RESEARCH PAPER

Blockade of adipocyte differentiation by cordycepin

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Keywords

cordycepin; adipocyte; adipogenesis; mTORC1; C/EBP β ; PPAR γ

Received

8 November 2011

Revised

23 March 2012

Accepted

16 April 2012

BACKGROUND AND PURPOSE

Cordyceps militaris has the potential to suppress differentiation of pre-adipocytes. However, the active entities in the extract and the underlying mechanisms of its action are not known. Hence, we investigated whether and how cordycepin (3'-deoxyadenosine), a constituent of *C. militaris*, inhibits adipogenesis.

EXPERIMENTAL APPROACH

Differentiation of 3T3-L1 pre-adipocytes and pre-adipocytes in primary cultures was induced by Insulin, dexamethasone and IBMX, and these were used as *in vitro* models of adipogenesis. The effects of cordycepin on adipogenesis were examined with particular focus on the regulation of CCAAT/enhancer-binding protein β (C/EBP β) and PPAR γ .

KEY RESULTS

Cordycepin suppressed the lipid accumulation and induction of adipogenic markers that occurred on differentiation of pre-adipocytes and also blocked the down-regulation of a pre-adipocyte marker. This anti-adipogenic effect was reversible and mediated by an adenosine transporter, but not A₁, A₂ or A₃ adenosine receptors. This effect of cordycepin was not reproduced by other adenosine-related substances, including ATP, ADP and adenosine. Early induction of the adipogenic C/EBP β -PPAR γ pathway was suppressed by cordycepin. Blockade of mTORC1 via inhibition of PKB (Akt) and activation of AMP kinase was identified as the crucial upstream event targeted by cordycepin. In addition to its negative effect on adipogenesis, cordycepin suppressed lipid accumulation in mature adipocytes.

CONCLUSIONS AND IMPLICATIONS

These results suggest that the anti-adipogenic effects of cordycepin occur through its intervention in the mTORC1-C/EBP β -PPAR γ pathway. Cordycepin, by blocking both adipogenesis and lipid accumulation, may have potential as a therapeutic agent for effective treatment of obesity and obesity-related disorders.

Abbreviations

4E-BP1, eukaryotic initiation factor 4E binding protein 1; AhR, aryl hydrocarbon receptor; AMPK, AMP-activated protein kinase; C/EBP, CCAAT/enhancer-binding protein; CHOP, C/EBP-homologous protein; Cl-IB-MECA, 2-chloro-N⁶-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide; CYP1B1, cytochrome P4501B1; DMEM-F12, Dulbecco's modified Eagle's medium/Ham's F-12; DN, dominant-negative; DPSPX, 1,3-dipropyl-8-(*p*-sulfophenyl)xanthine; DRESSA, dioxin responsive element-based sensing via secreted alkaline phosphatase; eIF2 α , eukaryotic initiation factor 2 α ; FBS, fetal bovine serum; GRP78, 78 kDa glucose-regulated protein; IDI, insulin, dexamethasone and IBMX; IRS-1, insulin receptor substrate-1; LAP, liver activating protein; LIP, liver-enriched inhibitory protein; MCP-1, monocyte chemoattractant protein 1; MEFs, mouse embryonic fibroblasts; MRS1523, 3-propyl-6-ethyl-5[(ethylthio)carbonyl]-2-phenyl-4-propyl-3-pyridinecarboxylate; mTORC1, mammalian target of rapamycin complex 1; NBTI, S-(4-Nitrobenzyl)-6-thioinosine; p70S6K, p70S6 kinase; PPRE, peroxisome proliferator responsive element; SEAP, secreted alkaline phosphatase; Sirt1, Sirtuin 1; TSC2, tuberous sclerosis complex 2; XRE, xenobiotic responsive element

Introduction

Obesity is common in advanced countries. It increases the risk of diseases including type 2 diabetes, cardiovascular diseases and cancers. Obesity is considered to be a major, global health target because of its pathogenic properties (National Task Force on the Prevention and Treatment of Obesity, 2000; James, 2008). The use of anti-obesity agents may be a possible approach to overcome this problem. Currently, however, there are no drugs for the safe and successful treatment of obesity (Tam *et al.*, 2011). The endeavour to find novel and safe substances is an important challenge towards the solution of obesity-related health problems.

The increase in the mass of the adipose tissue arises through an increase in cell size, an increase in cell number or both (Spiegelman and Flier, 1996). Blockade of adipocyte generation is a target for developing anti-obesity agents. Mycelial extracts have a variety of biological activities including anti-cancer, anti-inflammatory and anti-diabetic effects (Lo *et al.*, 2004; Park *et al.*, 2009; Shin *et al.*, 2009). We previously reported that among 10 mycelial extracts, extract of *Cordyceps militaris* exclusively suppressed differentiation of pre-adipocytes, in part, through activation of the aryl hydrocarbon receptor (AhR) (Shimada *et al.*, 2008). However, the active entities responsible for the anti-adipogenic effect of *C. militaris* are currently unknown.

Cordycepin (3'-deoxyadenosine) is a constituent of *C. militaris* and has a wide range of biological effects including anti-tumourigenic, pro-apoptotic, anti-thrombotic and anti-inflammatory activities (Cho *et al.*, 2006; Lee *et al.*, 2009; Shin *et al.*, 2009; Jen *et al.*, 2011). It exerts its biological effects in part through binding to adenosine receptors (Nakamura *et al.*, 2006; Won *et al.*, 2009). Currently, the effects of this agent on adipogenesis have not been reported, but cordycepin could be responsible for the anti-adipogenic effect of *C. militaris*. The present investigation was initiated to examine this possibility.

Adipogenesis is a process in which fibroblast-like pre-adipocytes differentiate into spherical adipocytes with abundant lipids, and the expression and activation of various molecules are dynamically regulated during this process (Fe've, 2005). Among these, CCAAT/enhancer-binding protein (C/EBP) and PPAR γ are particularly important and regarded as key regulators for adipocyte differentiation (Rosen *et al.*, 2000). In the present investigation, we first examined the effects of cordycepin on the differentiation of pre-adipocytes and then the molecular mechanisms underlying these effects with particular focus on C/EBP β and PPAR γ . Our results suggest the novel anti-adipogenic potential of cordycepin through intervention in the activation of the C/EBP β -PPAR γ pathway.

Methods

Reagents

Cordycepin, adenosine, insulin, IBMX, dexamethasone, oil red O, 1,3-dipropyl-8-(*p*-sulfophenyl)xanthine (DPSPX), S-(4-nitrobenzyl)-6-thioinosine (NBTI), MRS1523, 2-chloro-N⁶-(3-iodobenzyl)adenosine-5'-N-methylcarboxamide (CI-IB-

MECA), rapamycin, resveratrol, splitomicin and palmitic acid were purchased from Sigma-Aldrich Japan (Tokyo, Japan). ATP, ADP, 3,4-O-isopropylidene-D-mannitol, ergosterol and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin were obtained from Wako Pure Chemical (Osaka, Japan). The concentrations of these agents used for studies were not toxic to 3T3-L1 cells when examined by microscopic analysis and formazan assay. Throughout the experiments, 100 $\mu\text{g}\cdot\text{mL}^{-1}$ cordycepin was used, unless indicated otherwise.

Adipocytes and differentiation

3T3-L1 pre-adipocytes were purchased from Health Science Research Resources Bank (Osaka, Japan) and mouse embryonic fibroblasts (MEFs) were obtained from RIKEN BRC Cell Bank (Tsukuba, Ibaragi, Japan). These cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM-F12; Wako Pure Chemical) supplemented with 10% fetal bovine serum (FBS) (basal medium). For the induction of adipocyte differentiation, cells were: (i) precultured in basal medium for 2 days; (ii) treated with differentiation medium containing 10 $\mu\text{g}\cdot\text{mL}^{-1}$ insulin, 0.25 μM dexamethasone and 500 μM IBMX (IDI medium) for 2 days; and (iii) incubated in basal medium supplemented with insulin alone for 2 days. The cells were further incubated in basal medium for an additional 2 days and subjected to analyses. To examine effects of cordycepin, adenosine, ATP, ADP, DPSPX, MRS1523, NBTI, CI-IB-MECA, resveratrol, splitomicin, palmitic acid, 3,4-O-isopropylidene-D-mannitol and ergosterol, cells were exposed to these agents during the incubation with IDI for 48 h.

Establishment of stable transfectants

Using electroporation, 3T3-L1 cells were transfected with pEFBOS-AhR(Arg39) (Sogawa *et al.*, 2004) that encodes a dominant-negative mutant of AhR (AhR-DN) under the control of the elongation factor-1 α promoter (9 μg) together with pcDNA3.1 (3 μg ; Invitrogen, Carlsbad, CA, USA) that codes for neomycin phosphotransferase. Stable transfectants were selected by G418 (500 $\mu\text{g}\cdot\text{mL}^{-1}$), and 3T3-L1/AhR-DN cells were established. 3T3-L1/Neo cells were also established as a control by transfection of 3T3-L1 cells with pcDNA3.1 alone.

Primary cultures of pre-adipocytes

Primary cultures of pre-adipocytes were established as described previously (Boney *et al.*, 1994). Three wild-type C57BL/6J mice and 3 AhR-null mutant mice were used for primary cultures of pre-adipocytes according to the regulations and guidelines of the University of Yamanashi. Mice were maintained in a climate-controlled room at 22°C and fed with standard laboratory diet. The mice were killed by cervical dislocation. In brief, inguinal fat pads were obtained from the wild-type C57BL/6J mice and AhR-null mutant mice (Mimura *et al.*, 1997) and digested in DMEM-F12 containing 2 $\text{mg}\cdot\text{mL}^{-1}$ collagenase-1 (Sigma-Aldrich Japan) for 15 min at 37°C with gentle shaking. After gentle pipetting for a few minutes with a P-1000 micropipette, the tissues were passed through a 106 μm mesh, and the resulting cell suspension was centrifuged at 700 $\times g$ for 10 min to separate the stromal-vascular cells from adipocytes. The pellets were washed and

cultured using DMEM-F12 containing 10–20% FBS. All the studies involving animals are reported in accordance with the ARRIVE guidelines (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010).

Oil red O staining

To quantify lipid accumulation, cells were fixed with 10% formaldehyde in PBS for 10 min, rinsed with 60% isopropanol and stained by oil red O in 60% isopropanol for 20 min. After the staining, cells were rinsed several times with 60% isopropanol and subjected to microscopic analysis. To evaluate the amount of lipid quantitatively, cells were added with isopropanol containing 4% Nonidet P-40 and lysed by agitation for 5 min. Absorbance (520 nm wave length) was measured by a spectrophotometer.

Hoechst staining

Cells fixed in 4% formaldehyde were stained by Hoechst33258 (10 µg·mL⁻¹; Sigma-Aldrich Japan) for 2 h. The number of oval lipid-laden cells compared to the total number of nuclei was calculated to determine percentages of adipocytes.

Northern blot analysis

Northern blot analysis was performed as described previously (Kitamura, 1997). cDNAs for adiponectin, PPAR γ (purchased from Addgene, Cambridge, MA, USA), C/EBP β , monocyte chemoattractant protein 1 (MCP-1), 78 kDa glucose-regulated protein (GRP78), C/EBP-homologous protein (CHOP), AhR and cytochrome P4501B1 (CYP1B1) were used for preparation of radiolabelled probes. The level of 28S ribosomal RNA was used as a loading control.

Western blot analysis

Western blot analysis was performed by the enhanced chemiluminescent system (Amersham Biosciences, Buckinghamshire, UK). Primary antibodies used were: anti-p70S6K antibody, anti-phospho p70S6K (Thr389) antibody, anti-phospho AMPK antibody and anti-PPAR γ antibody from Cell Signaling Technology (Beverly, MA, USA); anti-C/EBP α antibody, anti-C/EBP β antibody and anti-phospho IRS-1 (Tyr632) antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA); and anti- β -actin antibody from Sigma-Aldrich Japan. Levels of phosphorylated PKB (Akt) and total PKB protein were evaluated using PhosphoPlus Akt (Ser⁴⁷³) Antibody Kit (Cell Signaling Technology).

Transient transfection

Using GeneJuice Transfection Reagent (Novagen, Madison, WI, USA), 3T3-L1 cells were transiently transfected with siRNA for tuberous sclerosis complex 2 (TSC2) or control siRNA purchased from TAKARA (Shiga, Japan). The nucleotide sequences are: siTSC2, 5'-GGCCUCACAGACAAUGGA-3'; and siControl, 5'-GCUGCAAUCGAUUGAUGC-3'.

Reporter assay

Using GeneJuice Transfection Reagent, 3T3-L1 cells were transiently transfected with pPPRE-Luc (provided by Dr Shunji Ishihara, Shimane University School of Medicine, Shimane,

Japan) (Rumi *et al.*, 2004) together with pcDNA3.1, pcDNA3.1-CHOPwt (provided by Dr Hidetoshi Hayashi; Nagoya City University, Nagoya, Japan) (Ohoka *et al.*, 2005), pcDNA-LAP or pcDNA-LIP (provided by Dr Jacob Friedman; University of Colorado, Denver, CO, USA) (Shao *et al.*, 2005). pcDNA-LAP and pcDNA-LIP encodes liver activating protein (LAP) and liver-enriched inhibitory protein (LIP), respectively. After 24 h, the cells were treated with test reagents for 24 h and subjected to luciferase assay.

Luciferase assay

The activity of luciferase was evaluated by Luciferase Assay System (Promega, Madison, WI, USA) (Hiramatsu *et al.*, 2006).

Formazan assay

The number of viable cells was assessed by the formazan assay using Cell Counting Kit-8 (Dojindo Laboratory; Kumamoto, Japan) (Hiramatsu *et al.*, 2006).

Dioxin responsive element-based sensing via secreted alkaline phosphatase (DRESSA) assay

The DRESSA bioassay was performed to evaluate the activity of AhR (Kasai *et al.*, 2004; 2006). In brief, 3T3-L1 cells were transfected with pXRE-SEAP that introduces the secreted alkaline phosphatase (SEAP) gene under the control of the dioxin responsive element (also called xenobiotic responsive element) (Kasai *et al.*, 2006). The DRESSA assay was also performed using HeDS49 cells to evaluate the activity of AhR (Kasai *et al.*, 2006). The cells were treated with cordycepin, and the activity of SEAP in culture media was evaluated by a chemiluminescent method using Great EscAPe SEAP Detection Kit (BD Biosciences, Palo Alto, CA, USA), as described previously (Kasai *et al.*, 2004).

Statistical analysis

Assays were performed in quadruplicate, and data are expressed as means $\pm$ SEM. Statistical analysis was performed using the non-parametric Mann-Whitney *U*-test to compare data in different groups. *P* values < 0.05 were considered to indicate statistically significant differences.

Results

Blockade of adipocyte differentiation by cordycepin

We previously reported that an extract of *C. militaris* suppressed differentiation of pre-adipocytes. To identify the active entities, we examined an effect of cordycepin on the differentiation of pre-adipocytes. 3T3-L1 pre-adipocytes were treated with differentiation medium [insulin, dexamethasone and IBMX (IDI)] in the absence or presence of cordycepin and subjected to microscopic analysis. Phase-contrast microscopy and oil red O staining showed that IDI medium caused differentiation of pre-adipocytes to lipid-laden adipocytes. Treatment with cordycepin inhibited this process in a dose-dependent manner (Figure 1A). Quantitative analysis showed

Figure 1

Blockade of adipocyte differentiation by cordycepin. (A–E) 3T3-L1 pre-adipocytes were treated with IDI medium in the absence or presence of cordycepin (0–100 $\mu\text{g}\cdot\text{mL}^{-1}$) and subjected to analyses. Cordycepin at 100 $\mu\text{g}\cdot\text{mL}^{-1}$ was generally used for experiments, unless indicated otherwise. (A) Gross photographs of culture plates after staining by oil red O (top), and phase-contrast microscopy and light microscopic analysis of cells stained with oil red O (bottom). (B) Percentages of lipid-laden adipocytes. (C) Quantitative analysis of lipid content. (D) Northern blot analysis of adipocyte and preadipocyte markers (Day 6). The level of 28S ribosomal RNA is shown at the bottom as a loading control. (E) Western blot analysis of adipocyte markers (Day 1). (F) Pre-adipocytes in primary cultures were treated with IDI in the absence or presence of cordycepin and subjected to oil red O staining (left) and quantitative analysis of differentiated adipocytes (right). (G,H) 3T3-L1 pre-adipocytes were incubated with IDI (first exposure) in the presence of cordycepin for 2 days and further treated with insulin for additional 2 days without cordycepin. The cells were then treated with or without IDI (second exposure) for 2 days, and after an additional 4 days, microscopic analysis was performed (G). Quantitative assessment of lipid is shown in (H). In (B), (C), (F) and (H), assays were performed in quadruplicate, and data are expressed as means $\pm$ SEM. Asterisks indicate statistically significant differences ($P < 0.05$).

that the percentage of adipocytes was markedly increased by IDI, and this was prevented by the treatment with cordycepin (Figure 1B). Accumulation of lipid was also induced by IDI, and this was suppressed by the treatment with cordycepin (Figure 1C). This result was further confirmed using adipocyte and pre-adipocyte markers. In differentiating adipocytes, the expression of *adiponectin* and *PPAR γ* (adipocyte markers) was up-regulated, whereas the expression of *MCP-1* (a pre-adipocyte marker) was down-regulated. This shift in the gene expression profile was reversed by the treatment with cordycepin (Figure 1D). Consistently, Western blot analysis also showed that C/EBP α , C/EBP β and PPAR γ proteins, alternative adipocyte markers, increased in response to IDI and this effect was completely suppressed by cordycepin (Figure 1E). Of note, the anti-adipogenic effect of cordycepin was not restricted to 3T3-L1 cells and was similarly observed in pre-adipocytes in primary cultures (Figure 1F).

We examined whether or not the anti-adipogenic effect of cordycepin is reversible. For this purpose, 3T3-L1 cells were incubated in IDI medium (first exposure) in the presence of cordycepin for 2 days and further incubated for an additional 2 days without cordycepin. The cells were then treated with or without IDI (second exposure) for 2 days, and after an additional 4 days, microscopic analysis was performed. As shown in Figure 1G, cordycepin-treated cells did not differentiate on their first exposure to IDI but underwent significant differentiation following the second exposure to IDI in the absence of cordycepin. Quantitative analysis also showed that accumulation of lipid was induced by the second exposure to IDI in the cordycepin-pretreated, initially undifferentiated cells (Figure 1H).

Roles of adenosine transporter, but not adenosine receptors, in the anti-adipogenic effect of cordycepin

A previous report suggested that cordycepin inhibits the growth of tumour cells by binding to the A_3 adenosine receptor (Nakamura *et al.*, 2006). To investigate involvement of the A_3 receptor in the anti-adipogenic effect of cordycepin, we tested the effect of the A_3 receptor antagonist MRS1523. Oil red O staining showed that the suppressive effect of cordycepin on adipogenesis was not reversed by the treatment with MRS1523 (Figure 2A). The lack of involvement of the A_3 receptor was further investigated by quantitative analysis of lipid content (Figure 2B). Of note, the concentration of MRS1523 used in this experiment was sufficient to inhibit the

anti-apoptotic effects of cordycepin (data not shown). To confirm this result, we further tested the effect of the A_3 receptor agonist Cl-IB-MECA on the differentiation of 3T3-L1 adipocytes. Microscopic analysis showed that, unlike cordycepin, Cl-IB-MECA did not suppress IDI-induced adipogenesis (Figure 2C,D). The concentration of Cl-IB-MECA we used was sufficient to inhibit proliferation of 3T3-L1 cells (Supporting Information Figure S1).

In a previous report it was suggested that A_1/A_2 adenosine receptors are involved in the cordycepin-induced suppression of mitogenesis in vascular smooth muscle cells (Won *et al.*, 2009). We therefore examined the effect of DPSPX, an A_1/A_2 adenosine receptor antagonist, on the anti-adipogenic effect of cordycepin. As shown in Figure 2E, DPSPX did not block the suppression of adipogenesis by cordycepin. The lack of involvement of the A_1/A_2 receptors was further confirmed by quantitative analysis of lipid content (Figure 2F).

Adenosine and other adenine nucleosides are transported across the cell membrane via adenosine transporter. Cordycepin may exert its biological actions not only extracellularly (via adenosine receptors) but also intracellularly through the adenosine transporter. To examine the latter possibility, 3T3-L1 cells were treated with IDI and cordycepin together with or without the adenosine transport inhibitor NBTL. Microscopic analysis showed that the anti-adipogenic effect of cordycepin was reversed by the treatment with NBTL (Figure 2G). The significant involvement of the adenosine transporter was further confirmed by quantitative analysis of lipid content (Figure 2H).

Cordycepin is an analogue of adenosine. Other adenosine-related substances may share its anti-adipogenic effect. To examine this possibility, the effects of adenosine, ADP and ATP were tested. 3T3-L1 cells were treated with IDI medium in the presence of adenosine, ADP or ATP, and subjected to microscopic analysis and quantitative assessment of lipid content. The results showed that none of these substances mimicked the anti-adipogenic effect of cordycepin even at high concentrations (Figure 2I).

Lack of involvement of AhR in the anti-adipogenic effect of cordycepin

We previously reported that extract of *C. militaris* suppresses the differentiation of 3T3-L1 pre-adipocytes, at least in part, through activation of AhR. We speculated that cordycepin may also inhibit adipocyte differentiation through the same mechanism. To examine this possibility, the potential of

Figure 2

Roles of adenosine receptors and adenosine transporter in the anti-adipogenic effect of cordycepin. (A–H) 3T3-L1 cells were treated with IDI medium and cordycepin (or C1-IB-MECA) in the presence of adenosine receptor antagonists or an adenosine transporter inhibitor. After 4 days, the cells were subjected to microscopic analysis (A, C, E, G) and quantitative analysis of lipid content (B, D, F, H). Concentrations used for individual agents were: 10 μ M MRS1523, 25 μ M CI-IB-MECA, 10 nM DPSPX and 10 μ M NBTI. (I) Cells were treated with IDI in the presence of 100 μ M adenosine (ADO), 100 μ M ADP or 100 μ M ATP and subjected to analysis of lipid content. Asterisks indicate statistically significant differences ($P < 0.05$); n.s., not statistically significant.

Figure 3

Lack of involvement of AhR in the anti-adipogenic effect of cordycepin. (A) 3T3-L1 pre-adipocytes were transfected with pXRE-SEAP, treated with cordycepin for 24 h and subjected to chemiluminescent assay to evaluate SEAP activity. (B) Cells were treated with cordycepin for indicated time periods and subjected to Northern blot analysis of *CYP1B1*. (C,D) Mock-transfected 3T3-L1/Neo cells (Mock) and 3T3-L1/AhR-DN cells were treated with IDI in the absence or presence of cordycepin, stained by oil red O and subjected to microscopic analysis (C) and quantitative analysis of differentiated adipocytes (D). (E,F) Pre-adipocytes in primary cultures derived from wild-type mice (WT) and AhR-null mutant mice (AhR-KO) were treated with IDI in the absence or presence of cordycepin, stained by oil red O and subjected to microscopic analysis (E) and quantitative analysis of differentiated adipocytes (F). Asterisks indicate statistically significant differences ($P < 0.05$).

cordycepin for activation of AhR was evaluated by the DRESSA bioassay (Kasai *et al.*, 2004; 2006). However, in contrast to *C. militaris*, cordycepin did not induce activation of AhR in 3T3-L1 cells (Figure 3A). Consistent with this result,

cordycepin did not induce expression of *CYP1B1*, an endogenous marker for AhR activation in pre-adipocytes (Figure 3B). To confirm the lack of involvement of AhR, we established 3T3-L1/AhR-DN pre-adipocytes overexpressing a

Figure 4

Suppressive effect of cordycepin on the induction of $PPAR\gamma$. (A) 3T3-L1 pre-adipocytes were treated with IDI in the absence or presence of cordycepin for indicated time periods and subjected to Northern blot analysis of $PPAR\gamma$ and $MCP-1$. (B) Cells were treated with IDI in the absence or presence of cordycepin and NBTI for 24 h and subjected to Northern blot analysis. (C) Cells were treated with IDI in the absence or presence of cordycepin for indicated time periods and subjected to Western blot analysis of $PPAR\gamma$. The level of β -actin is shown as a loading control. (D) Cells were transiently transfected with pPPRE-Luc, treated with IDI in the absence or presence of cordycepin for 24 h and subjected to luciferase assay. Asterisks indicate statistically significant differences ($P < 0.05$).

dominant-negative mutant of AhR (Shimada *et al.*, 2008). The transgene expression was confirmed by Northern blot analysis (data not shown). As shown in Figure 3C, as in mock-transfected 3T3-L1 cells, cordycepin substantially suppressed differentiation of 3T3-L1/AhR-DN cells. The inhibition of lipid accumulation by cordycepin observed in mock-transfected cells was similarly observed in 3T3-L1/AhR-DN cells (Figure 3D). Consistent with this result, the anti-adipogenic effect of cordycepin was similarly observed in pre-adipocytes derived from AhR-null mutant mice (Figure 3E). Suppression of lipid accumulation by cordycepin was also observed in AhR-knockout pre-adipocytes (Figure 3F). These results further confirm that the anti-adipogenic effect of cordycepin was independent of AhR. Of note, adipocyte differentiation by IDI was enhanced in 3T3-L1/AhR-DN cells and AhR-knockout cells, when compared with control pre-adipocytes (Figure 3D,F). Although AhR is not involved in the anti-adipogenic effect of cordycepin, basal activation of AhR by endogenous ligands might contribute to the attenuation of adipocyte differentiation.

Effect of cordycepin on the induction of $PPAR\gamma$

$PPAR\gamma$ is regarded as a transcription factor essential for adipogenesis *in vivo* and *in vitro* (Spiegelman *et al.*, 1997; Rosen

et al., 2000). We examined whether cordycepin suppresses adipogenesis by affecting $PPAR\gamma$ in the early phase of differentiation. Northern blot analysis showed that expression of $PPAR\gamma$ was induced by IDI within 12–24 h. However, this induction, as well as basal expression of $PPAR\gamma$, was abolished by the treatment with cordycepin (Figure 4A, top row). The suppressive effect of cordycepin was not due to non-specific transcriptional inhibition, as expression of $MCP-1$ was increased by cordycepin (Figure 4A, middle row). Consistent with the results shown in Figure 2G,H, inhibition of the adenosine transporter by NBTI reversed the suppressive effect of cordycepin on $PPAR\gamma$ mRNA (Figure 4B). The blockade of $PPAR\gamma$ expression by cordycepin was associated with the down-regulation of $PPAR\gamma$ protein (Figure 4C) and the reduced activation of $PPAR\gamma$, when examined by the peroxisome proliferator responsive element (PPRE)-based reporter assay (Figure 4D). These results suggest that cordycepin suppresses differentiation of pre-adipocytes through blockade of $PPAR\gamma$ induction in the early phase of adipogenesis.

Effect of cordycepin on the induction of C/EBP β

C/EBP β is regarded as another key regulator essential for adipogenesis upstream of $PPAR\gamma$ and C/EBP α (Rosen *et al.*, 2000; Tang *et al.*, 2003). Indeed, in the early phase of adipo-

Figure 5

Suppressive effect of cordycepin on the induction of *C/EBPβ* that induces *PPARγ*. (A) 3T3-L1 pre-adipocytes were treated with IDI in the absence or presence of cordycepin for indicated time periods and subjected to Northern blot analysis of *C/EBPβ*. (B) Cells were treated with IDI in the absence or presence of cordycepin and NBTI for 24 h and subjected to Northern blot analysis. (C) Cells were treated with IDI in the absence or presence of cordycepin for indicated time periods and subjected to Western blot analysis of *C/EBPβ* (LAP). (D) Cells were transfected with pPPRE-Luc together with empty vector (Control), *LIP* or *CHOP*, treated with IDI in the absence or presence of cordycepin for 24 h and subjected to luciferase assay. (E) Cells were transiently transfected with pPPRE-Luc together with empty vector or LAP. After 24 h, the cells were subjected to luciferase assay. Asterisks indicate statistically significant differences ($P < 0.05$).

genesis, the expression of *C/EBPβ* increased before the up-regulation of *PPARγ* and *C/EBPα* in 3T3-L1 cells (Supporting Information Figure S2). To examine whether or not *C/EBPβ* is an upstream target for the anti-adipogenic effect of cordycepin, Northern blot analysis was performed. As shown in Figure 5A, IDI induced the expression of *C/EBPβ* within 12 h. Like *PPARγ*, the induction of *C/EBPβ* was attenuated by the treatment with cordycepin (Figure 5A). Consistent with the results shown in Figure 2G,H and 4B, inhibition of the adenosine transporter reversed the suppressive effect of cordycepin on *C/EBPβ* mRNA (Figure 5B). The blockade of *C/EBPβ* expression was associated with a down-regulation of *C/EBPβ* protein (Figure 5C). Of note, like the kinetics of the mRNA level, the peak of *C/EBPβ* protein was earlier than that of *PPARγ* protein.

C/EBPβ is produced from *C/EBPβ* mRNA as three distinct protein isoforms, that is, 38/35 kDa LAP and 20 kDa LIP. LAP functions as a transcriptional activator, whereas LIP acts as a dominant-negative inhibitor of LAP (Zahnow, 2009). The transacting potential of LAP is also inhibited by another member of the C/EBP family, CHOP (Ramji and Foka, 2002). To confirm that induction of *C/EBPβ* is indeed an event upstream of *PPARγ*, 3T3-L1 pre-adipocytes were transfected with pPPRE-Luc together with *LIP* or *CHOP*, and activation of *PPARγ* was evaluated. The reporter assay showed that activation of *PPARγ* by IDI was blunted by co-transfection with *LIP* or *CHOP* (Figure 5D). Furthermore, transfection with *LAP* caused activation of *PPARγ* in 3T3-L1 cells (Figure 5E). These

results suggest that cordycepin suppressed differentiation of pre-adipocytes by intervention of the adipogenic *C/EBPβ*–*PPARγ* pathway.

Mammalian target of rapamycin complex 1 (mTORC1) as a proximal target for the anti-adipogenic effect of cordycepin

Insulin signalling plays a key role in adipogenesis *in vivo* and *in vitro* (Spiegelman and Flier, 1996). Binding of insulin to its receptor triggers activation of PI3K and PKB, leading to activation of mTORC1 and consequent phosphorylation of p70S6 kinase (p70S6K). Previous reports showed that inhibition of mTORC1 repressed adipocyte differentiation via blockade of clonal expansion of pre-adipocytes at the early stage of adipogenesis (Yeh *et al.*, 1995). To examine the possibility that cordycepin affects adipogenesis by an effect on mTORC1, 3T3-L1 pre-adipocytes were treated with IDI in the absence or presence of cordycepin. Western blot analysis showed that activation of mTORC1, indicated by phosphorylation of p70S6K, was rapidly induced by IDI, and it was completely suppressed by the treatment with cordycepin (Figure 6A). This suppressive effect was mediated by the adenosine transporter, as NBTI reversed the effect of cordycepin (Figure 6B). Microscopic analysis showed that, like cordycepin, inhibition of mTORC1 by rapamycin blocked adipocyte differentiation and accumulation of lipids in 3T3-L1 cells (Figure 6C,D).

Figure 6

mTORC1 as a proximal target for the anti-adipogenic effect of cordycepin. (A) 3T3-L1 pre-adipocytes were treated with IDI in the absence or presence of cordycepin for 24 h and subjected to Western blot analysis of phosphorylated p70S6K (p-p70S6K). (B) Cells were treated with IDI in the absence or presence of cordycepin and NBTI and subjected to Western blot analysis. (C,D) Cells were treated with IDI in the absence or presence of 100 nM rapamycin, stained with oil red O and subjected to microscopic analyses (C) and quantitative assessment of lipid content (D). (E) Cells were treated with IDI or 10 $\mu\text{g}\cdot\text{mL}^{-1}$ insulin in the absence or presence of rapamycin for 24 h and subjected to Northern blot analysis of *C/EBPβ* and *PPARγ*. (F) Cells were treated with IDI in the absence or presence of rapamycin for up to 24 h and subjected to Western blot analysis of *C/EBPβ* and *PPARγ*. (G–I, K) Cells were treated with cordycepin for 1 h (G), pretreated with cordycepin for 30 min and stimulated by IDI for 30 min (H), or pretreated with cordycepin and stimulated by IDI in the absence or presence of NBTI (I,K) and subjected to Western blot analysis of phosphorylated PKB (p-PKB), AMPK (p-AMPK) and IRS-1 (p-IRS-1). (J) 3T3-L1 cells were transfected with control siRNA or siTSC2, treated with IDI in the absence or presence of cordycepin and subjected to Western blot analyses of p-p70S6K, *C/EBPβ* and *PPARγ*. (L–N) MEFs were treated with IDI in the absence or presence of cordycepin and NBTI and subjected to oil red O staining (L, left), quantitative assessment of adipocyte differentiation (L, right), and Western blot analyses of p-PKB, p-AMPK and *C/EBPβ* (M, N). Asterisks indicate statistically significant differences ($P < 0.05$).

Figure 6

Continued

A previous report showed that long-term blockade of mTORC1 by rapamycin (for 12 days) reduces the expression of *PPARγ* mRNA in mature adipocytes (Cho *et al.*, 2004). However, currently, it is unclear whether and how mTORC1 regulates the C/EBPβ–PPARγ pathway in the early phase of adipogenesis. We tested the effects of rapamycin on the induction of C/EBPβ and PPARγ by IDI. Northern blot analysis showed that the expression of these genes was partially suppressed by rapamycin. A similar effect was also observed in the induction of *C/EBPβ* and *PPARγ* mRNAs by insulin (Figure 6E). Consistent with these results, induction of C/EBPβ and PPARγ proteins by IDI was attenuated by rapamycin (Figure 6F).

It is known that the activity of mTORC1 is regulated by PKB and AMP-activated protein kinase (AMPK) in an opposite direction. For example, PKB activates mTORC1 through inactivation of TSC2 within the TSC1–TSC2 complex (Inoki *et al.*, 2002). In contrast, AMPK activates TSC1/2, leading to suppression of mTORC1 (Shaw, 2009). To identify the upstream events involved in the regulation of mTORC1 by cordycepin, PKB and AMPK were investigated. In unstimulated conditions, basal activity of PKB was observed in 3T3-L1 cells, and it was attenuated by cordycepin. In contrast, cordycepin enhanced basal activity of AMPK (Figure 6G). Following stimulation with IDI, phosphorylated PKB was up-regulated, and this was attenuated by treatment with cordycepin. In contrast, treatment with IDI modestly decreased phosphorylated AMPK, whereas cordycepin reversed this suppressive effect (Figure 6H). The regulatory

effects of cordycepin on PKB and AMPK were abolished in the presence of NBTI (Figure 6I).

As described previously, TSC2 is a possible upstream target for cordycepin to suppress the mTORC1 pathway. Cordycepin could interfere with the mTORC1 pathway by reinforcing the function of TSC2. To examine this possibility, we tested the effects of siRNA-mediated knockdown of TSC2 on the suppression of C/EBPβ and PPARγ by cordycepin. As shown in Figure 6J, treatment with siTSC2 up-regulated the activity of mTORC1 (indicated by phosphorylation of p70S6K), whereas it did not reverse the suppressive effects of cordycepin on C/EBPβ and PPARγ. This result indicates that TSC2 is not a major target for cordycepin to inhibit the adipogenic pathway.

To further examine whether or not cordycepin affects insulin signalling at the level of the insulin receptor, IDI-triggered phosphorylation of insulin receptor substrate-1 (IRS-1) was evaluated. Western blot analysis showed that IDI caused phosphorylation of IRS-1. However, this process was not inhibited by cordycepin (Figure 6K, top row). The adenosine transport inhibitor NBTI also did not affect the level of IRS-1 phosphorylation. The lack of inhibition of IRS-1 was in contrast to the complete suppression of IDI-triggered PKB phosphorylation by cordycepin (Figure 6K, third row).

We confirmed that our findings are not specific to 3T3-L1 cells. It is known that MEFs have the potential to differentiate into adipocytes. We treated MEF with IDI in the absence or presence of cordycepin and NBTI and subjected them to oil red O staining and Western blot analyses of phosphorylated PKB, phosphorylated AMPK and C/EBPβ. As shown in

Figure 6 L, IDI induced adipocyte differentiation of MEF, and this was completely inhibited by cordycepin. Consistent with the result in 3T3-L1 cells, NBTI reversed the suppressive effect. Similarly, in MEFs, up-regulation of PKB phosphorylation and down-regulation of AMPK phosphorylation by IDI was suppressed by cordycepin, both of which were reversed by NBTI (Figures 6 M). Induction of downstream C/EBP β by IDI was also blocked in the presence of cordycepin in MEFs (Figure 6N). These results suggest that the anti-adipogenic mechanisms exerted by cordycepin are not restricted to 3T3-L1 cells and are similarly observed in other cell types.

Discussion

In the present investigation, we showed that cordycepin inhibited adipogenesis. This effect was via blockade of the C/EBP β –PPAR γ pathway. We also found that during adipogenesis, mTORC1 was rapidly activated, contributing to the induction of C/EBP β and PPAR γ . Cordycepin inhibited induction of C/EBP β and PPAR γ at mRNA and protein levels, which was dependent on the suppression of mTORC1. PKB and AMPK were identified as possible upstream targets for cordycepin to inhibit mTORC1 activation.

The insulin–PI3K–PKB pathway plays a crucial role in adipogenesis. mTORC1 is located downstream of PKB, and a previous report suggested a role for mTORC1 in insulin-induced adipogenesis (Cho *et al.*, 2004). In our experimental setting, inhibition of mTORC1 by rapamycin blocked IDI-induced differentiation of 3T3-L1 cells. However, it was unclear how mTORC1 promotes adipogenesis. We showed that rapamycin inhibited induction of C/EBP β and PPAR γ . mTORC1 facilitates general translation via activation of p70S6K and inhibition of eukaryotic initiation factor 4E binding protein 1 (4E-BP) (Burnett *et al.*, 1998). Recently, we also showed that cordycepin induced phosphorylation of eukaryotic initiation factor 2 α (eIF2 α) that may cause general translational suppression (Kitamura *et al.*, 2011). Based on these findings, it is reasonable to speculate that cordycepin inhibited induction of C/EBP β and PPAR γ via suppression of their translation. However, we also found that induction of C/EBP β and PPAR γ by IDI and insulin was inhibited at a transcriptional level. Previous reports suggested that cordycepin can inhibit transcriptional events through multiple mechanisms (Penman *et al.*, 1970; Müller *et al.*, 1977). For example, it inhibits mRNA polyadenylation, presumably by acting as a chain terminator (Müller *et al.*, 1977), and at high doses, it can affect the export, processing and stability of transcribed mRNA (Penman *et al.*, 1970). Hence, the transcriptional suppression of C/EBP β and PPAR γ by cordycepin, observed in the present study, might be through similar mechanisms.

The activity of mTORC1 is regulated by PKB (positive regulator) and AMPK (negative regulator) (Inoki *et al.*, 2002; Shaw, 2009). Several molecules including TSC may mediate these regulatory processes (Shackelford and Shaw, 2009). In the present investigation, we showed that, following stimulation with IDI, phosphorylation of PKB was markedly suppressed by the treatment with cordycepin. In contrast, cordycepin up-regulated the level of phosphorylated AMPK, possibly as a result of the dephosphorylation of PKB, because PKB is a negative regulator of AMPK (Kovacic *et al.*, 2003).

Figure 7

A hypothetical model for intervention in adipogenesis by cordycepin. Cordycepin interferes with adipogenesis via suppression of C/EBP β and PPAR γ . During adipogenesis, mTORC1 is rapidly activated via the PKB pathway, contributing to up-regulation of C/EBP β and PPAR γ . Cordycepin inhibits PKB phosphorylation and consequent activation of mTORC1, leading to translational suppression of C/EBP β (and PPAR γ) possibly via p70S6K and 4E-BP. Activation of AMPK via inhibition of PKB may also contribute to suppression of mTORC1 by cordycepin. In addition, cordycepin could directly affect transcription of C/EBP β (and PPAR γ). These molecular events could underlie the anti-adipogenic effect of cordycepin.

These results suggest that both inhibition of PKB and activation of AMPK contribute to the blockade of mTORC1 by cordycepin. Currently, it is not known how cordycepin inhibits phosphorylation of PKB. Our results indicated that the insulin receptor and its substrate IRS-1 were not affected by cordycepin. Further, the TSC1–TSC2 complex, a negative regulator of the mTORC1 pathway, was also not involved in the suppressive effect of cordycepin. Our current hypothesis as to how cordycepin affects each of the components involved in adipogenesis is schematically summarized in Figure 7.

Previous reports suggested that cordycepin exerts biological effects through adenosine receptors. For example, cordycepin inhibits platelet-derived growth factor-induced migration and proliferation of vascular smooth muscle cells through the A₁/A₂ adenosine receptor (Won *et al.*, 2009) and exerts an anti-tumour effect on melanoma cells through the A₃ adenosine receptor (Nakamura *et al.*, 2006). In contrast to these extracellular actions, little is known about intracellular action of cordycepin. In the present study, we demonstrated a crucial role of intracellular cordycepin in the regulation of adipogenesis, which is consistent with our recent report showing a role of adenosine transporter in the pro-survival action of cordycepin (Kitamura *et al.*, 2011).

Adipogenesis is inhibited by CHOP that is induced by endoplasmic reticulum (ER) stress. CHOP has the ability to dimerize with C/EBP, and CHOP-C/EBP heterodimers are unable to bind to the classical C/EBP binding site, leading to suppression of adipocyte differentiation (Batchvarova *et al.*, 1995). Under conditions of ER stress, CHOP is induced via the eIF2 α – activating transcription factor 4 pathway. Cordycepin induces the eIF2 α branch of the unfolded protein response in some cell types (Kitamura *et al.*, 2011), and therefore, the anti-adipogenic effect of cordycepin could be ascribed to induction of CHOP. However, Northern blot analysis showed that, like in NRK-52E cells (Kitamura *et al.*, 2011), cordycepin did not up-regulate *CHOP* as well as another ER stress marker *GRP78* in 3T3-L1 cells (Supporting Information Figure S3), excluding this possibility.

As we previously reported, extract of *C. militaris* suppressed differentiation of pre-adipocytes (Shimada *et al.*, 2008). Cordycepin is a component of *C. militaris* (Cunningham *et al.*, 1950), and cordycepin and *C. militaris* share several biological effects including anti-cancer, pro-apoptotic and anti-inflammatory activities. In the present investigation, we also showed that, like the extract of *C. militaris*, cordycepin has the potential to inhibit adipogenesis. However, the mechanisms underlying the inhibition of adipogenesis differ somewhat between *C. militaris* and cordycepin. *C. militaris* inhibits adipogenesis, at least in part, via activation of AhR (Shimada *et al.*, 2008), whereas the anti-adipogenic effect of cordycepin was not dependent on AhR. *C. militaris* contains a number of constituents that could cause AhR activation (Das *et al.*, 2010). Using silica gel column chromatographic purification, Rao *et al.* recently isolated and identified major constituents in *C. militaris*. These included cordycepin, D-mannitol, 3,4-o-isopropylidene-D-mannitol, palmitic acid, ergosterol palmitate, ergosterol and ergosterol peroxide (Rao *et al.*, 2010). Among these substances, we have checked the effects of D-mannitol, 3,4-o-isopropylidene-D-mannitol, palmitic acid and ergosterol on the activity of AhR in 3T3-L1 pre-adipocytes. Northern blot analysis showed that ergosterol, but not any of the others, modestly enhanced the expression of *CYP1B1*, an endogenous indicator for AhR activation (Supporting Information Figure S4A). Consistent with this result, the DRESSA assay also showed that only ergosterol induced a modest activation of AhR (Supporting Information Figure S4B). Furthermore, only ergosterol modestly attenuated lipid accumulation in IDI-treated 3T3-L1 cells (Supporting Information Figure S4C). These results indicate that ergosterol, but not cordycepin, is the candidate responsible for AhR-mediated suppression of adipogenesis by *C. militaris*. The cooperation of AhR activators and cordycepin possibly contributes to the anti-adipogenic effect of *C. militaris*.

A recent report indicated that cordycepin suppresses high fat diet-associated increases in adipose tissue mass (Guo *et al.*, 2010). Although the suppression of hyperlipidaemia was proposed as an underlying mechanism, our current results indicate direct suppression of adipogenesis by cordycepin through intervention in the mTORC1-mediated adipogenic pathway, especially via targeting upstream kinases PKB and AMPK. In addition, we found that, even in mature adipocytes, the lipid content was significantly reduced by the treatment with cordycepin (Supporting Information Figure S5). This reduction in lipid content was ascribed to both

inhibition of lipogenesis and facilitation of lipolysis (S. Takahashi *et al.*, unpublished observations).

Sirtuin 1 (Sirt1) affects a diverse range of cellular processes, in particular, mammalian metabolism, and Sirt1 activators have similar effects to cordycepin on adipogenesis, lipogenesis and lipolysis (Picard *et al.*, 2004). In general, Sirt1 represses PPAR γ by docking with its cofactors. Overexpression of Sirt1 attenuates adipogenesis, and RNA interference of Sirt1 enhances it. Furthermore, in differentiated adipocytes, up-regulation of Sirt1 also triggers lipolysis (Picard *et al.*, 2004). Recent reports suggested the potential of Sirt1 to inhibit the mTOR pathway similarly to cordycepin (Ghosh *et al.*, 2010). We also found that cordycepin activated AMPK, which may up-regulate Sirt1 by increasing NAD $^{+}$ (Cantó *et al.*, 2009). Based on these findings, activation of Sirt1 could be an alternative mechanism behind the anti-adipogenic and anti-lipogenic effects of cordycepin. To test this possibility, 3T3-L1 pre-adipocytes were treated with IDI in the absence or presence of resveratrol (Sirt1 activator), or cordycepin together with or without splitomicin (Sirt1 inhibitor) and subjected to oil red O staining, quantitative assessment of lipid content, and Western and Northern blot analyses of PPAR γ . As expected, treatment with resveratrol inhibited adipocyte differentiation, lipid accumulation and induction of PPAR γ at both mRNA and protein levels. However, treatment with splitomicin did not reverse the suppressive effects of cordycepin on adipogenesis, accumulation of lipid and induction of PPAR γ even at effective concentrations (Supporting Information Figure S6A–D). These results indicate that Sirt1 does not mediate the effects of cordycepin on adipogenesis.

In conclusion, we have shown for the first time that cordycepin inhibits adipocyte differentiation and accumulation of lipid in mature adipocytes. As cordycepin blocks both adipocyte differentiation and lipid accumulation, it has the potential to be an effective therapeutic agent for obesity and obesity-related disorders.

Acknowledgements

We thank Dr Shunji Ishihara (Shimane University School of Medicine), Dr Hidetoshi Hayashi (Nagoya City University) and Dr Jacob Friedman (University of Colorado) for providing us with plasmids. This work was supported, in part, by Grant-in-Aids for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology, Japan (No. 20390235) to M. Kitamura.

Conflicts of interest

None

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Inhibition of adipocyte proliferation by CI-IB-MECA. 3T3-L1 pre-adipocytes were treated with 25 μ M CI-IB-MECA for 48 h and subjected to formazan assay. * P < 0.05.

Figure S2 Kinetics of expression of adipocyte and preadipocyte markers following treatment with IDI. 3T3-L1 pre-adipocytes were treated with IDI medium for indicated time periods and subjected to Northern blot analysis of *C/EBP α* , *C/EBP β* , *PPAR γ* and *MCP-1*.

Figure S3 Lack of induction of ER stress by cordycepin. 3T3-L1 pre-adipocytes were treated with cordycepin for indicated time periods and subjected to Northern blot analysis of *GRP78* and *CHOP*.

Figure S4 Effects of major constituents of *Cordyceps militaris* on AhR activity and adipocyte differentiation. (A,B) 3T3-L1 pre-adipocytes (A) or HeDS49 cells (B) were treated with 10 nM 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; positive control), 200 μ g·mL⁻¹ D-mannitol, 200 μ g·mL⁻¹ 3,4-o-isopropylidene-D-mannitol (IPDM), 5–25 μ g·mL⁻¹ palmitic acid or 5–25 μ g·mL⁻¹ ergosterol for 12 h (A) or 24 h (B) and subjected to Northern blot analysis of *CYP1B1* (A) or luciferase assay to evaluate AhR activity (B). In (B), the values were normalized by the number of viable cells estimated by formazan assay. (C) 3T3-L1 cells were cultured in IDI medium in the presence of indicated agents. After 6 days, lipid content was evaluated by the oil red O method. * P < 0.05; n.s., not statistically significant.

Figure S5 Inhibition of lipid accumulation by cordycepin in mature adipocytes. Fully differentiated 3T3-L1 adipocytes (Day 6) were cultured in the absence or presence of cordycepin for 10 days (Day 16) and subjected to oil red O staining. left, light microscopy; right, quantitative analysis of lipid content. * P < 0.05

Figure S6 Lack of involvement of Sirt1 in the suppressive effect of cordycepin on the adipogenic pathway. 3T3-L1 pre-adipocytes were treated by IDI in the absence or presence of 100 μ M resveratrol, or cordycepin together with or without 100 μ M splitomicin and subjected to oil red O staining (A), quantitative assessment of lipid content (B), and Western and Northern blot analyses of PPAR γ (C,D). * P < 0.05; n.s., not statistically significant.

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