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Bioactive Substance from *Grifola frondosa* (Maitake) Mushroom Inhibits CCAAT Enhancer Binding Protein β and δ Expression on C3H10T1/2 B_2C_1 Adipocyte Cells

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Summary Bioactive compound substances from *Grifola frondosa* (maitake) inhibited adipocyte differentiation of B_2C_1 preadipocytes. This compound is not related to MAP kinase (ERK1 and ERK2) cascades or β-catenin, which inhibit the expression of Glut4, PPARγ and C/EBPα. The compound reduced the expression time of C/EBPβ and C/EBPδ. These results suggest that the inhibitory action of these bioactive compounds on adipocyte differentiation is exhibited through preadipocytes. They cannot induce the expression of PPARγ or C/EBPα because of the reduced expression time of C/EBPβ and C/EBPδ.

Key Words *Grifola frondosa* (maitake), bioactive compound, adipocyte, CCAAT/enhancer binding protein (C/EBP), mechanism regulation

The CCAAT/enhancer binding protein (C/EBP) family of leucine zipper-type transcription factors and the peroxisome proliferator-activated receptor (PPAR) family of ligand-dependent receptor-type transcription factors have been shown to play central roles in adipocyte differentiation (*1*, *2*). It has been generally thought that C/EBPβ and δ induce C/EBPα and PPARγ which trigger terminal differentiation (*3*, *4*).

On the other hand, increasing evidence indicates that the timing of gene expression during adipocyte differentiation is regulated not only by transcriptional activators, but also by release from negative control (*3*, *4*). Ross SE reported that the Wnt/β catenin inhibits the start of differentiation (5). Continuous activation of p42/p44 MAP kinase protein (ERK1 and ERK2) has been found to inhibit terminal differentiation via phosphorylation of PPARγ (*6*).

We have reported that *Grifola frondosa* (maitake) water extract inhibits the adipocyte conversion of C3H10T1/2 B_2C_1 (*7*). This inhibition is based on glycerol-3-phosphate dehydrogenase (GPDH) activity and leptin levels. This compound inhibits the expression of peroxisome proliferator-activated receptor γ (PPARγ), one of the master regulators expressed early in adipocyte differentiation cascades (*8*).

In this study, we examined the differentiation of preadipocytes to adipocytes in terms of their influence on adipocyte regulation factors (C/EBPα, β, γ, ERK1, ERK2, GLUT4, β-catenin) by a bioactive compound from *Grifola frondosa*.

MATERIALS AND METHODS

Sample preparation. *Grifola frondosa* powder was stirred with 10-fold distilled water for 3 h at 20°C, and then centrifuged at 14,000 $\times g$ for 20 min at 4°C. The supernatant was lyophilized and used in experiments as the water-soluble extract preparation. Before application to various experiments, the powder was dissolved in distilled water and filtered (0.45 μm Millipore Co.), and then purified by HPLC (TOSOH G-3000SW ϕ2.15×30.0 cm; Pharmacia DEAE Sepharose™ Fast Flow ϕ1.0×5.0 cm; Superdex Peptide HR 10/30 ϕ1.0×30.0 cm). We named the final purified sample a bioactive compound. We mention it in an article about the property of this sample (*7*).

Cell culture. C3H10T1/2 B_2C_1 cells were cultured under the 3T3L1 cell culture conditions. The cells were inoculated into 24-well plates and grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 50 units/mL penicillin, 50 μg/mL streptomycin and 1 mg/mL glucose (final concentration in medium 2 mg/mL) (normal DMEM-FCS). The culture cells were fed every other day and maintained at 37°C under a humidified CO_2-air (5 : 95) gas mixture until they became confluent. Adipocyte conversion was stimulated by feeding the cells DMEM-FCS containing 0.5 mM 1-methyl-3-isobutylxantin (MIX), 0.25 μM dexamethasone and 10 μg/mL insulin. When the culture cells reached confluence, 0.04 mL of the bioactive compound solution and 0.36 mL of the induction medium were added to each well of the 24-well plates. After incubation for 2 d, the medium was changed to normal DMEM-FCS and the cells were allowed to differentiate.

Measurement of triglycerides (TGs). In brief, culture cells on day 9 after induction into adipocytes in the plates were scraped with a policeman and collected by centrifugation. They were washed twice with 1 mL of CMF. The cell pellets were resuspended in CMF, and homogenized with a Dounce homogenizer. The homog-

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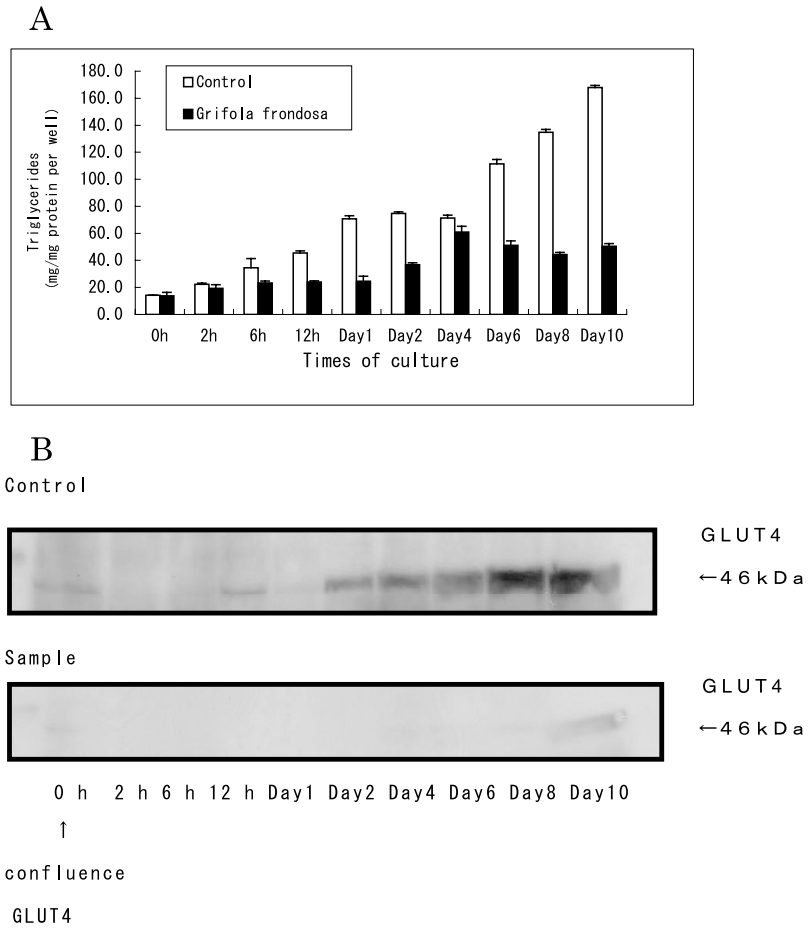

Fig. 1. Effect of *Grifola frondosa* bioactive compound on differentiation marker of adipocytes. A: Triglyceride accumulation by bioactive compound. The culture cells were used from Day 0 to Day 10 after induction. B: Western blot analyses of GLUT4 expression were performed from Day 0 to Day 10 after induction with the bioactive compound (bottom panel) and without it (top panel). Whole cell lysates were electrophoresed on 10% SDS-polyacrylamide gels. Separated proteins were transferred to PVDF membranes and incubated with specific anti-GLUT4.The values are means±SE, $n=4$.

enate was centrifuged at 12,000 $\times g$ for 10 min at 4˚C. The supernatant was used for the TG test. TGs were measured using the Triglyceride G-test Wako (Wako Pure Chemical Industries, Co., Ltd.).

SDS-PAGE and Western blot analysis. The protein concentration of the cell lysate was adjusted to 50 μg for each lane of SDS-PAGE, then electro-blotted onto polyvinylidene difluoride membranes. For immunodetection of proteins, the membranes were blocked for 1 h at 20˚C in 5% skim milk–0.1% Tween 20 in TBS (150 mM NaCl, 20 mM Tris-HCl (pH 7.5)). The blots were then incubated with primary antibody at 20˚C in 5% skim milk–0.1% Tween 20 in TBS for 1 h: rabbit polyclonal anti-C/EBPα SC-61; rabbit polyclonal anti-C/EBPβ SC-150; rabbit polyclonal anti-C/EBPδ SC-151; rabbit polyclonal anti-ERK SC-94; mouse monoclonal anti-P-ERK SC-783; rabbit polyclonal anti-Glut4 SC-7938; rabbit polyclonal anti-β-catenin SC-7963. All antibodies were from Santa Cruz Biotechnology, Inc.

After washing, the membranes were incubated with anti-rabbit IgG or anti-mouse IgG conjugated with horseradish peroxidase (Cell Signaling Technology, Inc.), and signals were developed with a Phototope-HRP Western Blot Detection kit (Cell Signaling Technology, Inc.).

RESULTS

The effect of triglycerides accumulation in cells, with addition of the bioactive compound is shown in Fig. 1A. The triglyceride accumulation in control cells gradually increased day by day and especially rapidly after Day 6. On the other hand, the accumulation in the cells induced with the bioactive compound was clearly very small.

Figure 1B shows the effect of Glut4 in cells with or without the bioactive compound. The expression of Glut4 first began to increase on Day 2 and reached a maximum level after Day 10. In the cells induced by adding the bioactive compound, Glut4 expression was inhibited.

The effects of ERK1 and ERK2, signaling factors in cells, are shown in Fig. 2. ERK1 and ERK2 were constantly expressed. p-ERK1 and p-ERK2 were activated from ERK1 and ERK2. p-ERK1 and p-ERK2 displayed two maximum peaks at 2 h and on Day 8. Thus, there was no difference between the control cells and cells induced with the bioactive compound for ERK1, ERK2, p-ERK1 or p-ERK2.

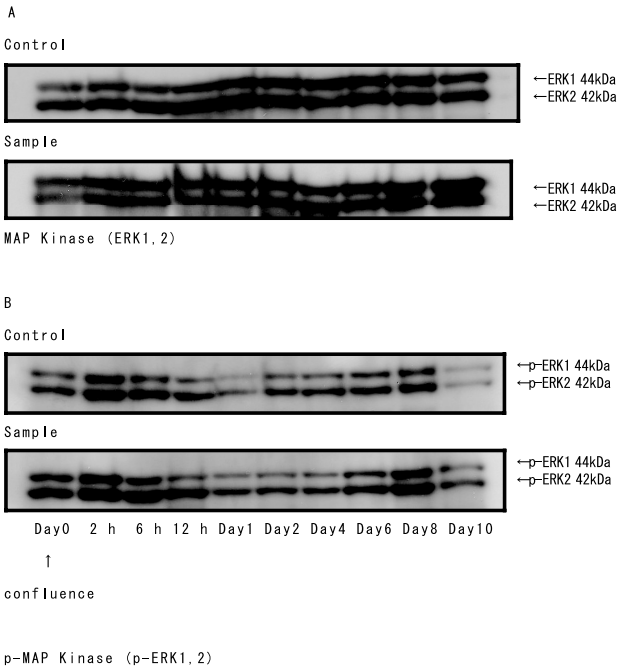

Fig. 2. Effect of *Grifola frondosa* bioactive substance on expression of ERK1, ERK2, p-ERK1 and p-ERK2 in B_2C_1 cells. Western blot analyses of ERK1 and ERK2 (A) and p-ERK1 and p-ERK2 (B) expression were performed from Day 0 to Day 10 after induction with the bioactive compound (bottom panel) and without it (top panel). Whole cell lysates were electrophoresed on 10% SDS-polyacrylamide gels. Separated proteins were transferred to PVDF membranes and incubated with specific anti-ERK and anti-p-ERK.

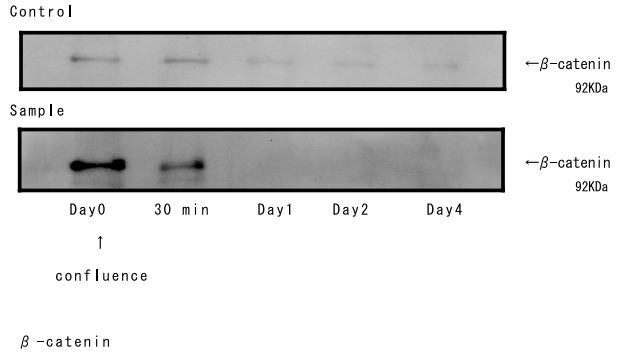

Fig. 3. Effect of *Grifola frondosa* bioactive compound on expression of β-catenin in B_2C_1 cells. Western blot analyses of β-catenin expression were performed from Day 0 to Day 4 after induction with the bioactive compound (bottom panel) and without it (top panel). Whole cell lysates were electrophoresed on 10% SDS-polyacrylamide gels. Separated proteins were transferred to PVDF membranes and incubated with specific anti-β-catenin. It was different in the light and shade, but the bottom panel and top panel were carried out under the same conditions.

One factor related to the inhibiting program. β-Catenin did not lead to a differentiation start in adipocytes. β-Catenin expression was inhibited within the first day (Fig. 3).

The effect of the bioactive compound on C/EBP family proteins, which serve as master regulators in cells, is shown in Fig. 4. C/EBPα first began to increase on Day 4 and reached a maximum level after Day 10. However, C/EBPα expression in cells induced with the bioactive compound was inhibited. (Fig. 4A).

C/EBPβ began to increase after induction, reached its maximum level at 2 h and decreased slightly until the end of the experiment. On the other hand, C/EBPβ expression of cells induced with the bioactive compound began to increase after induction and reached

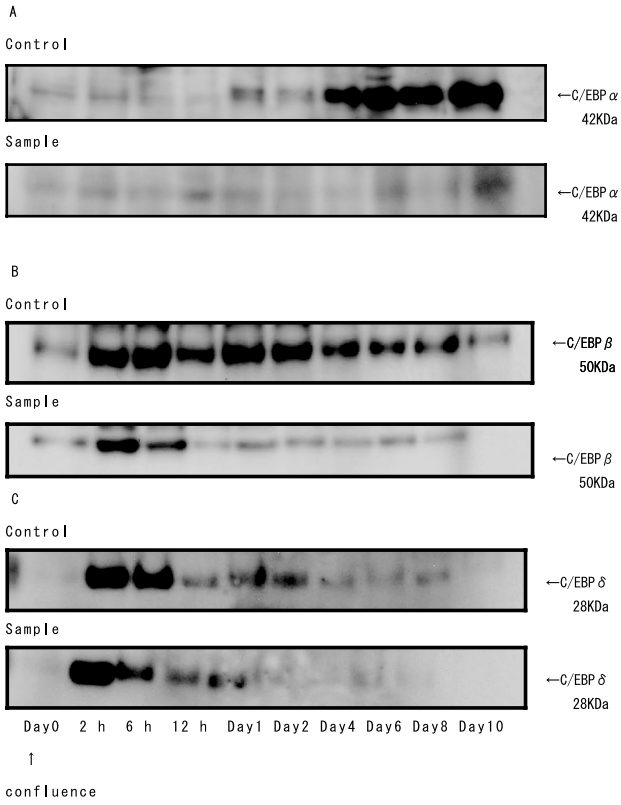

Fig. 4. Effect of *Grifola frondosa* bioactive compound on expression of C/EBP isoforms in B_2C_1 cells. Western blot analyses of C/EBPα (A), C/EBPβ (B) and C/EBPδ (C) expression were performed from Day 0 to Day 10 after induction with the bioactive compound (bottom panel) and without it (top panel). Whole cell lysates were electrophoresed on 10% SDS-polyacrylamide gels. Separated proteins were transferred to PVDF membranes and incubated with specific anti-C/EBPα, anti-C/EBPβ and anti-C/EBPδ.

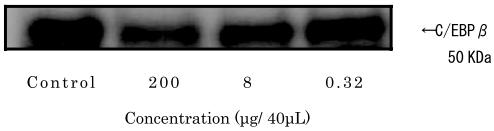

Fig. 5. Influence on expression of C/EBPβ by the density of bioactive compound dependence. C3H10T1/2 B_2C_1 cells (1.0×10^5 cells/well) were cultured in 0.4 mL of the inducing medium containing 0.5 mM 1-methyl-3-isobutylxantine, 0.25 μM dexamethasone and 10 μM/mL insulin for 6 h without or with bioactive compound; 200 to 0.32 μg/40 μL in total 400 μL of the culture medium.

maximum at 2 h, but decreased rapidly within the subsequent 6 h (Fig. 4B) and the activity of the bioactive compound deteriorated when we diluted it (Fig. 5). C/EBPδ began to increase after induction, reached a maximum at 2 h and decreased rapidly over the next 12 h. On the other hand, C/EBPδ expression of cells induced with the bioactive compound began to increase after induction and reached a maximum at 2 h, but decreased more rapidly than the control (6 h) (Fig. 4C).

DISCUSSION

We isolated a bioactive compound from *Grifola frondosa* (maitake) water extract which inhibited the expression of peroxisome proliferator-activated receptor γ (PPARγ) (*7*). PPARγ is induced early during the differentiation of the cultured adipocyte cell line and is specifically expressed at very high levels in adipose tissue (*8, 9*). Transcription factors such as PPARγ coordinate the expression of genes involved in creating and maintaining the adipocyte phenotype including GLUT4.

In this study, little triglyceride accumulation was observed in the cells induced with the bioactive compound (Fig. 1A). The bioactive compound inhibited Glut4 expression (Fig. 1B). This reduced triglyceride accumulation and inhibition of Glut4 expression may lead to the inhibition of PPARγ expression by the bioactive compound.

We postulate that this inhibition was caused by the inactivation of PPARγ or inhibition of PPARγ expression. PPARγ is phosphorylated and inhibited when ERK1 and ERK2 are continuously activated (*6*). Whether growth or differentiation will occur depends on the length of the activation time (*10*). Activation of ERK1 and ERK2 is thought to participate in controlling the activity of PPARγ. In our study, p-ERK1 and p-ERK2, second messenger signaling pathways and the activated form, showed similar changes in the control cells and the cells induced with the bioactive compound. Continuous activity was not observed (Fig. 2). These findings suggest that the cause of this inhibition is not the continuous inactivation of PPARγ by the p-ERK1 and p-ERK2.

On the other hand, the intercellular accumulation of β-catenin was the cause of inhibition of differentiation (5). In this study, no difference in β-catenin accumulation was detected on Day 1 between the control cells and the cells induced with the bioactive compound (Fig. 3). This observation suggests that the cause of the inhibition is not β-catenin accumulation.

C/EBPα cross-regulates with PPARγ and then is induced by C/EBPβ and δ. They are related to adipose conversion (*4*). In our present study, C/EBPβ and δ began to increase after induction and reached a maximum at 2 h, but decreased rapidly after 6 h in cells induced by the bioactive compound. C/EBPα expressed later was suppressed (Fig. 4).

C/EBP has multiple roles in adipocyte differentiation including coordinate transcriptional activation of a group of adipocyte genes (*11, 12*).

C/EBP plays an important role in adipocyte tissue and regulates the insulin-responsive glucose transporter gene; GLUT4 is expressed specifically on adipose tissue and skeletal muscle. In this study, the decreases of C/EBPβ and δ expression caused by the bioactive compound repressed the induction of differentiation factors such as PPARγ and GLUT4.

In summary, when adipocyte cells were exposed to the bioactive compound, the expression of PPARγ in B_2C_1 cells was weak, because the expression of C/EBPβ and δ, which are expressed prior to PPARγ, were suppressed. The differentiated factors such as C/EBPα were also suppressed (Fig. 6, Table 1).

In addition, this effect may be regarded as cytotoxicity, but we do not think so because clonal expansions occur and expression of MAPK was normal.

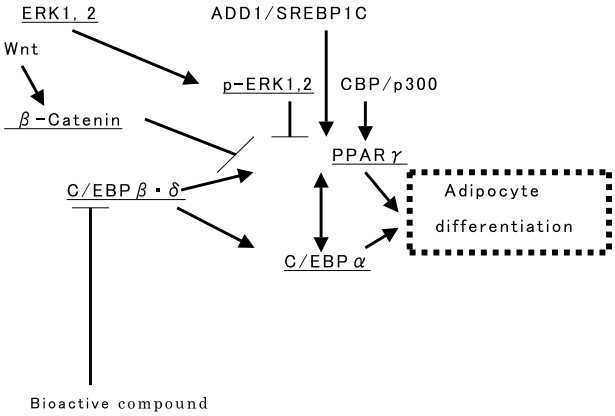

Fig. 6. Second messenger pathways and transcription factors during an adipocyte development program. ADD1, adipocyte determination and differentiation-dependent factor 1; SREBP1, sterol regulatory element binding protein 1; ERK, extracellular-signal related kinase.

Table 1. Effect of *Grifola frondosa* on adipocyte development factor at different program stages.

	Control	*Grifola frondosa*
TGs	+	−
GLUT4	+	−
C/EBPα	+	−
C/EBPβ	+	−
C/EBPδ	+	−
ERK	+	+
p-ERK	+	+
β-Catenin	+	+

(+) means normal expression, (−) means the bioactive compounds inhibited several factor expressions.

Acknowledgments

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