentages of the control (mean $\pm$ S.D.; ** P < 0.01, *** P < 0.001 vs. control).

cumulation in retroperitoneal fat wall associated with elevated fat intake. Furthermore, serum lipid metabolism symptoms associated with a HFD, such as increased serum TC, TG, and LDL-c, were all significantly improved by cordycepin treatment.

TC and TG are important components of blood and tissue lipids, and elevated levels of TC and TG are closely linked to cardiovascular diseases (25). In an effort to uncover the potential mechanisms by which cordycepin prevents hyperlipidemia, we evaluated the effect of cordycepin on the biosynthesis of these two substances in HepG2 cells using an [14 C]acetate acid absorption assay. Cordycepin significantly inhibited the biosynthesis of TC and TG in a wide concentration range (0.1–10 μ M). Furthermore, the efficacy of 1 μ M cordycepin rivaled that of 1 mM AICAR, a specific activator of AMPK and a known inhibitor of TC and TG biosynthesis (26). These data indicate that the anti-hyperlipidemia effects of cordycepin is likely attributable to a direct inhibition

Fig. 5. Specificity of cordycepin in AMPK activation. A) Increased phosphorylation of AMPK and ACC in HepG2 cells upon treatment with 1 μ M cordycepin for 6 h was abrogated by pretreatment with 40 μ M compound C for 30 min. B) Decreases in triglyceride and cholesterol biosynthesis after treatment with 1 μ M cordycepin for 6 h were completely inhibited by pretreatment with 40 μ M compound C for 30 min. Values are expressed as percentages of the control (mean $\pm$ S.D.; *** P < 0.001 vs. control).

Fig. 6. Cordycepin treatment improves insulin sensitivity in the abnormal metabolic mice. A) Insulin sensitivity assessed by the insulin tolerance test. B) Glucose tolerability assessed by the oral glucose tolerance test. # P < 0.05, ## P < 0.01, ### P < 0.001 vs. control group; * P < 0.05, ** P < 0.01, *** P < 0.001 vs. HFD group.

Table 2. Effects of cordycepin on serum insulin levels in the abnormal metabolic mice

	Control	HFD	HFD + cordycepin	HFD + rosiglitazone
Insulin (mIU/L)	11.2 $\pm$ 0.7	68.9 $\pm$ 5.1***	44.9 $\pm$ 5.1##	28.9 $\pm$ 8.1##
HOMA-IR (mM/L mIU/L)	2.12 $\pm$ 0.8	20.2 $\pm$ 2.2***	7.89 $\pm$ 0.7##	5.23 $\pm$ 0.6##

Values are means $\pm$ S.D. *** P < 0.001 vs. control group; ## P < 0.01 vs. HFD group.

of TC and TG synthesis in the liver.

The activation of AMPK plays a critical role in fat metabolism. Previously, adenosine has been reported to be an effective activator of AMPK (17). As cordycepin is a structural analogue of adenosine, it is reasonable to speculate that it is also an AMPK activator. Western blotting demonstrated that cordycepin (≥ 0.01 μ M) stimulated the phosphorylation of AMPK, which in turn triggered the activation of the fatty acid biosynthesis enzyme ACC, thus demonstrating that cordycepin is a potent AMPK activator. Furthermore, pretreatment with Compound C (40 μ M) almost completely suppressed cordycepin-induced phosphorylation of AMPK and ACC. Similarly, the ability of cordycepin to reduce the biosynthesis of TC and TG contents in HepG2 cells was almost abolished by pretreatment with Compound C. These results indicate that the reduced of lipid levels by

cordycepin is mainly achieved through AMPK activation in HepG2 cells. In addition, the capacity of inducing AMPK expression by 1 μ M cordycepin was equivalent to that of 1 mM AICAR, a well-known AMPK activator. As AMPK activity can have profound effects on preventing hyperlipidemia as well as cardiovascular diseases, these results suggest that cordycepin could be used in the treatment of hyperlipidemia.

It has been widely reported that AMPK plays an important role in insulin signaling (27 – 29). In this work, we also tested the effect of cordycepin on insulin resistance in abnormal metabolic mice. Animals fed a HFD for 20 weeks showed high serum insulin levels and were resistant to insulin injection. Treatment with 50 mg/kg cordycepin greatly enhanced the insulin sensitivity and improved the oral glucose tolerance. These results indicated that cordycepin may be also effective for the treat-

ment of insulin resistance.

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