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Suppressive effect of hot water extract of wasabi (*Wasabia japonica* Matsum.) leaves on the differentiation of 3T3-L1 preadipocytes

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ABSTRACT

This study investigated the effect of hot water extract of wasabi (*Wasabia japonica* Matsum.) leaves (WLE), without its specific pungent constituents, such as allyl isothiocyanate, on the differentiation of 3T3-L1 preadipocytes. WLE suppressed the increase in glycerol-3-phosphate dehydrogenase (GPDH) activity and triglyceride (TG) accumulation, markers of adipogenesis, in a dose-dependent manner. Quantitative real time RT-PCR results showed that WLE significantly reduced the mRNA expression levels of peroxisome proliferator-activated receptor (PPAR) γ and CCAAT/enhancer-binding protein (C/EBP) α, both key adipogenic transcription factors, as subsequently were the mRNA expression levels of their target genes, such as adipocyte fatty acid binding protein 2 (aP2). Western blot analysis results showed that the protein expression levels of both PPARγ and C/EBPα were also inhibited by WLE. Thus, WLE suppressed the differentiation of 3T3-L1 preadipocytes, and the suppressive effect was mediated, in part, through the altered regulation of PPARγ, C/EBPα, and other specific genes, such as aP2. These results suggest that WLE may prevent obesity and insulin resistance by inhibiting the differentiation of preadipocytes.

1. Introduction

In humans, obesity has become a serious medical problem in developed countries as it is intimately implicated in metabolic syndrome and type 2 diabetes, which ultimately lead to atherosclerosis (Kopelman, 2000; Zimmet, Magliano, Matsuzawa, Alberti, & Shaw, 2005). Therefore, the prevention of obesity should naturally decrease the risk for developing these diseases. Obesity results from an imbalance between energy intake and expenditure, and is mainly characterized at the cell biological levels by an increase in the size of adipocytes differentiated from preadipocytes, through the conversion of extra energy to lipids and pooling in adipose tissues. In trials of obesity prevention, researchers have investigated whether some food components exhibit an ability to suppress intracellular lipid accumulation. For example, tea catechin has been reported to be effective in the inhibition of adipogenesis in 3T3-L1 cells (Furuyashiki et al., 2004), and *in vivo* studies have shown that oral administration of green tea decreases the weight of adipose tissue in rats and mice (Han, Takaku, Li, Kimura, & Okuda, 1999; Sayama, Lin, Zheng, & Oguni, 2000; Sayama, Ozeki, Taguchi, & Oguni, 1996). Also, the same suppressive effects have been demonstrated with mioga extract (*Zingiber mioga* Rosc.) (Iwashita, Yamaki & Tsushida, 2001), red yeast rice extract (Jeon et al., 2004), flavonoids (Harmon & Harp, 2001; Iwashita, Yamaki & Tsushida, 2001), and mushroom extracts (Ohtsuru, Horio, & Masui, 2000) on 3T3-L1 or C3H10T1/2 B_2C_1 adipocyte differentiation.

Wasabi (*Wasabia japonica* Matsum.) is a native Japanese plant and was used as a medicinal herb in ancient times. In most parts, wasabi is used as a spice or as pickles as it contains strong pungent constituents, including isothiocyanates such as allyl isothiocyanate. In previous studies, isothiocyanates have been reported to have various physiological properties, including antimicrobial activity (Isshiki, Tokuoka, Mori, & Chiba, 1992), antioxidative activity (Fukuchi et al., 2004), anti-platelet aggregative activity (Kumagai et al., 1994), and anti-carcinogenic activity (Fuke, Haga, Ono, Nomura, & Ryoyama, 1997). However, there have been few studies of the functional properties of other components, particularly in summer leaves, which are too late of stage, and have little pungency (Mochida & Ogawa, 2008).

To develop an effective use for summer leaves, which are discarded as an agricultural waste, we investigated the possible

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existence of any anti-obesity function of wasabi leaves, *i.e.* the effect of their hot water extract (WLE), without its specific pungent constituents, such as allyl isothiocyanate, on the differentiation of 3T3-L1 preadipocytes, measuring glycerol-3-phosphate dehydrogenase (GPDH) activity and triglyceride (TG) content in 3T3-L1 cells as markers of adipogenesis. To determine whether WLE affected the alteration in the differentiation programme, we also investigated the expression of PPARγ and C/EBPα, both of which act as central regulators of adipocyte differentiation, and their target genes, such as aP2.

2. Materials and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from AGC Techno Grass Co. Ltd. (Chiba, Japan). Dexamethazone (DEX), gentamicin sulfate and insulin were purchased from Sigma–Aldrich Co. (St. Louis, MO). 3-Isobuthyl-1-methylxanthine (IBMX), (−)-epigallocatechin (EGC) and allyl isothiocyanate (AIT) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). ISOGEN, for total RNA isolation from cells, was purchased from Nippon Gene (Tokyo, Japan). ReverTra Ace -α-, for cDNA synthesis, was purchased from TOYOBO Co., Ltd. (Osaka, Japan). The PCR reagents and the oligonucleotide primers of mouse (Table 1) were obtained from Takara Bio Inc. (Shiga, Japan). Rabbit polyclonal antibodies against PPARγ and C/EBPα were purchased from Upstate (Lake Placid, NY) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. As secondary antibodies, horseradish peroxidase-conjugated goat anti-rabbit IgG, and chemiluminescent substrate (SuperSignal West Femto Maximum Sensitivity Substrate), to detect immunoreactive band, were obtained from Thermo Fisher Scientific Inc. (Rockford, IL). A polyvinylidene fluoride (PVDF) membrane (Immun-Blot PVDF Membrane) was purchased from Bio-Rad Laboratories (Hercules, CA). All other chemicals used were of analytical grade.

2.2. Preparations of wasabi extract

Wasabi (*W. japonica* Matsum.) leaves were harvested in Shimane Prefecture, Japan, in July, 2004, and pooled at −50 °C until used for experiments. After lyophilisation in a vacuum at room temperature for 3 days and milling with a vibrating sample mill TI-100 (Heiko Seisakusho Ltd., Tokyo, Japan), the samples were ready for experiment. One gramme of lyophilized sample of wasabi leaves in 10 ml of distilled water was boiled for 10 min in a hot water bath, and then centrifuged at 12,000*g* for 10 min at 4 °C; the supernatants were then filtered through a 0.45 μm membrane filter (Adovantec Toyo Kaisha Ltd., Tokyo, Japan). The solid contents of the extract were calculated by evaporating the sample in a vacuum and subsequently measuring the dry weight. Wasabi rhizomes and stalks were also used in this study. To examine the differences of suppressive effects of WLE, from samples of different harvesting periods, on adipocyte differentiation, wasabi leaves harvested from July, 2004, to June, 2005, were also used. The extract from these leaves, as well as from the rhizomes and stalks, was prepared as described above.

2.3. Cell culture and treatment

Murine 3T3-L1 cells (JCRB 9014) were obtained from the Health Science Research Resources Bank (Osaka, Japan). The cells were grown in maintenance medium (MM, DMEM supplemented with 10% FBS and 50 μg/ml of gentamicin) at 37 °C in 5% CO_2. The medium was changed every two or three days. For adipocyte differentiation, the cells were cultured in 6-well plates to full confluence for 2 days and were then treated with differentiation medium containing 0.25 μM DEX, 0.5 mM IBMX, and 1.5 μg/ml of insulin (DMI). After 2 days of induction (day 2), the medium was changed to MM containing 1.5 μg/ml of insulin, and WLE, at final concentrations of 333, 667 or 1333 μg/ml, was added every 2 days. In the case of examining the differences of suppressive effects on adipocyte differentiation of WLE from samples of the various harvesting periods, 15 μl of WLEs were added to 1.5 ml of MM containing 1.5 μg/ml of insulin. After 6 days of incubation from the initiation of differentiation, the cells were harvested. For quantitative real time RT-PCR and Western blot analysis, the cells were treated with WLE, or EGC as positive control, at the same time as DMI induction of differentiation (day 0), and then incubated in the same manner as described above. All samples were prepared in triple cell culture wells.

2.4. Measurement of GPDH activity and quantification of TG accumulation

The cells were washed twice with ice-cold 0.9% saline solution, collected in 1 ml of cold sonication buffer (25 mM Tris buffer

Table 1
PCR primers used in this study.

Gene name[a]	Sequences[b]	GenBank accession number
PPARγ	F: TGTCGGTTTCAGAAGTGCCTTG R: TTCAGCTGGTCGATATCACTGGAG	NM_011146
C/EBPα	F: TGCGCAAGAGCCGAGATAAAG R: TCACGGCTCAGCTGTTCCAC	NM_007678
aP2	F: TGGGAACCTGGAAGCTTGTCTC R: GCTGATGATCATGTTGGGCTTG	NM_024406
GLUT4	F: ACGACGGACACTCCATCTGTTG R: GGAGACATAGCTCATGGCTGGAA	NM_009204
LPL	F: TCCGAGTGAAAGCCGGAGA R: TGGCATTTCACAAACACTGCTG	NM_008509
SCD1	F: TCTTGTCCCTATAGCCCAATCCAG R: AGCTCAGAGCGCGTGTTCAA	NM_009127
CD36	F: GATGGCCTTACTTGGGATTGGA R: GGCTTTACCAAAGATGTAGCCAGTG	N M_007643
GAPDH	F: AAATGGTGAAGGTCGGTGTG R: TGAAGGGGTCGTTGATGG	NM_001001303

[a] PPARγ, peroxisome proliferator-activated receptorγ; C/EBPα, CCAAT/enhancer-binding proteinα; aP2, adipocyte fatty acid binding protein 2; GLUT4, glucose transporter 4; LPL, lipoprotein lipase; SCD1, stearoyl coenzyme A desaturase 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
[b] F: forward, R: reverse.

containing 1 mM EDTA, pH 7.5) using a cell scraper, and then sonicated. Twenty microlitres of suspension were used to measure TG concentrations, using a commercial kit (Triglyceride E-test Wako, Wako Pure Chemical Industries, Ltd., Osaka, Japan) according to manufacturer's instructions. The remaining portion of the suspension was centrifuged at 8000g for 10 min at 4 °C, and the supernatant was assayed for GPDH activity. This was done by measuring the decrease in the absorbance of NADH at 340 nm at 37 °C. Protein concentrations were determined using Protein Quantification Kit-Rapid (Dojindo Molecular technologies, Inc., Rockville, MD). The GPDH activity and the TG concentration per mg of protein in the cells were calculated and expressed as ratios (%) of the control value.

2.5. Analysis of allyl isothiocyanate (AIT) content in hot water extract of wasabi

The AIT contents of the test samples were measured by high-performance liquid chromatography (L-7100, Hitachi, Ltd., Tokyo, Japan), using the following analytical conditions: column, TSKGEL ODS-80TS (φ4.6 × 250 mm); mobile phase, acetonitrile–water = 50/50; flow rate, 1.0 ml/min; detection, UV 240 nm. On the HPLC analysis, limit of detection (LOD) and limit of quantification (LOQ) of AIT were 0.01 mM and 0.02 mM, respectively.

2.6. Quantitative real time RT-PCR

Total RNA was isolated from the cells using ISOGEN, according to the manufacturer's instructions. One microgramme of total RNA was used for the single strand cDNA synthesis with a cDNA synthesis kit, ReverTra Ace -α-. Gene expression levels were analyzed by quantitative real time RT-PCR, using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA). The cDNA was denatured at 95 °C for 10 s, followed by 40 cycles of PCR (95 °C, 5 s; 60 °C, 31 s). Results were obtained from at least three independent experiments. The mRNA levels of all genes were normalized, using GAPDH as an internal control.

2.7. Western blot analysis

The cells were washed twice with ice-cold PBS, scraped with RIPA buffer, and allowed to stand on ice for 1 h to permit lysis. After centrifugation at 18,000g for 20 min at 4 °C, protein content in the supernatant was determined, using a DC-protein assay kit (Bio-Rad Laboratories, Hercules, CA), and aliquots (20 μg) of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred onto a PVDF membrane. The membrane was blocked with 0.3% (w/v) non-fat dried milk in TBST (25 mM Tris–HCl, pH 7.4, 150 mM NaCl, and 0.1% (v/v) Tween 20) for 1 h, and then incubated for 1 h with primary antibody in TBST containing 0.3% (w/v) non-fat dried milk. The blots were treated with horseradish peroxidase-conjugated secondary antibody in TBST containing 0.3% (w/v) non-fat dried milk for 1 h, and then reacted with a chemiluminescent substrate. An immune complex was detected using a multiimager (Bio-Rad SU-115, Bio-Rad Laboratories, Hercules, CA).

2.8. Statistical analysis

Statistical analysis of the data was done with SPSS statistical analysis software (Version 17.0J, SPSS Inc., Tokyo, Japan). One-way ANOVA was used to assess the differences between the individual groups, and *post hoc* analyses were performed by the Bonferroni test for two independent variables. Differences were considered significant at $p < 0.025$. The significance of differences between two groups (samples and control) were analyzed by the Student's *t*-test, and were determined at $p < 0.05$.

3. Results

3.1. Suppressive effects of wasabi extract on GPDH activity and TG accumulation

The extracts from all parts of the wasabi plant suppressed the increase in GPDH activity and TG accumulation in a dose-dependent manner (Fig. 1). Particularly, the cells treated with 667 μg/ml of extract from leaves and stalks showed suppression of increase in GPDH activity up to 36% and 25%, respectively, in comparison with control cells. However, in the case of treatment with 1333 μg/ml of extract from them, the cells were damaged (#), so GPDH activity and TG accumulation could not be evaluated clearly. From the results obtained above, the adipocyte differentiation-inhibitory activities of the extracts of leaves and stalks were higher than those of rhizomes. Table 2 shows AIT content of the extracts. The extracts of all wasabi parts tested had little or no AIT, so we surmised that the results obtained here were not because of isothiocyanates, such as AIT. The differences in suppressive effects on adipocyte differentiation of WLE, from samples of the various harvesting periods were also examined (Fig. 2). The effects of WLE from samples harvested in winter were greater, on the whole, but the summer samples (July/2004) also showed significant effects. Hence we focused on WLE in this study, which also had inhibitory activity on adipogenesis.

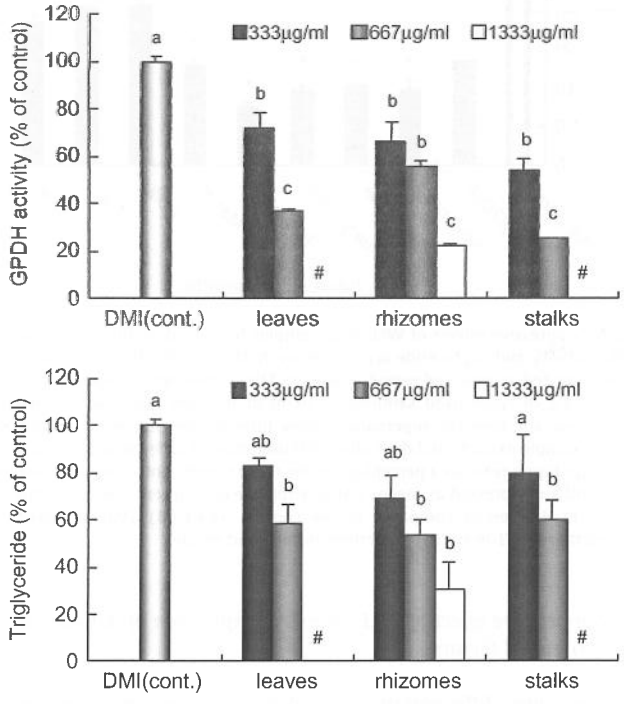

Fig. 1. Hot water extracts of wasabi leaves, rhizomes and stalks suppress glycerol-3-phosphate dehydrogenase (GPDH) activity and triglyceride accumulation in 3T3-L1 cells in a dose-dependent manner. Samples are used at indicated concentrations. Cells were treated with samples at 2 days after DMI induction of differentiation (day 2). All values are represented as a percentage of the results from control which is taken as 100%, and are expressed as means ± SE of triplicate experiments. Different letters between samples and control, or among samples in each part, indicate significant differences by the Student's *t*-test ($p < 0.05$) and the Bonferroni test ($p < 0.025$), respectively. #: GPDH activity and TG accumulation of the cells treated with 1333 μg/ml of extract from both leaves and stalks were not evaluated clearly because these treatments damaged the cells. DMI: a mixture of dexamethazone, 3-isobuthyl-1-methylxanthine and insulin.

Table 2
AIT content in hot water extract of the wasabi parts.

Parts	AIT contents (mM)
Leaves	ND[a]
Rhizomes	0.03
Stalks	0.04

The sample extracts were obtained as follows: 1 g of lyophilized samples in 10 ml of distilled water was boiled and centrifuged, and then the supernatants were filtered. AIT contents in the extracts were analyzed by HPLC. LOD: 0.01 mM, LOQ: 0.02 mM.
[a] Not detected.

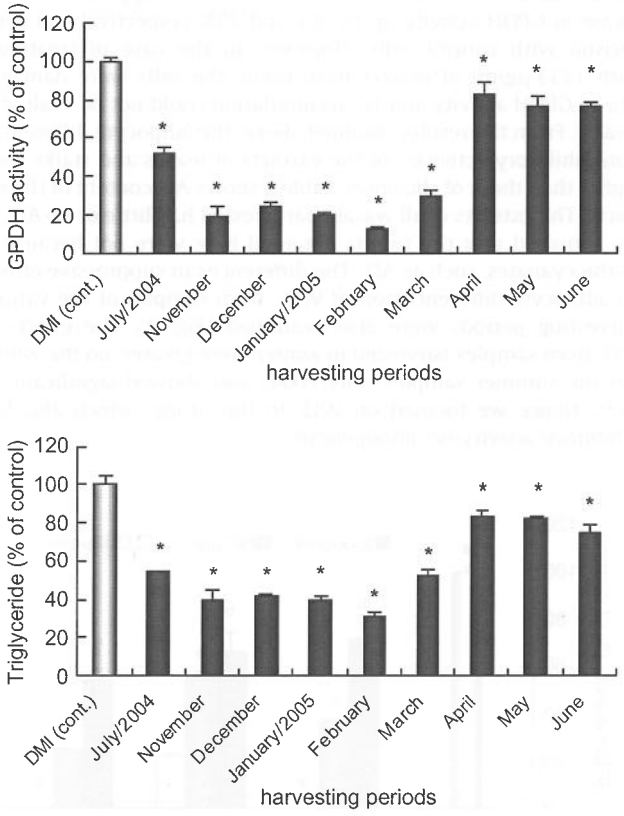

Fig. 2. Suppressive effects of WLE from samples harvested in different periods on GPDH activity and triglyceride accumulation in 3T3-L1 cells. Wasabi leaf samples were harvested at indicated periods and used. The sample extracts were obtained as follows: 1 g of lyophilized samples in 10 ml of distilled water was boiled and centrifuged, and then the supernatants were filtered. The cells were treated with 15 μl of sample extracts at 2 days after DMI induction of differentiation (day 2). All values are represented as a percentage of the results from control, which is taken as 100%, and are expressed as means ± SE of triplicate experiments. Asterisk indicates significant differences compared to the control ($*p < 0.05$). DMI: a mixture of dexamethazone, 3-isobuthyl-1-methylxanthine and insulin.

3.2. Suppressive effect of WLE on mRNA expression of adipogenic transcriptional factors

Adipocyte differentiation involves a series of programmed changes in gene expression. To determine whether the suppressed increase in GPDH activity and reduced TG accretion resulted from a WLE-mediated alteration in the differentiation programme, the expression of a number of adipogenic genes was investigated by quantitative real time RT-PCR. In the cells treated with WLE, as in those treated with EGC, we noted a significant decrease in the mRNA expression levels of PPARγ and C/EBPα, both key transcriptional factors, and followed by reduced mRNA expression levels of their target genes, such as aP2, in a dose-dependent manner (Fig. 3). For example, the mRNA expression level of PPARγ in cells treated with 333 or 667 μg/ml of WLE decreased to 76% and 40%, respectively, of that of the control cells.

3.3. Suppressive effect of WLE on protein expression of PPARγ and C/EBPα

During DMI induction of 3T3-L1 adipocyte differentiation, PPARγ and C/EBPs are activated by DMI (Rangwala & Lazar, 2000; Rosen & Spiegelman, 2000). To investigate the inhibitory mechanism of WLE, protein expression levels of PPARγ and C/EBPα were examined. We found that the WLE-associated attenuation of PPARγ and C/EBPα gene expressions was accompanied by a decrease in the corresponding proteins. For example, at 667 μg/ml of WLE, protein expression levels of PPARγ and C/EBPα decreased to 43% and 30%, respectively, of the control levels (Fig. 4).

4. Discussion

In the present study, we demonstrated that hot water extracts of several parts of the wasabi plant, suppressed the adipocyte differentiation in 3T3-L1 cells, while preventing increases in GPDH activity and TG accumulation, markers of adipogenesis, in a dose-dependent manner (Fig. 1). Iwashita et al. (2001) reported that only mioga (*Z. mioga* Rosc.) extract, among 15 edible-plant PBS-soluble extracts examined, proved effective in inhibiting 3T3-L1 adipocyte differentiation without cell damage, and that cells treated with 200 or 500 μg/ml of extract suppressed increases in GPDH activity to about 60% and 20%, respectively, of that of the control cells. Our results, where cells treated with 667 μg/ml of WLE suppressed increase in activity to about 40% compared to control cells, showed nearly the same as or slightly less than mioga extract.

At the molecular level, cells treated with WLE reduced mRNA and protein expression levels of PPARγ and C/EBPα, both of which act as key adipogenic transcription factors (Figs. 3 and 4). During subsequent terminal differentiations of preadipocytes into mature adipocytes with mitotic clonal expansion in adipose tissues, the adipocyte differentiation programme is regulated by transcriptional activators, such as C/EBPα, PPARγ2, fatty acid-activated receptor (FAAR), and transcriptional receptors, such as preadipocyte repressor element binding protein (PRE) and C/EBP undifferentiated protein (CUP) (MacDougald & Lane, 1995). For example, under induced preadipocyte differentiation into adipocyte in the presence of IBMX in 3T3-L1 cell cultures, C/EBPβ, belonging to a basic leucine zipper family of transcriptional factors, is induced temporarily at the early stage of differentiation (Ko et al., 2005), which, in turn, C/EBPβ induces downstream transcriptional factors, PPARγ and C/EBPα (Wu, Bucher, & Farmer, 1996), followed by the expression of genes, including ap2, GLUT4, LPL, SCD1 and CD36, which mediate fatty acid or glucose uptake into adipocyte, triglyceride hydrolysis and lipogenesis (Abumrad, El-Maghrabi, Amri, Lopez, & Grimaldi, 1993; Brun, Kim, Hu, Altiok, & Spiegelman, 1996; Long & Pekala, 1996; Miller & Ntambi, 1996; Tontonoz, Hu, Graves, Budavari, & Spiegelman, 1994). Thereafter, adipocytes incorporate glucose and free fatty acids to synthesize and accumulate lipids as energy, resulting in an increase in cell size. Huang et al. (2006) reported that berberine (BBR), a compound purified from *Cortidis rhizoma*, inhibited differentiation of 3T3-L1 adipocytes through PPAR pathways; not only were the mRNA and protein expression levels of PPARγ and C/EBPα directly affected, but levels of C/EBPβ were inhibited by BBR, resulting in the down-regulation of the target genes, such as CD36. In our study, in which the treatment with WLE suppressed adipocyte differentiation in 3T3-L1 cells, we supposed that PPARγ and C/EBPα gene expressions might be down-regulated directly, as with BBR, by reason that their expressions were also inhibited after treatment with WLE at 2 days after DMI induction of 3T3-L1 cells (day 2, data not shown). However, it re-

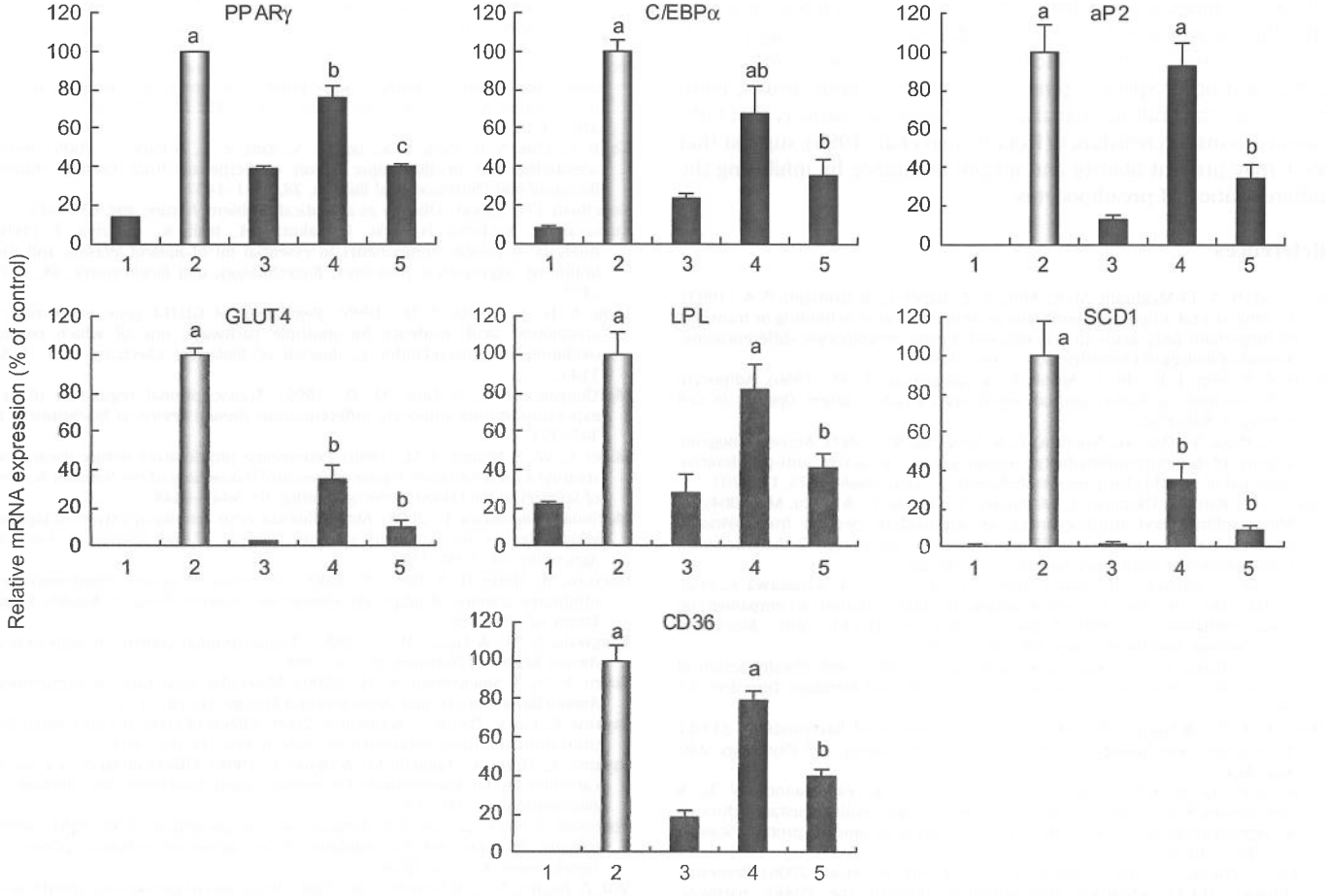

Fig. 3. WLE suppresses mRNA expressions of PPARγ, C/EBPα and their target genes in 3T3-L1 cells. The cells were treated with WLE at the same time as DMI induction of differentiation (day 0). Quantitative real time RT-PCR results are shown for (1) undifferentiated cells, (2) control cells differentiated by DMI, and cells treated with (3) 30 μM of EGC, (4) 333 μg/ml of WLE, and (5) 667 μg/ml of WLE, respectively. The mRNAs of GAPDH in the same samples were used as an internal control. All values are represented as a percentage of the results from control which is taken as 100%, and are expressed as means ± SE of triplicate experiments. Different letters among WLE samples and control indicate significant differences ($p < 0.025$). DMI: a mixture of dexamethazone, 3-isobuthyl-1-methylxanthine and insulin. EGC: (−)-epigallocatechin (positive control).

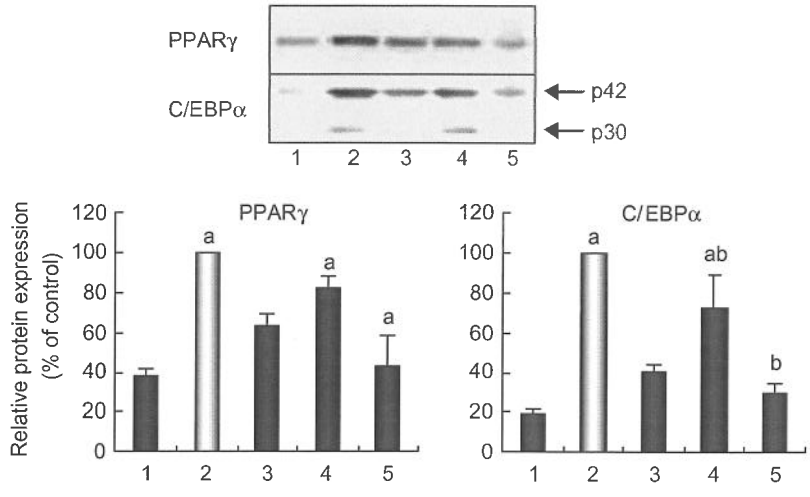

Fig. 4. WLE suppresses protein expressions of PPARγ and C/EBPα in 3T3-L1 cells. The cells were treated with WLE at the same time as DMI induction of differentiation (day 0). A set of representative Western blots for PPARγ and C/EBPα is shown in the upper panel. The quantitative changes of the target proteins are determined by densitometry, as shown in the lower panel. Lanes 1–5 are samples from (1) undifferentiated cells, (2) control cells differentiated by DMI, and cells treated with (3) 30 μM of EGC, (4) 333 μg/ml of WLE and (5) 667 μg/ml of WLE, respectively. All values are represented as percentages of the results from the control, which is taken as 100%, and are expressed as means ± SE of triplicate experiments. Different letters among WLE samples and control indicate significant differences ($p < 0.025$). DMI: a mixture of dexamethazone, 3-isobuthyl-1-methylxanthine and insulin. EGC: (−)-epigallocatechin (positive control).

mains unclear whether WLE affects upstream transcriptional factors, such as C/EBPβ.

In conclusion, we have demonstrated, in the present study, that WLE suppressed the differentiation of 3T3-L1 preadipocytes by

inhibiting increases in GPDH activity and TG accumulation, and that the suppressing effects were mediated, in part, through the altered regulation of adipogenic transcriptional factors, PPARγ and C/EBPα, and other specific genes, such as aP2. These results, combined with the findings that aP2 is central to the pathway that links obesity to insulin resistance (Hotamisligil et al., 1996), suggest that WLE may prevent obesity and insulin resistance by inhibiting the differentiation of preadipocytes.

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