Intervention in Genotoxic Stress–Induced Senescence by Cordycepin Through Activation of eIF2α and Suppression of Sp1

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In this study, we show that cordycepin (3′-deoxyadenosine), a major nucleoside isolated from Cordyceps species, attenuates genotoxic stress–induced senescence. Etoposide- or doxorubicin-treated cells exhibited senescent morphology, growth arrest, and positive staining for senescence-associated β-galactosidase. The induction of the senescent phenotype was inhibited by the treatment of cordycepin. This suppression was correlated with blunted activation of the p16INK4a and p21WAF1/CIP1 gene promoters, as well as a decreased level of p21 WAF1/CIP1 mRNA. Other adenosine-related substances including ATP, ADP, and adenosine did not mimic the suppressive effect of cordycepin. The antisenescence effect of cordycepin was mediated by activation of eukaryotic translation initiation factor 2α (eIF2α) because (1) cordycepin induced phosphorylation of eIF2α, (2) selective activation of eIF2α mimicked the suppressive effect of cordycepin on senescence, and (3) functional knockdown of eIF2α reversed the effect of cordycepin. Unexpectedly, induction of p53 by etoposide was not inhibited by cordycepin, whereas (1) expression of Sp1 (required for the induction of p21 WAF1/CIP1 by genotoxic stress) was attenuated by cordycepin, (2) DNA binding activity of Sp1 was also inhibited, and (3) selective inhibition of Sp1 reproduced the suppressive effect of cordycepin on senescence. These results suggest that cordycepin interferes with senescence signaling via activation of eIF2α and suppression of Sp1 without affecting the level of p53.

Key Words: senescence; cordycepin; eIF2α; Sp1; p21WAF1/CIP1; p16INK4a.

Senescence is associated with a wide range of pathologies such as Alzheimer’s disease, cancers, diabetes, and cardiovascular diseases. To delay onset of these disorders, one possible approach may be to develop antiaging agents that attenuate cellular senescence (Sikora et al., 2011). Previous reports showed that Cordyceps sinensis and Cordyceps taii, medicinal mushrooms in oriental medicines, may contain some substances that improve function of the brain and the immune system in mice with D-galactose-induced senescence (Ji et al., 2009; Xiao et al., 2012). Other group also showed that polysaccharides isolated from fruiting bodies of Cordyceps militaris have the antiaging potential (Li et al., 2010). However, currently, active entities responsible for the antiaging property of Cordyceps species have not been identified yet.

Cordycepin (3′-deoxyadenosine) is a major nucleoside isolated from Cordyceps species (Cunningham et al., 1950). It has a wide range of biological potential including antitumor, proapoptotic/antiapoptotic, antithrombotic, antiadipogenic, and anti-inflammatory activities (Kitamura et al., 2011; Takahashi et al., 2012). It exerts these biological effects through adenosine transporters and adenosine receptors (Kitamura et al., 2011; Nakamura et al., 2006; Takahashi et al., 2012). Currently, effects of this agent on senescence have not been reported yet, but cordycepin could be responsible for the antiaging action of Cordyceps species.

Senescence is a state of irreversible growth arrest initially described by Hayflick and Moorhead (1961). Senescent cells are characterized by several morphological and biochemical features including large, flat, and multinucleated morphology, expression of senescence-associated β-galactosidase (SA-β-gal) (Dimri et al., 1995), and activation of p53–p21WAF1/CIP1 and p16INK4a–retinoblastoma (RB) signaling cascades (Kuilman et al., 2010). Cellular senescence is closely correlated with aging of organisms and may contribute to age-related disorders (Collado et al., 2007; Kuilman et al., 2010; Sikora et al., 2011). For example, it drives aging not only by net accumulation of senescent cells in tissues but also by impairing the regenerative potential of stem cells (Collado et al., 2007). Furthermore, senescent cells secrete various proteins including inflammatory cytokines, chemokines, and growth factors. It is called “senescence-associated secretory phenotype (SASP)” and contributes to chronic inflammation observed in a variety of organs during aging (Freund et al., 2010). SASP thereby contributes to development of age-related pathologies such as carcinogenesis, atherogenesis, and tissue fibrosis/sclerosis.

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Cellular senescence is induced by telomere shortening (replicative senescence), oncogene activation, and DNA damage (premature senescence) (Kuilkman et al., 2010). It is known that various genotoxic agents induce senescence. For example, a low concentration of etoposide, an inhibitor of DNA topoisomerase, induces senescence in fibroblasts and epithelial cells (Leontieva and Blagosklonny, 2010; Gu and Kitamura, 2012). Low concentrations of doxorubicin also induce histone acetylation and senescence in different cell types (Demarchi et al., 2010, Tong et al., 2011). In this investigation, we aimed at examining an effect of cordycepin on genotoxic stress–induced senescence. We found that cordycepin has the antisenescence potential, and the effect of cordycepin was accompanied by blunted induction of p16(NPκC) and p21(WAF1/CIP1), without suppression of p53 levels. We also demonstrated that activation of eukaryotic translation initiation factor 2α (eIF2α) and suppression of Sp1 are involved in the effect of cordycepin on cellular senescence.

**MATERIALS AND METHODS**

**Reagents.** Cordycepin, adenosine, actinomycin D, N-acetyl-L-cysteine (NAC), and rapamycin were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Etoposide, doxorubicin, 5-azacytidine, mithramycin A, ATP, and ADP were obtained from Wako Pure Chemical (Tokyo, Japan), and salubrinal was from Tocris Bioscience (Ellisville, MO). Cordycepin at 5 μg/ml was generally used for experiments.

**Cell culture.** The rat renal tubular epithelial cell line NRK-52E was purchased from American Type Culture Collection (Manassas, VA) and generally used for studies. WI-38 human fibroblasts purchased from RIKEN BRC Cell Bank (Tsukuba, Ibaragi, Japan) were also used in some experiments. Cells were cultured in Dulbecco’s Modified Eagle’s Medium/Ham’s F-12 (Gibco-BRL, Gaithersburg, MD) supplemented with 5–10% fetal bovine serum.

**Transient transfection.** Using electroporation, NRK-52E cells were transfected with pGL3-p21(WAF1/CIP1) (provided by Dr Naoko Ohtani; Japanese Foundation for Cancer Research, Tokyo, Japan) or pGL2-p16(NPκC) (provided by Dr Kiyoshi Nose; Showa University School of Pharmaceutical Sciences, Tokyo, Japan), treated with etoposide in the absence or presence of cordycepin or salubrinal, and subjected to luciferase assay. pGL3-p21(WAF1/CIP1) and pGL2-p16(NPκC) introduce a luciferase gene under the control of the p21(WAF1/CIP1) promoter and the p16(NPκC) promoter, respectively. In some experiments, pcDNA3.1-GADD34 was cotransfected with pGL3-p21(WAF1/CIP1), pGL3-SV40/GC that introduces a luciferase gene under the control of the simian virus 40 (SV40)/GC box promoter (provided by Dr Takeshi Omasa; Osaka University, Osaka, Japan) was cotransfected with pGL3-p21(WAF1/CIP1), pGL3-SV40/GC that introduces a luciferase gene under the control of the simian virus 40 (SV40)/GC box promoter (provided by Dr Kimitoshi Kohno; University of Occupational and Environmental Health, Fukuoka, Japan) was used to evaluate activity of Sp1 binding to the GC box.

**Luciferase assay.** Activity of luciferase was evaluated by Luciferase Assay System (Promega, Madison, WI) according to the manufacturer’s protocol. Assays were performed in quadruplicate.

**SA-β-gal assay.** SA-β-gal activity was evaluated as described by Dimri et al. (1995). In brief, cells were fixed in 3% formaldehyde for 4 min and incubated at 37°C overnight in 5-bromo-4-chloro-3-indolyl-D-galactopyranoside (X-gal) solution containing 1 mg/ml X-gal (Sigma-Aldrich Japan), 40 mM citric acid/sodium phosphate (pH 6.0), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 150 mM NaCl, and 2 mM MgCl₂. Assays were performed in quadruplicate.

**Evaluation of viable cells.** The number of viable cells was assessed by trypan blue exclusion. Water-soluble tetrazolium salt (WST) assay (formazan assay) was also performed using Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan).

**Western blot analysis.** Western blot analysis was performed using following antibodies. Anti-p53 antibody, antiphospho eIF2α antibody (Ser51), and antiphospho-p70S6K antibody (Thr389) were purchased from Cell Signaling (Beverly, MA), and anti-eIF2α antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphorylation of mitogen-activated protein (MAP) kinases was assessed using PhosphoPlus p44/42 MAP Kinase (Thr202/Tyr204) Antibody Kit and PhosphoPlus p38 MAP Kinase (Thr180/Tyr182) Antibody Kit (Cell Signaling), following the protocol provided by the manufacturer. The level of β-actin was assessed using anti-β-actin antibody (Sigma-Aldrich Japan) as a loading control. Blots were visualized using the enhanced chemiluminescent system (Amersham Biosciences, Buckinghamshire, UK).

**Northern blot analysis.** Total RNA was extracted by a single-step method, and Northern blot analysis was performed as described before (Kitamura et al., 2011). cDNAs for p21(WAF1/CIP1) (provided by Dr Konrad Huppi; National Institutes of Health, Bethesda, MD) and Sp1 (provided Dr Soichi Kojima; RIKEN, Wako, Japan) were used to prepare radiolabeled probes. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and 28S ribosomal RNA were used as loading controls.

**Statistical analysis.** Individual reporter assays, formazan assay, and cell counting assay were performed in quadruplicate. Each experiment was repeated 2–3 times, and a representative result was shown. Data were expressed as means ± SE. Statistical analysis was performed using the nonparametric Mann-Whitney U test to compare data in different groups. A value of p < 0.05 was considered to indicate a statistically significant difference.

**RESULTS**

**Suppression of Etoposide-Triggered Senescence by Cordycepin**

Cellular senescence is induced by genotoxic reagents such as etoposide, a DNA topoisomerase inhibitor (Gu and Kitamura, 2012; Leontieva and Blagosklonny, 2010). To examine whether etoposide-triggered senescence is inhibited by cordycepin, NRK-52E cells were treated with 1 μg/ml etoposide with or without cordycepin for up to 3 days, and microscopic analysis was performed. The etoposide-treated cells exhibited a large, extended and flattened cell shape typical of senescence. This morphological change was attenuated by the treatment with cordycepin (Fig. 1A). Quantitative analysis showed that percentages of senescent cells were reduced by cordycepin in a dose-dependent manner (Fig. 1B). Consistent with this result, activity of SA-β-gal was reduced by cordycepin (Fig. 1C). Percentages of SA-β-gal-positive cells significantly decreased from 75.6 ± 7.6% to 34.5 ± 4.8% by the treatment with 2.5 μg/ml cordycepin (Fig. 1D). Of note, cell viability was affected by neither etoposide nor cordycepin, and treatment with cordycepin alone did not induce senescent morphology and SA-β-gal expression (data not shown). The suppressive effect of cordycepin on the induction of SA-β-gal was not only in etoposide-treated cells but also in cells treated with other genotoxic agents. For example, treatment of NRK-52E cells with 20nM doxorubicin induced expression of SA-β-gal, and it was attenuated by the treatment with cordycepin (Figs. 1E and F).
**FIG. 1.** Suppression of etoposide-triggered senescence by cordycepin. (A–D) NRK-52E cells were treated with 1 μg/ml etoposide (Etop) together with indicated concentrations of cordycepin (Cor) for 24 h (A and B) or 3 days (C and D) and subjected to phase-contrast microscopy (A), quantification of senescent morphology (B), SA-β-gal staining (C), and quantitative assessment of SA-β-gal-positive cells (D). In (B), relative percentages of senescent cells versus etoposide (alone)–treated group (100%) are shown. Assays were performed in quadruplicate. Data are expressed as means ± SE, and asterisks indicate statistically significant differences (*p* < 0.05). (E and F) Cells were exposed to doxorubicin (Doxo; 20nM) in the absence or presence of 5 μg/ml cordycepin for 3 days and subjected to SA-β-gal staining (E) and quantitative analysis of SA-β-gal-positive cells (F). (G and H) Cells were treated with etoposide with or without cordycepin for 3 days. After the treatment, cells were reseeded with fresh growth medium (without etoposide and cordycepin) at 1 × 10^4 cells per well into 24-well plates, incubated for additional 8 days, and subjected to phase-contrast microscopy (G) and quantitative assessment of total cell number (H). (I and J) Cells were treated with etoposide in the presence of adenosine (ADS), ADP, or ATP at 100μM for 3 days and subjected to phase-contrast microscopy (I) and quantitative analysis of SA-β-gal-positive cells (J). N.S., not statistically significant.
Growth arrest is another important feature of cellular senescence. We further tested whether cordycepin has the potential to attenuate genotoxic stress–induced growth arrest. For this purpose, NRK-52E cells were treated first with etoposide in the absence or presence of cordycepin for 3 days. The cells were washed repeatedly and reseeded in fresh culture medium without etoposide and cordycepin. After 8 days, cells treated with etoposide alone maintained the senescent morphology, whereas cells cotreated with cordycepin regained mitogenic activity, leading to an increase in the cell number (Fig. 1G). Quantitative analysis confirmed that treatment with etoposide completely inhibited cell proliferation, whereas cotreatment with cordycepin partially reversed this suppressive effect (Fig. 1H).

Cordycepin is an analogue of adenosine. We examined whether other adenosine-related substances have similar effects to cordycepin. For this purpose, influences of adenosine, ADP, and ATP (100 μM, individually) on etoposide-induced senescence were tested. The results showed that, in contrast to cordycepin, adenosine and ATP did not affect induction of senescent morphology by etoposide (Fig. 1I). Although ADP reduced the percentage of senescent cells significantly, the effect was only minimal (Fig. 1J).

Influences of Cordycepin on p16<sup>INK4α</sup>–RB and p53–p21<sup>WAF1/CIP1</sup> Signaling

The p16<sup>INK4α</sup>–RB signal cascade plays a critical role in mediating activation of the senescence program. Signaling components involved in this process, including p16<sup>INK4α</sup>, are commonly used as biomarkers for cellular senescence (Hara et al., 1996; Kuilman et al., 2010). To further confirm our finding and to elucidate underlying mechanisms, we tested effects of cordycepin on senescence-induced p16<sup>INK4α</sup>. Reporter assay showed that a time-dependent increase in p16<sup>INK4α</sup> promoter activity was observed in the cells treated by etoposide (Fig. 2A). When cells were cotreated with cordycepin, the induction of p16<sup>INK4α</sup> was significantly attenuated (Fig. 2B). Consistent with the results shown in Figures 1I and J, adenosine, ADP, and ATP did not significantly affect etoposide-induced activation of the p16<sup>INK4α</sup> promoter (Fig. 2C).

Cellular senescence is dependent on another important pathway, p53–p21<sup>WAF1/CIP1</sup> signaling. p21<sup>WAF1/CIP1</sup> is a major mediator of p53-induced G1 arrest caused by DNA damage. Cells lacking p21<sup>WAF1/CIP1</sup> fail to arrest the cell cycle in response to DNA damaging agents (Brown et al., 1997). We further tested whether cordycepin affects induction of p21<sup>WAF1/CIP1</sup> by genotoxic stress. Northern blot analysis showed that etoposide induced expression of p21<sup>WAF1/CIP1</sup> mRNA, and it was markedly suppressed by the treatment with cordycepin (Fig. 2D). Consistent with this result, activity of the p21<sup>WAF1/CIP1</sup> gene promoter was induced by etoposide in a time-dependent manner (Fig. 2E), and it was significantly inhibited by cordycepin (Fig. 2F). In contrast, ADP and ATP did not affect etoposide-induced activation of the p21<sup>WAF1/CIP1</sup> gene (Fig. 2G). Although adenosine reduced the induction of p21<sup>WAF1/CIP1</sup> significantly, the effect was only minimal and much less than the effect of cordycepin.

Phosphorylation of eIF2α Mediates the Antisenescence Effect of Cordycepin

We recently found that cordycepin induces phosphorylation of eIF2α in NRK-52E cells (Kitamura et al., 2011). A previous report showed that the level of eIF2α phosphorylation declined in various organs during aging in rats (Hussain and Ramaiah, 2007). Other reports also suggested that eIF2 family members are involved in the regulation of aging and life span (Kimball et al., 1992; Tohyama et al., 2008). We, therefore, tested whether activation of eIF2α is involved in the antisenescence effect of cordycepin. For this purpose, salubrinal, a selective inhibitor of eIF2α dephosphorylation, was utilized (Boyce et al., 2005). As expected, cordycepin induced phosphorylation of eIF2α at concentrations higher than 2.5 μg/ml, and it was mimicked by 50 μM salubrinal (Fig. 3A).

To examine whether activation of eIF2α by salubrinal reproduces the antisenescence effect of cordycepin, cells were exposed to etoposide in the absence or presence of salubrinal and subjected to analyses of senescent phenotypes. Microscopic analyses showed that salubrinal attenuated etoposide-induced senescent morphology and induction of SA-β-gal (Fig. 3B). Quantitative analysis showed that the percentage of cells with senescent morphology was reduced from 57.6 ± 5.6% to 39.3 ± 2.7% by the treatment with salubrinal (Fig. 3C). The percentage of SA-β-gal-positive cells was also significantly reduced from 74.6 ± 14.0% to 15.6 ± 2.2% by salubrinal (Fig. 3D). Consistent with these results, salubrinal suppressed etoposide-triggered activation of the p16<sup>INK4α</sup> promoter and the p21<sup>WAF1/CIP1</sup> promoter (Figs. 3E and F). The downregulation of the p21<sup>WAF1/CIP1</sup> promoter activity was correlated with attenuated expression of p21<sup>WAF1/CIP1</sup> mRNA (Fig. 3G).

To further confirm that the antisenescence effects of cordycepin and salubrinal are mediated by activation of eIF2α, function of eIF2α was impaired by overexpression of GADD34 that dephosphorylates eIF2α. NRK-52E cells were cotransfected with pGL3-p21<sup>WAF1/CIP1</sup> together with GADD34, exposed to etoposide with or without cordycepin or salubrinal, and subjected to analysis. Reporter assay showed that, in mock-transfected cells, both drugs significantly inhibited activation of the p21<sup>WAF1/CIP1</sup> promoter and that the functional inhibition of eIF2α reversed the suppressive effects of these agents (Fig. 3H).

Sp1 Is a Target for Antisenescence Effect of Cordycepin

Tumor suppressor p53 plays an important role in cellular responses to genome instability, e.g., genotoxic stress–induced senescence. p21<sup>WAF1/CIP1</sup> is one of the most famous targets regulated by p53 (Reinhardt and Schumacher, 2012). We tested whether p53 is an upstream target for cordycepin to suppress p21<sup>WAF1/CIP1</sup> and p16<sup>INK4α</sup>. For this purpose, protein levels of p53 were examined in cordycepin- and salubrinal-treated cells. Etoposide increased the level of p53, whereas this induction
was inhibited neither by cordycepin nor salubrinal (Fig. 4A). Of note, cordycepin rather enhanced the induction of p53 by etoposide. These results indicate that some factors other than p53 mediate the effect of cordycepin.

Global DNA methylation decreases during senescence (Wilson and Jones, 1983). Cordycepin may inhibit gene expression by induction of hypermethylation. To test this possibility, 5-azacytidine, an inhibitor of methylation, was used. Cells were treated with cordycepin in the absence or presence of 5-azacytidine, and activity of the p16^{INK4a} promoter was tested. The result showed that inhibition of methylation failed to reverse the suppressive effect of cordycepin on p16^{INK4a} expression.

**FIG. 2.** Influences of cordycepin on p16^{INK4a}-RB and p53–p21^{WAF1/CIP1} signaling. (A) NRK-52E cells were transfected with pGL2-p16^{INK4a}, treated with etoposide for indicated time periods, and subjected to luciferase assay to evaluate p16^{INK4a} promoter activity. Luciferase activity was normalized by the number of viable cells estimated by formazan assay, and relative values are shown. Assays were performed in quadruplicate. Data are expressed as means ± SE, and asterisks indicate statistically significant differences (p < 0.05). (B and C) Cells transfected with pGL2-p16^{INK4a} were exposed to etoposide in the absence or presence of cordycepin (B), or ADS, ADP, or ATP (C) for 12 h and subjected to luciferase assay and formazan assay. (D) Cells were treated with etoposide in the absence or presence of cordycepin for 6 h and subjected to Northern blot analysis of p21^{WAF1/CIP1}. The level of GAPDH mRNA is shown at the bottom as a loading control. (E) Cells were transfected with pGL3-p21^{WAF1/CIP1}, treated with etoposide for up to 72 h, and subjected to formazan assay and luciferase assay to evaluate p21^{WAF1/CIP1} promoter activity. (F and G) Cells transfected with pGL3-p21^{WAF1/CIP1} were exposed to etoposide in the absence or presence of cordycepin (F) or indicated adenosine analogues (G) for 24 h and subjected to luciferase assay and formazan assay. N.S., not statistically significant.
promoter activity (Supplementary fig. S1), excluding this possibility.

Previous reports showed that six Sp1 binding sites are present in the promoter region of the p21WAF1/CIP1 gene and that these sites are required for p21WAF1/CIP1 induction by genotoxic agents such as histone deacetylase inhibitor (Gartel and Tyner, 1999; Sowa et al., 1999). The regulatory region of the p16INK4a gene also contains Sp1 binding sites essential for the
induction of p16\(^{INK4a}\) by toyocamycin (Kurihara et al., 2002). We, therefore, tested a role of Sp1 in the regulation of p21\(^{WAF1/CIP1}\) and p16\(^{INK4a}\) by cordycepin. An Sp1 inhibitor mithramycin A was used for this purpose. Northern blot analysis showed that etoposide-induced expression of p21\(^{WAF1/CIP1}\) was abrogated by the treatment with mithramycin A (Fig. 4B). Similarly, reporter assay demonstrated that etoposide-induced activation of the p16\(^{INK4a}\) promoter was inhibited by mithramycin A (Fig. 4C).

These results suggest that etoposide induces activation of Sp1, which is essential for genotoxic stress–triggered induction of p21\(^{WAF1/CIP1}\) and p16\(^{INK4a}\).

To further examine whether cordycepin inhibits activity of Sp1, we evaluated binding of Sp1 to the Sp1 sites (Niina et al., 2007). Reporter assay showed that the DNA binding activity of Sp1 was induced by etoposide, and it was significantly suppressed by the treatment of cordycepin (Fig. 4D). This suppressive effect of cordycepin was mediated, at least in part, by transcriptional suppression of basal Sp1 mRNA (Fig. 4E).

**DISCUSSION**

In the present investigation, we demonstrated that cordycepin, a bioactive substance in *Cordyceps* species, interferes with genotoxic stress–induced senescence via suppression of p16\(^{INK4a}\) and p21\(^{WAF1/CIP1}\). We found that the suppressive effect was mediated by activation of eIF2α. Furthermore, cordycepin inhibited expression and activity of Sp1, which was responsible for the induction of p16\(^{INK4a}\) and p21\(^{WAF1/CIP1}\). Our current hypothesis was schematically presented in Figure 5. Previous reports showed that *Cordyceps* species contain substances that may have the antiaging potential (Ji et al., 2009; Li et al., 2010; Xiao et al., 2012). Our current findings raise a possibility that cordycepin is an active entity responsible for the antiaging property of *Cordyceps* species.

In the present investigation, we found that cellular senescence caused by either etoposide or doxorubicin was attenuated by cordycepin. It indicates that genotoxic stress–induced cellular senescence (premature senescence) is generally attenuated by this agent. Our preliminary data using WI-38 normal human fibroblasts also suggest that cellular senescence induced by telomere shortening (replicative senescence) is similarly inhibited by cordycepin, when assessed by SA-β-gal expression (Supplementary fig. S2) and growth arrest (data not shown). The antisenescent effect of cordycepin is, possibly, not restricted to premature senescence.

In this study, we elucidated that the antisenescent effect of cordycepin was ascribed to activation of eIF2α and suppression of Sp1. Important questions are how cordycepin affects these molecules. Currently, it is unclear how 3′-deoxyadenosine activates eIF2α. One possibility is its suppressive effect on GADD34.
involved in dephosphorylation of eIF2α. As we previously reported, GADD34 is constitutively expressed in NRK-52E cells, and it was suppressed by the treatment with cordycepin (Kitamura et al., 2011). The downregulation of GADD34 might be a possible mechanism underlying increased phosphorylation of eIF2α by cordycepin. Of note, previous reports suggested that GADD34 is an important regulator that facilitates expression of p21	extsuperscript{WAF1/CIP1} (Minami et al., 2007; Yagi et al., 2003).

The suppression of Sp1 by cordycepin is not mediated by activation of eIF2α. It is because neither expression of Sp1 mRNA nor Sp1 promoter activity was suppressed by the treatment of salubrinal (our unpublished data). As demonstrated, cordycepin suppressed Sp1 expression at a transcriptional level. Previous reports suggested that cordycepin has the potential to inhibit transcriptional events through multiple mechanisms (Müller et al., 1977; Penman et al., 1970). It inhibits mRNA polyadenylation presumably by acting as a chain terminator (Müller et al., 1977). At high doses, cordycepin also inhibits mRNA synthesis by interfering with the incorporation of ATP (Penman et al., 1970). In this report, we showed that the steady-state level of Sp1 mRNA was markedly decreased by the treatment with cordycepin. The suppression of Sp1 by cordycepin may be through inhibition of mRNA synthesis. However, individual mRNAs have different susceptibility to cordycepin. We observed that the transcriptional expression of luciferase driven by viral or housekeeping gene promoters was not depressed by cordycepin. Sp1 mRNA may be a preferential target of cordycepin. Of note, the suppressive effect of cordycepin on senescence was not mimicked by actinomycin D (Supplementary fig. S3). This result further supports that the effect of cordycepin is not ascribed to global inhibition of RNA synthesis.

p21	extsuperscript{WAF1/CIP1} is a key mediator of senescence. We demonstrated that both p21	extsuperscript{WAF1/CIP1} promoter activity and p21	extsuperscript{WAF1/CIP1} mRNA level were suppressed by cordycepin. In contrast, Lee et al. (2010) showed that cordycepin induced G2/M cell cycle arrest by induction of p21	extsuperscript{WAF1/CIP1} expression in tumor cells. Currently, the reason for the discrepancy is unclear, but pharmacological action of cordycepin may be different depending on its concentrations and cell types. In the present investigation, we used cordycepin at 5 μg/ml (approximately 20μM), whereas the study by Lee et al. used 10 times higher concentration, 200μM. Of note, the authors showed that expression of p21	extsuperscript{WAF1/CIP1} was not induced by cordycepin at 50μM.

Oxidative stress is considered to be an important event underlying aging processes. Cellular activity and capacity of antioxidant systems decline with increased age, leading to oxidative cell injury. 	extit{Cordyceps} species and cordycepin have the potential to scavenge free radicals (Xiao et al., 2012; Yu et al., 2006). Recently, Ramesh et al. (2012) reported that cordycepin attenuated age-related oxidative stress and improved antioxidant capacity in rats. They showed that age-associated declines in the activities of superoxide dismutase, catalase, and glutathione peroxidase levels were observed in the liver, kidney, heart, and lung and that it was attenuated by the treatment with cordycepin in rats. Genotoxic stress caused, for example, by etoposide is known to trigger generation of reactive oxygen species (Oh et al., 2007). The antisenescence potential of cordycepin may be ascribed to its antioxidant property. However, our data showed that treatment with antioxidant NAC did not attenuate etoposide-induced senescent morphology and SA-β-gal induction (Supplementary fig. S4), excluding this possibility.
In cellular senescence, some kinases including MAP kinases and mammalian target of rapamycin complex 1 (mTORC1) play crucial roles in the induction of senescent phenotypes. Oncogenic and environmental stresses induce premature cellular senescence in which Ras/Raf/extracellular signal–regulated kinase (ERK) pathway and the p38 MAP kinase pathway are involved (Debacq-Chainiaux et al., 2010). We examined effects of cordycepin on etoposide-induced activation of ERK and p38 MAP kinase in NRK-52E cells. Our data showed that phosphorylation of p38 MAP kinase, but not ERK, was induced by etoposide (Supplementary fig. S5A). However, the etoposide-induced phosphorylation of p38 MAP kinase was suppressed by neither cordycepin nor salubrinal (Supplementary fig. S5B). This result exclude a possibility that p38 MAP kinase is a target for the antisenescent effect of cordycepin. Similarly, although mTORC1 is an important regulator for aging and longevity (Evans et al., 2011) and activation of mTORC1 is induced by etoposide (Supplementary fig. S6A), 100nM rapamycin (inhibitor of mTORC1) did not affect etoposide-induced senescent morphology and SA-β-gal expression (Supplementary fig. S6B).

In summary, our results suggest that cordycepin interferes with senescence via activation of eIF2α and suppression of Sp1 without inhibition of p53. These mechanisms may underlie the antiaging property of Cordyceps species that abundantly contain cordycepin.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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