Suppression of adipocyte differentiation by *Cordyceps militaris* through activation of the aryl hydrocarbon receptor

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Mycelial extracts have a variety of biological effects that modulate functions of mammalian cells, especially antiinflammatory and immunomodulating activities (45). However, presently little is known about effects of mycelia on the adipose tissue. We sought to identify antiadipogenic mycelia with an in vitro model of adipogenesis and found that the extract of *Cordyceps militaris* exclusively suppressed differentiation of 3T3-L1 preadipocytes. *C. militaris* is a fungus that parasitizes Lepidoptera larvae and has benefits in the human body including in the circulatory, immune, and metabolic systems. Previous studies showed that *C. militaris* inhibits angiogenesis and proliferation of normal and malignant cells (27, 47, 50). Other investigators also reported the antiinflammatory, antidiabetic, antiangiogenic, and hypocholesterolemic potential of this mycelium (8, 25, 30, 46). However, to date, the antiadipogenic potential of *C. militaris* has never been reported. In the present investigation, we demonstrate that *C. militaris* suppresses differentiation of 3T3-L1 preadipocytes into mature adipocytes with the use of several adipocyte and preadipocyte markers. We investigate molecular mechanisms involved in the suppressive effect and provide evidence that *C. militaris* interferes with adipocyte differentiation through activation of the aryl hydrocarbon receptor (AhR).

MATERIALS AND METHODS

Reagents. Mycelial extracts of 10 Basidiomycetes strains, *Phellinus linteus*, *C. militaris*, *Lyophyllum decastes*, *Macrolepita gracilenta*, *Agaricus blazei*, *Grifola frondosa*, *Ganoderma lucidum*, *Inonotus obliquus*, *Lentinula edodes*, and *Pleurotus nebrodensis*, were prepared by IBI as described previously (16). In brief, 20 g of individual dried mycelia were suspended in 140 ml of distilled water and boiled at 105°C for 60 min. After centrifugation at 15,000 rpm for 5 min, the supernatants were filtrated with serial filters (5 μm → 1 μm → 0.45 μm → 0.2 μm), freeze-dried, dissolved in sterile water at a concentration of 5%, and used for experiments. Insulin, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, oil red O, and 3-methylcholanthrene (3MC) were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Benzo[α]pyrene (B[α]P) was obtained from Wako Pure Chemical Industries (Osaka, Japan).

Induction of adipocyte differentiation. 3T3-L1 preadipocytes purchased from Health Science Research Resources Bank (Osaka, Japan) were maintained in Dulbecco’s modified Eagle’s medium-F-12 (Wako Pure Chemical Industries) supplemented with 10% fetal bovine serum (basal medium). For the induction of adipocyte differentiation, cells were 1) precultured in basal medium for 2 days, 2) treated with differentiation medium containing 10 μg/ml insulin, 0.25 μM dexamethasone, and 500 μM IBMX (IDI medium) for 2 days, or 3) treated with differentiation medium containing 10 μg/ml insulin, 0.25 μM dexamethasone, and 500 μM IBMX (IDI medium) for 2 days, and then treated with 10 μg/ml insulin, 0.25 μM dexamethasone, and 500 μM IBMX (IDI medium) for 2 days.
days, and J) incubated in basal medium supplemented with insulin alone (insulin medium) for 2 days, as described previously (36). The cells were further incubated in basal medium for an additional 2 days and subjected to analyses. To examine effects of mycelial extracts and AhR agonists, cells were exposed to the individual agents only during incubation in IDI medium.

Establishment of stable transfectants. With electroporation, 3T3-L1 cells were transfected with pEFBOS-AhR(Arg39), which encodes a dominant-negative mutant of AhR (AhR-DN) under the control of the elongation factor-1α promoter (9 µg; a gift from Dr. Kazuhiro Sagawa) (40) together with pcDNA3.1 (3 µg; Invitrogen, Carlsbad, CA), which codes for neomycin phosphotransferase. Stable transfectants were selected by G418 (500 µg/ml), 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.

Oil red O staining. To quantify lipid accumulation, cells were fixed with 10% formalin in PBS for 10 min, rinsed with 60% isopropanol, and stained with 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 with agitation for 5 min. Absorbance (520-nm wavelength) was measured by a spectrophotometer.

Northern blot analysis. Northern blot analysis was performed as described previously (24). cDNAs for adiponectin (42), PPARγ (purchased from Addgene, Cambridge, MA) (13), C/EBPα (3), monocyte chemoattractant protein-1 (MCP-1) (33), 78-kDa glucose-regulated protein (GRP78) (22), C/EBP-homologous protein (CHOP) (44), AhR (40), and cytochrome P-450 1B1 (CYP1B1) (37) were used for preparation of radiolabeled probes. The levels of 28S ribosomal RNA and glyceraldehyde-3-phosphate dehydrogenase mRNA were used as loading controls.

Western blot analysis. Levels of total Akt protein and phosphorylated Akt were evaluated by Western blot analysis using anti-Akt antibody (Ab) and anti-phospho-Akt Ab (1/200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA).

Dioxin-responsive element-based sensing via secreted alkaline phosphatase assay. Dioxin-responsive element-based sensing via secreted alkaline phosphatase (DRESSA) bioassay was performed with HeXS34 cells to evaluate activity of AhR (19–21). Activity of secreted alkaline phosphatase (SEAP) in culture medium was evaluated by a chemiluminescent method using a Great EscApE SEAP Detection Kit (BD Biosciences, Palo Alto, CA) as described previously (20).

Endoplasmic reticulum stress-responsive alkaline phosphatase assay. Induction of endoplasmic reticulum (ER) stress was evaluated by ER stress-responsive alkaline phosphatase (ES-TRAP) assay (14). Activity of ES-TRAP secreted by transfected cells is rapidly and sensitively downregulated in response to ER stress independently of transcriptional regulation. This phenomenon is observed in a wide range of cell types triggered by various ER stress inducers (14). To evaluate induction of ER stress in 3T3-L1 cells, the cells were transiently transfected with pSEAP2-Control (BD Biosciences) with the use of GeneJuice Transfection Reagent (Novagen, Madison, WI) and treated with test reagents. Activity of ES-TRAP in culture medium was evaluated with the Great EscApE SEAP Detection Kit.

Formazan assay. The number of viable cells was assessed by formazan assay with Cell Counting Kit-8 (Dojindo Laboratory, Kumamoto, Japan) (49).

Statistical analysis. Data are expressed as means ± SE. Statistical analysis was performed with the nonparametric Mann-Whitney U-test to compare data in different groups. A P value of <0.05 was considered to indicate a statistically significant difference.

RESULTS

Blockade of adipocyte differentiation and lipid accumulation by C. militaris. To seek antiadipogenic mycelia, we tested effects of 10 mycelial extracts (see MATERIALS AND METHODS) on differentiation of 3T3-L1 preadipocytes. 3T3-L1 cells were cultured in IDI medium in the absence or presence of various mycelial extracts at a final concentration of 0.2%. After 2 days, the cells were cultured in insulin medium for 2 days, further incubated in basal medium for 2 days, and subjected to microscopic analyses. Among the mycelial extracts tested, only C. militaris markedly blocked adipocyte differentiation (Fig. 1A). In contrast to P. nebrodensis, which induced mild cell injury and modest inhibition of adipogenesis, C. militaris did not cause any cellular damage. Oil red O staining revealed that accumulation of lipid was almost completely suppressed by C. militaris (Fig. 1B).

During differentiation of 3T3-L1 preadipocytes, expression of adiponectin, PPARγ, and C/EBPα increases dramatically (6, 7, 15), whereas basal expression of MCP-1 declines (10). To confirm the inhibitory effect of C. militaris on adipocyte differentiation, differentiation of 3T3-L1 cells was induced in the absence or presence of C. militaris, and expression levels of those markers were evaluated. Northern blot analysis revealed that expression of adipocyte markers adiponectin, PPARγ, and C/EBPα was induced by IDI, whereas the induction was abrogated by C. militaris. In contrast, expression of the preadipocyte marker MCP-1 was abrogated by IDI, whereas this inhibitory effect was reversed by C. militaris (Fig. 1F). Together, these results demonstrated that C. militaris inhibits adipocyte differentiation.

We examined whether or not suppression of adipocyte differentiation by C. militaris is reversible. For this purpose, 3T3-L1 cells were cultured in IDI medium (first exposure) in the presence of C. militaris for 2 days and further incubated in insulin medium for an additional 2 days. The cells were then treated with or without IDI for 2 days, and after an additional 4 days microscopic analyses were performed. As shown in Fig. 2A, C. militaris-primed cells that did not differentiate by the first exposure to IDI underwent significant differentiation by the second exposure to IDI. Quantitative analysis showed that intracellular lipid significantly increased from 7.9 ± 0.5% to 56.1 ± 0.8% by the second exposure to IDI in C. militaris-primed cells (P <0.05) (Fig. 2B).

To further examine whether C. militaris inhibits not only differentiation of preadipocytes into adipocytes but also lipid accumulation in mature adipocytes, fully differentiated 3T3-L1 cells (2 days in IDI medium, 2 days in insulin medium, and 2 days in basal medium) were incubated in the absence or presence of 0.2% C. militaris for 12 days, and microscopic analyses were performed. As shown in Fig. 2C, the size of lipid droplets in C. militaris-treated adipocytes was smaller than that of untreated adipocytes. Quantitative analysis revealed that accumulation of intracellular lipid significantly decreased to 71.0 ± 1.2% vs. 100 ± 2.7% in untreated controls (Fig. 2D),
indicating that *C. militaris* may also inhibit the process of hypertrophy in mature adipocytes. Hypertrophic adipocytes are known to be resistant to insulin. To confirm the antihypertrophic effect of *C. militaris*, we examined insulin-triggered phosphorylation of Akt in mature adipocytes treated with or without *C. militaris* for 7–14 days. Northern blot analysis showed that prolonged culture caused expression of *MCP-1*, a marker of hypertrophic adipocytes, within 4 days, and its

![Image](http://ajpendo.physiology.org/)

Fig. 1. Blockade of adipocyte differentiation by *Cordyceps militaris*. 3T3-L1 preadipocytes were treated with mycelial extracts (0.05–0.2%) in differentiation medium containing 10 μg/ml insulin, 0.25 μM dexamethasone, and 500 μM 3-isobutyl-1-methylxanthine (IDI) for 2 days. The cells were then incubated in basal medium supplemented with insulin alone (insulin medium) for 2 days, cultured in basal medium for an additional 2 days, and subjected to phase-contrast microscopy and oil red O staining. A: effects of extracts (0.2%) from 10 mycelia, *Phellinus linteus* (PL-1), *C. militaris* (CM), *Lyophyllum decastes* (LD), *Macrolepista gracilenta* (MGR), *Agaricus blazei* (AB), *Grifola frondosa* (GF), *Ganoderma lucidum* (GL), *Inonotus obliquus* (IOB), *Lentinula edodes* (LE), and *Pleurotus nebrodensis* (PNE), by phase-contrast microscopy. B–E: effects of *C. militaris* evaluated by microscopic analyses (B and D; phase-contrast microscopy, top; oil red O staining, bottom) and quantification of intracellular lipid (C and E). In C and E, data are expressed as relative % (means ± SE). *Statistically significant differences (P < 0.05). Assays were performed in quadruplicate. F: Northern blot analysis of adipocyte and preadipocyte markers. Differentiation of 3T3-L1 cells was induced in the absence or presence of *C. militaris*, and expression levels of adipocyte markers *adiponectin*, peroxisome proliferator-activated receptor-γ (PPARγ), CCAAT/ enhancer binding protein α (C/EBPα), and the preadipocyte marker monocyte chemoattractant protein-1 (*MCP-1*) were examined. The level of 28S ribosomal RNA is shown at bottom as a loading control.
Fig. 2. Reversibility of the suppressive effect of *C. militaris* on adipocyte differentiation and its effect on hypertrophy in mature adipocytes. A and B: 3T3-L1 cells were cultured in IDI medium (1st exposure) in the presence of *C. militaris* (0.2%) for 2 days and further incubated in insulin medium for an additional 2 days. The cells were then treated with or without IDI for 2 days (2nd exposure) and, after an additional 4 days, subjected to analyses. A: microscopic analyses (phase-contrast microscopy, top; oil red O staining, bottom). B: quantitative analysis of lipid content. In B and D, data are expressed as relative % (means ± SE). *Statistically significant differences (P < 0.05). Assays were performed in quadruplicate. C and D: 3T3-L1 cells were fully differentiated by incubation for 2 days in IDI medium, 2 days in insulin medium, and 2 days in basal medium and then treated with or without 0.2% *C. militaris* for 12 days. C: microscopic analyses. D: quantitative analysis of lipid content. E: fully differentiated adipocytes were cultured in basal medium for 7–14 days, stimulated by 50 μg/ml insulin for 30 min, and subjected to Western blot analysis of phosphorylated Akt (p-Akt). Level of total Akt protein is shown at bottom as a loading control.
expression level increased thereafter (data not shown). Western blot analysis revealed that, after stimulation with insulin for 30 min, hypertrophic adipocytes showed weak phosphorylation of Akt, whereas adipocytes treated with \textit{C. militaris} showed a substantial response to the stimulus (Fig. 2E). Of note, CM per se did not induce Akt phosphorylation. This result was observed consistently at both day 7 and day 14.

\textit{Inhibition of adipogenesis by \textit{C. militaris} through activation of AhR.} Various chemical and bioactive substances have the potential to perturb function of the ER, leading to accumulation of unfolded proteins within the ER (26). This ER stress triggers cascades of signal transduction pathways, known as the unfolded protein response, and affects various cell functions (35).

Recently, we reported (36) that K-7174, a GATA inhibitor, suppresses adipocyte differentiation and that it is associated with induction of ER stress. We speculated that \textit{C. militaris} may suppress adipocyte differentiation through induction of ER stress. To examine this possibility, 3T3-L1 cells were treated with 0.2\% \textit{C. militaris} for up to 9 h, and expression of endogenous indicators for ER stress, GRP78 and CHOP, was examined. Northern blot analysis revealed that expression of these genes was not induced by the treatment with \textit{C. militaris} (Fig. 3A). To further confirm this conclusion, we performed the ES-TRAP assay, which can detect ER stress with high sensitivity and specificity (14). Under ER stress conditions, activity of extracellular ES-TRAP is rapidly downregulated in ES-TRAP-transfected cells regardless of triggers for ER stress. 3T3-L1 preadipocytes were transiently transfected with an ES-TRAP gene and treated with 0.2\% \textit{C. militaris} for up to 24 h. The culture medium and cells were subjected to chemiluminescent assay and formazan assay, respectively. Activity of ES-TRAP was then normalized by the number of viable cells estimated by formazan assay. As shown in Fig. 3B, ES-TRAP activity was not affected by \textit{C. militaris} throughout the course of experiments, confirming the lack of induction of ER stress.

We and others showed that natural products including herbal medicines contain various agonists of AhR (2, 18). Previous reports suggested that activation of AhR may suppress adipocyte differentiation (1, 39). We hypothesized that \textit{C. militaris} may inhibit adipocyte differentiation through activation of AhR. To examine this possibility, the potential of \textit{C. militaris} for activation of AhR was evaluated by DRESSA, a reporter assay that we previously established (19–21). Reporter cells that secrete SEAP under the control of AhR were treated with 0.1\% \textit{C. militaris} for 24 h, and activity of SEAP in the culture medium was evaluated. As shown in Fig. 3C, activity of AhR was significantly increased in response to \textit{C. militaris}. In contrast, other tested mycelial extracts did not induce activation of AhR (data not shown). This result was further confirmed by using an endogenous indicator of AhR activation in adipocytes, \textit{CYP1B1} (1). Consistent with the result from the DRESSA assay, Northern blot analysis revealed that \textit{C. militaris} rapidly induced expression of \textit{CYP1B1} (Fig. 3D), confirming the induction of AhR activation.

To examine whether activation of AhR is responsible for inhibition of adipogenesis by \textit{C. militaris}, we first examined effects of AhR agonists on the differentiation of 3T3-L1 preadipocytes. 3T3-L1 cells were treated with IDI in the presence of 5 \mu M 3MC or 1 \mu M B[a]P for 2 days, and morphological examination was performed after 4 days. As shown in Fig. 3E, the inhibitory effect of \textit{C. militaris} was reproduced by these AhR agonists. Quantitative analysis revealed that intracellular lipid was significantly reduced to 24.8 \pm 1.3\% by 3MC and to 12.5 \pm 2.4\% by B[a]P vs. 100\% in \textit{C. militaris}-untreated, IDI-treated cells (Fig. 3F).

To confirm the role of AhR in the suppression of adipocyte differentiation by \textit{C. militaris}, we established 3T3-L1 cells overexpressing a dominant-negative mutant of AhR (Fig. 4A), and the suppressive effect of \textit{C. militaris} was retested. As shown in Fig. 4B, \textit{C. militaris} inhibited adipocyte differentiation in mock-transfected 3T3-L1/Neo cells. However, this inhibitory effect was abolished in 3T3-L1/AhR-DN cells overexpressing the dominant-negative mutant of AhR (Fig. 4B). Quantitative analysis using oil red O staining revealed that the suppression of lipid accumulation by \textit{C. militaris} was significantly reversed by dominant-negative inhibition of AhR from 25.9 \pm 1.2\% to 94.5 \pm 4.4\% vs. 100\% in \textit{C. militaris}-untreated, IDI-treated 3T3-L1/Neo cells (Fig. 4C). These results confirmed that \textit{C. militaris} suppresses adipocyte differentiation through activation of AhR.

\textbf{DISCUSSION}

\textit{C. militaris} has been considered an entomopathogenic fungus that may be potentially useful for prevention and treatment of various pathologies in humans including cancers, inflammation, fibrosis, diabetes, and hypercholesterolemia (8, 25, 27, 30, 46, 47, 50). However, in the majority of previous studies only phenomenological evidence was provided, and molecular mechanisms underlying the beneficial, therapeutic effects of \textit{C. militaris} have never been reported. In the present investigation, we demonstrated for the first time that \textit{C. militaris}, but not other mycelia, uniquely suppresses adipogenesis in vitro. We also elucidated that \textit{C. militaris} interferes with adipocyte differentiation through activation of the AhR.

Currently, the mechanisms underlying the unique, antiadipogenic property of \textit{C. militaris} are not fully determined, but several possibilities can be postulated. Previous reports demonstrated that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the ligand of AhR, inhibited adipocyte differentiation in several cell types including 3T3-L1 cells, C3H10T1/2 cells, and primary mouse embryonic fibroblasts (MEFs) (1, 5, 31). Subsequent investigation suggested possible involvement of mitogen-activated protein kinases and tyrosine kinases. For example, using 3T3-L1 cells, Shima et al. (39) reported that cells overexpressing AhR exhibited enhanced extracellular signal-regulated kinase (ERK) activity and that pharmacological inhibition of ERK abrogated the inhibitory action of TCDD on adipogenesis. Hanlon et al. (12) demonstrated that low levels of ERK activation cooperate with activated AhR to induce a transcriptional suppressor of PPAR\gamma, the crucial differentiation factor during adipogenesis. Vogel and Matsumura (43) reported that \textit{J} TCDD did not suppress differentiation of c-Src-deficient MEFs into adipocytes and \textit{2} TCDD induced \textit{C/EBP}b and \textit{C/EBP}x mRNA and their DNA binding activity in wild-type MEFs but not in c-Src-deficient MEFs. These data indicated that suppression of adipocyte differentiation by TCDD requires ERK activation, functional c-Src, and/or induction of \textit{C/EBP}b and \textit{C/EBP}x. Similar mechanisms might also be involved in the suppression of adipogenesis by \textit{C. militaris} observed in this report.

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**C. militaris** may also inhibit adipogenesis in other ways. In the induction of adipocyte differentiation, we used IDI medium that contained insulin. A previous report showed that activation of the phosphatidylinositol 3-kinase-protein kinase B/Akt signal cascade triggered by insulin and insulin-like growth factor plays a crucial role in adipocyte differentiation (48). Cordycepin is a major component of **C. militaris** with antiviral, anticancer, and immunomodulatory activities (9, 29, 52). A recent study using lipopolysaccharide-activated macrophages showed that cordycepin markedly inhibits phosphorylation of Akt (23). Cordycepin in **C. militaris** may be responsible for suppression of adipogenesis by interfering with insulin signaling.

Currently, the active entity responsible for the activation of AhR by **C. militaris** is unknown. However, several previous
reports showed that nucleosides and polysaccharides are major components in Cordyceps and possess a broad range of biological and pharmacological properties (28, 45). These substances may be the ingredients to activate AhR, and further investigation will be required to examine this possibility.

In this report, we showed that C. militaris suppressed not only adipocyte differentiation but also accumulation of lipid in differentiated adipocytes. Treatment of adipocytes with C. militaris prevented hypertrophy and preserved the responses of the cells to insulin in prolonged culture. These results suggest the possibility that administration with C. militaris may be useful for prevention of insulin resistance in type 2 diabetes (17). Currently, it is unclear how C. militaris inhibits lipid accumulation and hypertrophy in mature adipocytes. A previous report suggested that the level of AhR is downregulated during adipocyte differentiation and that adipocytes are relatively insensitive to TCDD when compared with preadipocytes (38). In contrast to the effect of C. militaris on adipocyte differentiation, its effect on adipocyte hypertrophy might be independent of AhR.

Fig. 4. Involvement of AhR activation in the antiadipogenic effect of C. militaris. 3T3-L1 cells were stably transfected with a dominant-negative mutant of AhR (AhR-DN), and 3T3-L1/AhR-DN cells were established. A: expression of endogenous AhR and exogenous AhR-DN in 3T3-L1/AhR-DN cells and mock-transfected 3T3-L1/Neo cells was examined by Northern blot analysis. B and C: established cells were treated with IDI in the absence or presence of 0.2% C. militaris and subjected to microscopic analyses (B) and quantitative analysis of lipid content (C). In C, assays were performed in quadruplicate. Data are expressed as means ± SE. *Statistically significant difference (P < 0.05).
Previous reports evidenced antiobesity effects of *C. militaris* in pancreaticectomized diabetic rats and streptozotocin-induced diabetic mice (8, 51). Although the molecular mechanisms involved have not been elucidated, our present results indicate the possibility that the antiadipogenic effect of *C. militaris* on preadipocytes as well as its antihypertrophic effect on mature adipocytes may, at least in part, explain the antiobesity effect of this mycelium in vivo. Our present findings raise the possibility that *C. militaris* may be useful for treatment of obesity and obesity-related metabolic disorders. In addition, several putative endogenous AhR ligands have been identified to date, e.g., tryptophan photooxidation products, lyposin A4, indirubin, bilirubin, biliverdin, and an indole derivative (32). These substances per se or some substances that trigger elevation of these AhR agonists may also be useful as antiobesity agents.

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