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

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ABSTRACT

Mycelial extracts have a wide range of biological activities that modulate functions of mammalian cells. In this report, we sought anti-adipogenic mycelia using 3T3-L1 cells and found that the extract of *Cordyceps militaris* (*C. militaris*) exclusively suppressed differentiation of 3T3-L1 preadipocytes into mature adipocytes without affecting cell viability. This inhibitory effect was dose-dependent, reversible, and associated with: 1) decreases in lipid accumulation, 2) blunted induction of adipocyte markers including adiponectin, peroxisome proliferator-activated receptor γ and CCAAT/enhancer binding protein α, and 3) sustained expression of a preadipocyte marker monocyte chemoattractant protein 1. *C. militaris* also significantly decreased accumulation of lipid and hypertrophy in mature adipocytes and preserved their response to insulin (phosphorylation of Akt) during prolonged culture. Subsequent experiments revealed that *C. militaris* has the potential to activate the aryl hydrocarbon receptor (AhR). In 3T3-L1 cells, treatment with AhR agonists including benzo[a]pyrene and 3-methylcholanthrene reproduced the anti-adipogenic effect of *C. militaris*. Furthermore, dominant-negative inhibition of AhR abrogated the suppressive effect of *C. militaris* on adipocyte differentiation. These results suggested that *C. militaris* has the potential for interfering with adipocyte differentiation through activation of AhR.

**Keywords:** *Cordyceps militaris*, adipocyte, differentiation, aryl hydrocarbon receptor
Abbreviations: C/EBP, CCAAT/enhancer binding protein; PPARγ, peroxisome proliferator-activated receptor γ; C. militaris, Cordyceps militaris; AhR, aryl hydrocarbon receptor; IBMX, 3-isobutyl-1-methylxanthine; 3MC, 3-methylcholanthrene; B[a]P, benzo[a]pyrene; IDI, insulin, dexamethasone and IBMX; MCP-1, monocyte chemoattractant protein 1; GRP78, 78 kDa glucose-regulated protein; CHOP, C/EBP-homologous protein; CYP1B1, cytochrome P4501B1; DRESSA, dioxin responsive element-based sensing via secreted alkaline phosphatase; ER, endoplasmic reticulum; SEAP, secreted alkaline phosphatase; ES-TRAP, ER stress-responsive alkaline phosphate; TCDD, 2,3,7,8-tetrachlorodibenzo-α-dioxin; MEF, mouse embryonic fibroblast; ERK, extracellular signal-regulated kinase
INTRODUCTION

The incidence of obesity and its related disorders is increasing rapidly in advanced countries. Obesity predisposes individuals to an increased risk of developing various diseases including diabetes and cardiovascular disease. The increase in the mass of the adipose tissue during the development of obesity arises through an increase in cell size, an increase in cell number, or both (4, 41). Adipocyte differentiation is a process in which fibroblast-like preadipocytes are converted into mature, spherical adipocytes that contain lipids. Expression of various molecules is altered during this process, which may be regulated by a variety of extracellular factors and transcription factors (11). CCAAT/enhancer binding protein (C/EBP) family of transcription factors and peroxisome proliferator-activated receptor γ (PPARγ) are regarded as key regulators for adipocyte differentiation (34).

Mycelial extracts have a variety of biological effects that modulate functions of mammalian cells, especially anti-tumor and immunomodulating activities (45). However, currently, little is known about effects of mycelia on the adipose tissue. We sought anti-adipogenic mycelia using an in vitro model of adipogenesis and found that the extract of Cordyceps militaris (C. 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 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 anti-fibrotic, anti-diabetic, anti-inflammatory and
hypocholesterolemic potential of this mycelium (8, 25, 30, 46). However, to date, the
anti-adipogenic potential of *C. militaris* has never been reported. In the present investigation, we
demonstrate that *C. militaris* suppress differentiation of 3T3-L1 preadipocytes into mature
adipocytes using 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*, *Cordyceps militaris*, *Lyophyllum decastes*, *Macrolepita gracilenta*, *Agaricus blazei*, *Grifola frondosa*, *Ganoderma lucidum*, *Inonotus obliquus*, *Lentinula edodes* and *Pleurotus nebrodensis* were prepared by IBI Co., Ltd. (Nirasaki, Yamanashi, Japan), as described previously (16). In brief, 20 g of individual dried mycelia were suspended in 140 ml distilled water and boiled at 105°C for 60 min. After centrifugation at 15,000 rpm for 5 min, the supernatants were filtrated using 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[a]pyrene (B[a]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, Tokyo, Japan) 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, and 3) incubated in basal medium supplemented with insulin alone (insulin medium) for 2 days, as described before (36). The cells were further incubated in basal medium for 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. Using electroporation, 3T3-L1 cells were transfected with pEFBOS-AhR(Arg39) that encodes a dominant-negative mutant of AhR under the control of the elongation factor-1α promoter (9 μg; a gift from Dr. Kazuhiro Sogawa) (40) together with pcDNA3.1 (3 μg; Invitrogen, Carlsbad, CA) that 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 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 with agitation for 5 min. Absorbance (520 nm wavelength) was measured by a spectrophotometer.

Northern blot analysis. Northern blot analysis was performed as described before (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 P4501B1 (CYP1B1) (37) were used for preparation of radio-labeled 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 Ab and anti-phospho-Akt Ab (1/200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA).

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

Endoplasmic reticulum (ER) stress-responsive alkaline phosphate (ES-TRAP) assay. Induction of ER stress was evaluated by 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) by using GeneJuice Transfection Reagent (Novagen, Madison, WI) and treated with test reagents. Activity of ES-TRAP in culture medium was evaluated using Great EscAPe SEAP Detection Kit.

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

Statistical analysis. Data were expressed as means ± SE. Statistical analysis was performed using the non-parametric Mann-Whitney U test to compare data in different groups.
\( P \text{ value } < 0.05 \) was considered to be a statistically significant difference.
RESULTS

Blockade of adipocyte differentiation and lipid accumulation by *C. militaris*. To seek anti-adipogenic 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 day, 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 *Pleurotus nebrodensis* that 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). Quantitative analysis showed that intracellular lipid was significantly reduced to 6.3 ± 0.9 % vs. 100 ± 5.1 % in *C. militaris*-untreated cells (means ± SE, *P* <0.05) (Fig. 1C). The anti-adipogenic effect of *C. militaris* was dose-dependent (Fig. 1D). Significant inhibition of adipocyte differentiation was observed even at 0.05 %, and a linear, concentration-dependent effect was observed at 0.05 - 0.2 % (Fig. 1E).

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 a preadipocyte marker MCP-1 was abrogated by IDI, whereas this inhibitory effect was reversed by C. militaris (Fig. 1F). Taken 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 additional 2 days. The cells were then treated with or without IDI for 2 days, and after 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 control (Fig. 2D), indicating that C. militaris may also inhibit the process of hypertrophy in mature adipocytes.
adipocytes. Hypertrophic adipocytes are known to be resistant to insulin. To confirm the anti-hypertrophic 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 expression level increased thereafter (data not shown). Western blot analysis revealed that, following stimulation with insulin for 30 min, hypertrophic adipocytes showed weak phosphorylation of Akt whereas adipocytes treated with *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.

Inhibition of adipogenesis by *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 function (35). Recently, we reported that K-7174, a GATA inhibitor, suppresses adipocyte differentiation and that it was associated with induction of ER stress (36). We speculated that *C. militaris* may suppress adipocyte differentiation through induction of ER stress. To examine this possibility, 3T3-L1 cells were treated with 0.2% *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 *C. militaris* (Fig. 3A). To further confirm this conclusion, we performed the ES-TRAP assay that can detect ER stress with
high sensitivity and specificity (14). Under ER stress conditions, activity of extracellular ES-TRAP is rapidly down-regulated 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% *C. militaris* for up to 24 h. The culture media 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 *C. militaris* throughout the course of experiments, confirming lack of induction of ER stress.

We and others showed that natural products including herbal medicines contains various agonists of AhR (2, 18). Previous reports suggested that activation of AhR may suppress adipocyte differentiation (1, 39). We hypothesized that *C. militaris* may inhibit adipocyte differentiation through activation of AhR. To examine this possibility, the potential of *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% *C. militaris* for 24 h, and activity of SEAP in culture medium was evaluated. As shown in Fig. 3C, activity of AhR was significantly increased in response to *C. militaris*. In contrast, some other tested mycelial extracts including *Phellinus linteus* did not induce activation of AhR (data not shown). This result was further confirmed by using an endogenous indicator of AhR activation in adipocytes, *CYP1B1* (1). Consistent with the result from DRESSA assay, Northern blot analysis revealed that *C. militaris* rapidly induced expression of *CYP1B1* (Fig. 3D), confirming the induction of AhR activation.
To examine whether activation of AhR is responsible for inhibition of adipogenesis by *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 μM 3MC or 1 μM B[a]P for 2 days, and morphologic examination was performed after 4 days. As shown Fig. 3E, the inhibitory effect of *C. militaris* was reproduced by these AhR agonists. Quantitative analysis revealed that intracellular lipid was significantly reduced to 24.8 ± 1.3 % by 3MC and to 12.5 ± 2.4 % by B[a]P vs. 100 % in *C. militaris*-untreated, IDI-treated cells (Fig. 3F).

To confirm the role of AhR in the suppression of adipocyte differentiation by *C. militaris*, we established 3T3-L1 cells overexpressing a dominant-negative mutant of AhR (Fig. 4A), and the suppressive effect of *C. militaris* was re-tested. As shown in Fig. 4B, *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 *C. militaris* was significantly reversed by dominant-negative inhibition of AhR from 25.9 ± 1.2 % to 94.5 ± 4.4 % vs. 100 % in *C. militaris*-untreated, IDI-treated 3T3-L1/Neo cells (Fig. 4C). These results confirmed that *C. militaris* suppresses adipocyte differentiation through activation of AhR.
DISCUSSION

*C. militaris* has been considered as entomopathogenic fungi 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 has been provided, and molecular mechanisms underlying the beneficial, therapeutic effects of *C. militaris* have never been reported. In the present investigation, we demonstrated for the first time that *C. militaris*, but not other mycelia, uniquely suppress adipogenesis *in vitro*. We also elucidated that *C. militaris* interferes with adipocyte differentiation through activation of the AhR.

Currently, mechanisms underlying the unique, anti-adipogenic property of *C. militaris* are not 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, Shimba *et al.* 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 (39). Hanlon *et al.* demonstrated that low levels of ERK activation cooperate with activated AhR to induce a transcriptional suppressor of PPARγ, the crucial differentiation factor during adipogenesis (12). Vogel *et al.*
reported that 1) TCDD did not suppress differentiation of c-Src-deficient MEFs into adipocytes, and 2) TCDD induced C/EBPβ and C/EBPδ mRNA and their DNA binding activity in wild-type MEFs but not in c-Src-deficient MEFs (43). These data indicated that suppression of adipocyte differentiation by TCDD requires ERK activation, functional c-Src, and/or induction of C/EBPβ and C/EBPδ. Similar mechanisms might also be involved in the suppression of adipogenesis by *C. militaris* observed in this report.

*C. militaris* may also inhibit adipogenesis in other ways. In the induction of adipocyte differentiation, we used IDI medium that contain 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 anti-viral, anti-cancer and immunomodulatory activities (9, 29, 52). Using lipopolysaccharide-activated macrophages, a recent report showed that cordycepin markedly inhibits phosphorylation of Akt (23). Cordycepin in *C. militaris* may be responsible for suppression of adipogenesis via interfering with the insulin signaling.

Currently, 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 the 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 from hypertrophy and preserved responses of the cells to insulin in prolonged culture. These results suggest a 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 that in preadipocytes (38). In contrast to the effect of *C. militaris* on adipocyte differentiation, its effect on adipocyte hypertrophy might be independent of AhR.

Previous reports evidenced anti-diabetic effects of *C. militaris* in pancreatectomized diabetic rats and streptozotocin-induced diabetic mice (8, 51). Although molecular mechanisms involved have not been elucidated, our present results indicated a possibility that the anti-adipogenic effect of *C. militaris* on preadipocytes as well as its anti-hypertrophic effect on mature adipocytes may, at least in part, explain the anti-diabetic effect of this mycelium *in vivo*. Our present findings raise a possibility that *C. militaris* may be useful for the treatment with obesity and obesity-related metabolic disorders. In addition, several putative, endogenous AhR ligands have been identified to date; *e.g.*, tryptophane photooxidation products, lypoxin 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 anti-obesity agents.
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REFERENCES


8. Choi SB, Park CH, Choi MK, Jun DW, Park S. Improvement of insulin resistance and insulin secretion by water extracts of Cordyceps militaris, Phellinus linteus, and


32. Puga A, Tomlinson CR, Xia Y. Ah receptor signals cross-talk with multiple


FIGURE LEGENDS

Fig. 1. Blockade of adipocyte differentiation by *Cordyceps militaris* (*C. 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 day, cultured in basal medium for 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), *Cordyceps militaris* (CM), *Lyophyllum decastes* (LD), *Macrolepita gracilenta* (MGR), *Agaricus blazei* (AB), *Grifola frondosa* (GF), *Ganoderma lucidum* (GL), *Inonotus obliquus* (IOB), *Lentinula edodes* (LE) and *Pleurotus nebrodensis* (PNE). Phase-contrast microscopy. (B-E) Effects of *C. militaris* evaluated by microscopic analyses (B,D; top row, phase-contrast microscopy; bottom row, oil red O staining) and quantification of intracellular lipid (C,E). In C and E, data are expressed as relative percentages (means ± SE), and asterisks indicate 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 (*PPARG*) CCAAT/enhancer binding protein α (*C/EBPα*) and a preadipocyte marker monocyte chemoattractant protein 1 (*MCP-1*) was examined. The level of 28S ribosomal RNA is shown at the
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,B) 3T3-L1 cells were cultured in IDI medium (first exposure; 1st) in the presence of *C. militaris* (0.2%) for 2 days and further incubated in insulin medium for additional 2 days. The cells were then treated with or without IDI for 2 days (second exposure; 2nd), and after additional 4 days, subjected to analyses. (A) Microscopic analyses (top, phase-contrast microscopy; bottom, oil red O staining). (B) Quantitative analysis of lipid content. In (B), data are expressed as relative percentages (means ± SE), and asterisks indicate statistically significant differences (*P* <0.05). Assays were performed in quadruplicate. (C,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. Level of total Akt protein is shown at the bottom as a loading control.

**Fig. 3.** Activation of aryl hydrocarbon receptor (AhR) and lack of induction of endoplasmic reticulum (ER) stress by *C. militaris*. (A) 3T3-L1 preadipocytes were treated with 0.2% *C. militaris* for indicated time periods and subjected to Northern blot
analysis of 78 kDa glucose-regulated protein (GRP78) and C/EBP-homologous protein (CHOP). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is shown at the bottom as a loading control. (B) 3T3-L1 cells transiently transfected with a gene encoding ER stress-responsive alkaline phosphate (ES-TRAP) were treated with (+) or without (-) 0.2 % C. militaris for 3 - 24 h, and the cells and culture media were subjected to formazan assay and chemiluminescent assay to evaluate ES-TRAP activity. The values of ES-TRAP activity were normalized by the number of viable cells estimated by formazan assay. Assays were performed in quadruplicate, and data are presented as means ± SE. RLU, relative light unit. NS, not statistically significant. (C) Reporter cells that produce secreted alkaline phosphatase (SEAP) following activation of AhR were exposed to 0.1 % C. militaris for 24 h, and activity of SEAP in culture media was evaluated by chemiluminescent assay. An asterisk indicates a statistically significant difference (P<0.05). (D) 3T3-L1 cells were treated with 0.2 % C. militaris for indicated time periods and subjected to Northern blot analysis of cytochrome P4501B1 (CYP1B1). (E,F) 3T3-L1 cells were treated with IDI in the presence of AhR agonists, 5 μM 3-methylcholanthrene (3MC) or 1 μM benzo[a]pyrene (B[a]P) and subjected to phase-contrast microscopy (E) and quantitative analysis of lipid content (F).

Fig. 4 Involvement of AhR activation in the anti-adipogenic 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) The 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, and an asterisk indicates a statistically significant difference (*P*<0.05). NS, not statistically significant.