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Inhibitory activity of *Phellodendri cortex* extracts on differentiation of 3T3-L1 preadipocytes

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Abstract−The purpose of the present study was to investigate the inhibitory effect of beberine-rich fraction of *Phellodendri cortex* extract (PC) on adipogenesis in 3T3-L1 cells. PC effectively prevented TG accumulation in differentiation of 3T3-L1 preadipocytes in a dose-dependent manner. Compared to controls, PC at a concentration of 75 μg/mL significantly decreased lipid droplets by 79.5%. Beberine exhibited similar inhibitory effect to that of PC extract, suggesting that fractionated PC extract contained mostly berberine. The expression levels of PAARγ and C/EBPα were significantly reduced by PC treatment. This result consequently led to suppression of expression of LPL, FABP4, CD36, and SCD2, which are target genes of PPARγ and C/EBPα. PC also inhibited protein levels of PPARγ, FABP4, and GLUT4. Accordingly, these results suggest that PC inhibited adipogenesis in 3T3-L1 preadipocyte cells through PPARγ and CEBPα-related pathways at both transcript and protein levels.

Key words: *Phellodendri cortex*, Berberine, C/EBPα, PPARγ, 3T3-L1 Preadipoctye

INTRODUCTION

Obesity is a risk factor for arteriosclerosis, diabetes, and hyperlipidemia, and is a major public health problem in many countries. Obesity and its complications are closely associated with adipocyte differentiation and subsequent extent of fat accumulation [18,31]. Obesity results from growth and expansion of adipose tissue in which lipid storage and energy metabolism are tightly controlled [9]. Therefore, reduction of adipose tissue by inhibition of adipocyte differentiation could be a possible method for prevention of obesity. Adipose tissue is now apparently regarded as an endocrine organ that secretes various types of bioactive molecules known as adipocytokines that play a prominent role in energy metabolism [3].

The murine-derived 3T3-L1 preadipocyte cell line is frequently used for the study of differentiation and function of adipocytes *in vitro* [11] due to distinct expression of obesity-related genes, such as PPARγ, C/EBPα, LPL, CD36, and FABP4, which have critical functions in induction of adipocyte differentiation. In particular, PPARγ, the ligand-activated nuclear transcription factor, regulates expression of adipocyte differentiation, lipogenesis, and glucose metabolism, and is involved in type 2 diabetes mellitus (T2DM), dyslipidemia, atherosclerosis, obesity, and other metabolic diseases [10,29].

Ligands of PPARs have been sought for use as agonists. As a result, the thiazolidinedione (TZD) class of insulin-sensitizing compounds, such as rosiglitazone and pioglitazone, which lower hyperglycemia, were found to be PPARγ agonists [2,33]. Since these drugs have limitations and side-effects, some researchers have attempted to find new drugs for use in treatment of some of these metabolic disorders [4,5,26]. Expression of PPARγ is induced by two transcription factors, C/EBPβ and C/EBPδ, which are upstream proteins of C/EBPα that regulate expression of adipose-specific genes with PPARγ, this leads the adipose phenotype during adipocyte differentiation.

To date, plenty of natural compounds are known to have inhibitory activity on 3T3-L1 preadipocyte differentiation. For example, *Undaria pinnatifida* (brown algae) [23], garlic [1], and *Humulus lupulus* (Common hop) [25] showed inhibition of 3T3-L1 preadipiocyte differentiation. Meanwhile, *Garcina cambogia* [13] and *Cordyceps militaris* [32] exhibited decreased accumulation of lipids or triglycerides (TG) in 3T3-L1 preadipocytes.

Phellodendri cortex (PC) contains a number of alkaloids, e.g., berberine, palmatine, phellodendrine, and jatrorrhizine [19]. PC is well known for its anti-inflammatory effect [27,28], and has been studied for its effect on a variety of conditions, such as hyperglycemia and diabetic nephropathy [19], and for its immune-stimulating properties [30]. Berberine, one of the major components of PC, has shown an inhibitory effect on accumulation of fibronectin and collagen in rat glomerular mesangial cells [22], expression of adipogenic enzymes and inflammatory molecules of 3T3-L1 adipocytes [6], aldose reductase and oxidative stress in rat mesangial cells [21], and 3T3-L1 adipocyte differentiation [14], as well as activated effects on expression of GATA-2 and GATA-3 during inhibition of adipocyte differentiation [14], and AMP-activated protein kinase with beneficial metabolic effects in diabetic and insulin-resistant states [20].

In the present study, we screened possible anti-obesity materials among numerous natural plants and found that beberine-rich fraction of PC has the most powerful inhibitory effect on adipocyte differentiation in 3T3-L1 cells by Oil Red O staining and TG content assay. Using immunoblotting and real-time RT-PCR analysis, we further investigated molecular pathways of inhibition of adipocyte differentiation. To the best of our knowledge, this is the first report to identify PPARγ and CEBPα-related molecular mechanisms of suppression of PC on adipocyte differentiation.

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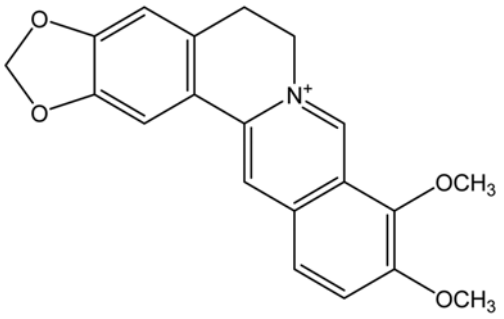

Fig. 1. Chemical structure of berberine in *Phellodendri cortex* (PC).

MATERIALS AND METHODS

1. Preparation of standardized fraction of *Phellodendri cortex* (PC)

Dried and pulverized PC (50 g) was extracted with 70% (w/v) hot ethanol for 2 h and filtered with microfilter (Millipore 0.22 mm). The filtrate was evaporated *in vacuo* to produce an ethanolic extract (9.2 g). The extract was suspended with distilled water (100 mL), followed by filtration for removal of the precipitate. After the filtrate was adsorbed in an Amberlite XAD-2 column (5×15 cm), distilled water (1,500 mL) and 100% ethanol (1,500 mL) were eluted successively. The resulting ethanol-eluted fraction was evaporated *in vacuo* to afford 3.91 g of the beberine-rich fraction of PC (hereafter referred to as PC), which was deposited at the New Natural Material Bank, sponsored by the Ministry of Education, Science and Technology of Korea. The standardized fraction of PC was evaluated based on the content of berberine (Fig. 1), one of the major constituents of PC, determined by using high-performance liquid chromatography (HPLC). HPLC separation was carried out using a Cosmosil $5C^{18}$-AR-II column (5 m; 4.6 mm×150 mm; Tacalai Tesque, Kyoto, Japan). Elution was performed initially with acetonitrile-water (0.34 g of potassium dihydrogenphosphate and 0.17 g of sodium lauryl sulfate dissolved in 100 mL of water; 80 : 20), which was changed according to linear gradient over 40 min to acetonitrile-water. The flow rate was 1 mL/min, 0.01 mL aliquots of samples were injected for analysis, and UV detection was carried out at 230 nm. Berberine was found in the standardized fraction of PC at a level of 25.2±0.4%. PC used in this study was kindly provided by the Natural Product Research & Development supported by NRF, College of Pharmacy, Wongwang University, Korea.

2. Preparation and Differentiation of Adipocytes

3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM; Thermo, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; PAA Laboratories, Pasching, Austria) and 100 μg/mL penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA) at 37 °C in a 5% CO_2 atmosphere. After 3T3-L1 cells reached to confluence, they were incubated for two days and then the culture medium was changed to a differentiation-induction medium containing 10 μg/mL insulin (Sigma, St Louis, MO, USA), 0.25 μM dexamethasone (Sigma), and 0.5 mM 3-isobutyl-1-methylxanthine (IBMX; Sigma) in DMEM with 10% FBS, and then treated with PC of 10, 25, 50, and 75 μg/mL every two days. After two days (day 2), the differentiation-induction medium without dexamethasone and IBMX was replaced. After two more days (day 4), cells were cultured in growth medium without insulin. On day 5 after induction of differentiation, at least 95% of the cells displayed an adipocyte phenotype like accumulation of lipid droplets. Cells in these conditions were harvested for further studies.

3. Oil Red O Staining

On day 6 of adipocyte differentiation induction, formation of lipid droplets in cells was analyzed by an Oil Red O staining method as follows. Cells were washed with phosphate buffered saline (PBS), fixed with 10% formaldehyde for 1 h at 4 °C, and washed three times with deionized water. The cells were then incubated with a filtered Oil Red O solution (Sigma) at 60 °C for 30 min, and washed three times with deionized water. Fat droplets in adipocytes were stained red. Photos were then taken using an Olympus microscope (Tokyo, Japan).

4. Measurement of Cytotoxicity

The MTT (3′-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay was used for assessment of cell toxicity. 3T3-L1 preadipocyte cells were treated with PC of 0, 10, 25, 50, 75, 100, 150, 200, and 300 μg/mL. After 24 h, MTT solution was added, and cells were incubated at 37 °C for 4 h. Thereafter, the supernatant was completely removed, and 0.1% dimethyl sulphoxide (DMSO, Sigma) was used to dissolve the formazan crystal. Absorbance was read at 540 nm using a microtiter plate reader.

5. TG Content Assay

On day 7 of induction of adipocyte differentiation, differentiated cells in 60 mm plates were harvested in 0.5 mL of cold PBS, and then mixed. TG content in the supernatant was measured with the TG test kit (Asan pharm. Co., Yeongcheon, Kyungbuk, Korea), according to the manufacturer's instructions. Absorbance was measured at 550 nm, and TG was normalized to protein content as determined by the Bradford assay.

6. Quantitative Real-time RT-PCR

Total RNA was isolated from 3T3-L1 preadipocytes using QIAZOL reagent (Qiagen, MA, USA) and the RNeasy Mini Kit (Qiagen, Valencia, CA, USA); 2 μg of extracted RNA was reverse-transcripted using the cDNA Reverse Transcription Kit. Transcript levels of genes were determined using quantitative real-time PCR, which was performed using the Applied Stratagene mx 3000p QPCR System (San Diego, CA, USA) by SYBR Green I Dye Chemistry, according to the manufacturer's protocol. Reaction mixtures were preheated at 95 °C for 10 min; temperature conditions were as follows for 40 cycles: melting at 95 °C for 30 sec, annealing at 60 °C for 1 min, and elongation at 72 °C for 1 min. Sequences of used primer sets are listed in Table 1. Transcript levels of each gene were normalized to those of GAPDH.

7. Immunoblot Analysis

Cultured and differentiated cells, harvested using a cell scrape, were rinsed with PBS and lysed with cold RIPA buffer containing 150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris (pH 8), and protease inhibitor cocktail (Sigma). Cell lysates were mixed for 30 sec and centrifuged at 14,000 ×*g* for 20 min at 4 °C for removal of insoluble materials. Protein content of the supernatant was determined using the Bradford protein assay kit. The protein extract (10 μg) was diluted in 5× sample buffer (60 mM Tris of pH 6.8, 2% SDS, 25% glycerol (Bio Basic, Ontario, Canada), 0.1% bromophenol blue (Sigma),

Table 1. Sequences of primers used for real-time PCR

Gene name	Accession No.	Forward	Reverse
GAPDH	NM_008084.2	GGTCTCGCTCCTGGAAAGA	GTATGACTCCACTCACGGCAA
PPARγ	NM_011146	GGTGAAACTCTGGGAGATTC	CAACCATTGGGTCAGCTCTT
C/EBPα	NM_007678	AGGTGCTGGAGTTGACCAGT	CAGCCTAGAGATCCAGCGAC
LPL	NM_008509	GGCCAGATTCATCAACTGGAT	GCTCCAAGGCTGTACCCTAAG
FABP4	NM_024406	CATGGCCAAGCCCAACAT	CGCCCAGTTTGAAGGAAATC
CD36	NM_007643	TTGTACCTATACTGTGGCTAAATGAGA	CTTGTGTTTTGAACATTTCTGCTT

and 14.4 mM β-mercaptoethanol (Sigma), and heated for 5 min at 95 °C before SDS-polyacrylamide gel (10%) electrophoresis (SDS-PAGE). Gels were subsequently transferred to polyvinylidene fluoride (PVDF; Santa Cruz Biotechnology, Santa Cruz, CA, USA) membranes, and were then blocked for 2 h at room temperature with TBS containing 0.1% Tween 20 (TBS-T) and 5% skim milk. The membranes were then washed with TBS-T three times every 15 min, and incubated with a 1 : 1,000 dilution of antibodies to PPARγ, GLUT4, and FABP4 (Santa Cruz Biotechnology), and a 1 : 2,000 dilution of antibody to β-actin (Santa Cruz Biotechnology) in TBS-T containing 1% skim milk at 4 °C overnight. After three washes, the membrane was incubated for 2 h with horseradish peroxidase (HRP)-conjugated secondary antibody (1 : 1,000; Santa Cruz Biotechnology) and developed using enhanced chemiluminescence (ECL; Amersham Biosciences). Western blot was analyzed by scanning with a UMAX PowerLook 1120 (Maxium Technologies, Akron, OH, USA) and digitalized using image analysis software (KODAK 1D, Eastman Kodak, Rochester, NY, USA).

8. Statistical Analysis

All experimental results were compared by one-way analysis of variance (ANOVA) using the Statistical Package of Social Science (SPSS) program; data were expressed as the mean±SE. Group means were considered significantly different at $p<0.05$, as determined by the technique of protective least-significant difference (LSD) when ANOVA indicated an overall significant treatment effect *$p<0.05$, **$p<0.01$.

RESULTS

1. Effect of PC on Morphological Change in Adipocyte Differentiation

As shown in Fig. 2, PC was found to have a strong inhibitory effect on preadipocyte differentiation in 3T3-L1 cells by Oil Red O staining on the 6th day. PC displayed inhibitory action against differentiation of adipocytes in a dose-dependent manner, and the most effective dosage for inhibition was 75 μg/mL. These results indicate that PC obviously suppressed adipocyte differentiation visually and showed a potential anti-obesity effect in 3T3-L1 cells.

2. Effect of PC on Toxicity and TG-secretion on 3T3-L1 Preadipocyte Cells

To assess the possibility of PC as an anti-obesity agent, cytotoxicity of PC was examined using 3T3-L1 preadipocyte cells; results are shown in Fig. 3(a). Cells were seeded in a 96-well plate and cultured under various concentrations (0, 10, 25, 50, 75, 100, 150, 200, 300 μg/mL) of PC for 24 h. Cell toxicity was then assessed using the MTT assay. PC up to 75 μg/mL was confirmed to have no significant cytotoxicity against preadipocyte cells, whereas PC over 100 μg/mL cells decreased cell viability. Next, we also evaluated the effect of PC on TG-secretion of 3T3-L1 preadipocytes during differentiation. As shown in Fig. 3(b), the results indicated that PC effectively prevented TG accumulation in differentiation of 3T3-L1 preadipocytes in a dose-dependent manner. Compared to controls, PC at a concentration of 75 μg/mL significantly decreased lipid droplets by 79.5%. We also measured the inhibitory effect of berberine on TG-secretion of 3T3-L1 preadipoctes during differentiation. Interestingly, beberine showed a similar inhibitory effect on lipid accumulation to those of PC extracts with two specific concentrations (10 and 25 μg/mL). Taken together, these results imply that the inhibitory effect of PC resulted from mainly berberine. However, we could not exclude the possibility that other constituents in PC also might affect inhibitory activity on adipogenesis.

3. Effect of PC on Expression of Adipogenic Genes

Various transcription factors and adipocyte-specific genes can induce adipogenesis and lipogenesis. To examine the inhibitory mech-

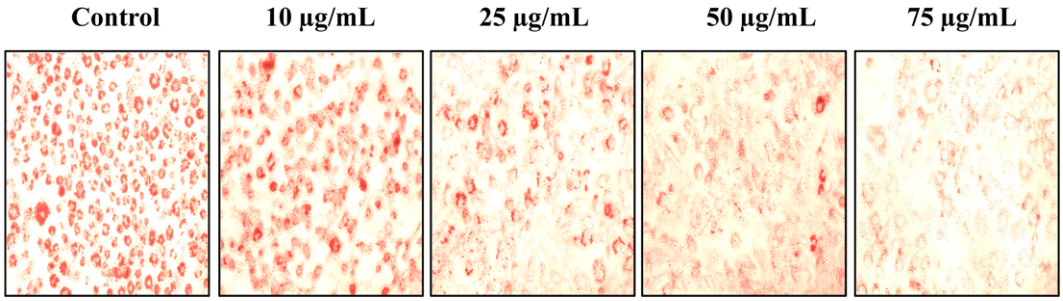

Fig. 2. Effect of PC on morphological change in adipocyte differentiation. After treatment of cells with PC, Oil Red O staining was performed on day 6 of differentiation. Fat droplets in adipocytes were stained red, and were then monitored by microscope and photographed (200×).

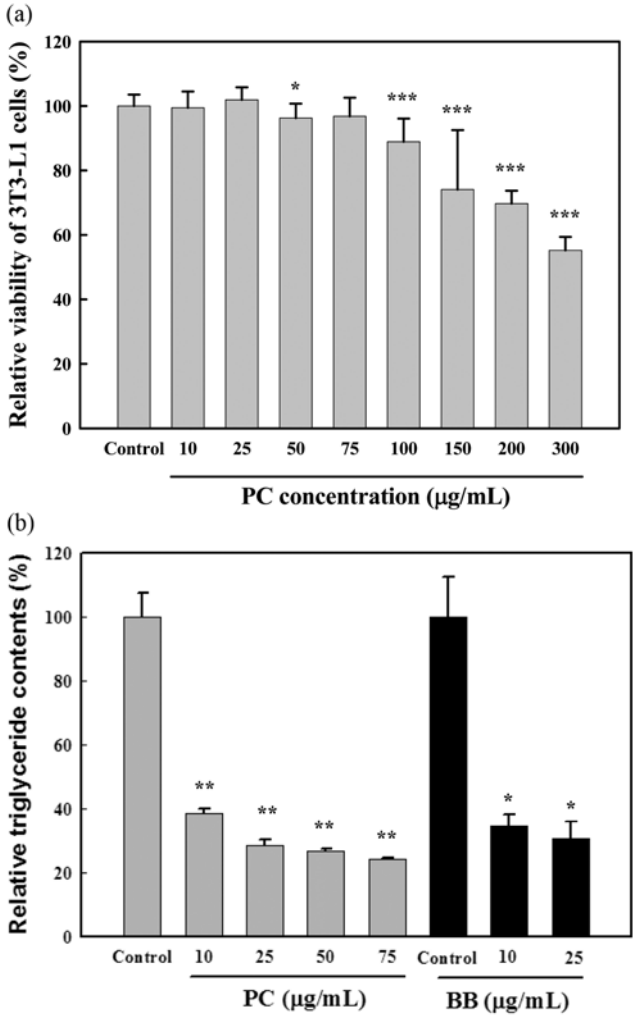

Fig. 3. Effect of PC on toxicity and TG-secretion on 3T3-L1 preadipocyte cells. (a) Toxicity of 3T3-L1 preadipocytes by PC was tested in various concentrations for 24 h using the MTT assay. (b) Inhibitory effect of PC and berberine on triglyceride accumulation of 3T3-L1 cells was determined by TG content assay on day 7. Data are representative of two independent experiments and presented as mean±SD. Student's *t* test was employed (*p<0.05, **p<0.01, ***p<0.001).

anism of PC during adipocyte differentiation, expression levels of PPARγ and C/EBPα, key transcriptional factors for adipocyte differentiation, were investigated before and after PC treatment. On day 5, expression patterns of genes with involvement in the adipogenesis pathway were measured by quantitative real time RT-PCR. PPARγ and C/EBPα were up-regulated during adipogenic differentiation, and were significantly down-regulated following treatment with PC (Fig. 4(a)). Because PC significantly decreased the expression levels of PPARγ, we hypothesized that expression on target genes of PPARγ might also be down-regulated and tested. As a result, mRNA levels of LPL, FABP4, SCD2, and CD36 were also significantly decreased during adipocyte differentiation (Fig. 4(b)). These results demonstrated that PC may reduce adipocyte differentiation through decreased expression levels of adipogenic genes.

4. Effect of PC on Expression of Adipogenic Proteins

The effects of PC on protein expression of PPARγ, FABP4, and GLUT4 in 3T3-L1 preadipocytes were investigated; the results are shown in Fig. 5. Protein levels of PPARγ, FABP4, and GLUT4 were uniquely expressed in the process of adipogenesis. We confirmed that protein expression of PPARγ, FABP4, and GLUT4 decreased dose-dependently during adipocyte differentiation when treated with PC. The most effective concentration of PC for differentiation inhibition was 75 μg/mL. These results suggest that PC obviously inhibited lipogenesis and adipogenesis by down-regulation of adipogenic marker proteins.

DISCUSSION

Because some chemically synthetic compounds have noxious side effects, natural resources have been used as protective agents against obesity, hypertension, T2DM, hyperlipidemia, hypercholesterolemia, and atherosclerosis. In the present study, we demonstrated for the first time that *Phellodendri cortex* inhibits differentiation of 3T3-L1 presdipocytes into mature adipocytes through reduction of TG accumulation. Moreover, PC was found to significantly inhibit levels of PPARγ, C/EBPα, CD36, LPL, FABP4, and SCD2 mRNA expression and their target proteins during adipocyte differentiation (Figs. 4 and 5).

C/EBPα and PPARγ, transcriptional activators, play key roles in adipocyte differentiation and induce expression of many adipogenic genes [8]. PPARγ is predominantly expressed in adipose tissue, and is a member of the nuclear receptor superfamily of transcription factors; restraint of PPARγ expression can shut out adipocyte differentiation. Furthermore, fatty acid synthesis, oxidation, transport storage and energy expenditure are mediated by PPARs [15,16]. C/EBPs (C/EBPα, β, and δ) appertain to the basic leucine zipper family of transcription factors [16,34]. C/EBPα has been reported as a possible candidate transcription factor for direct regulation of adipogenesis [34].

As mentioned above, these two factors together accelerate markers of terminal differentiation by activation of adipogenic gene with involvement in making and maintaining adipocyte phenotype. Therefore, PPARγ and C/EBPα induce downstream adipocyte specific-gene expression, including FABP4, LPL, CD36, and SCD2, which control fatty acid and glucose metabolism [12]. Berberine was shown to suppress adipocytes differentiation and reduce lipid accumulation in 3T3-L1 adipocytes [6,14]. Choi et al. [6] demonstrated that berberine has both anti-adipogenic and anti-inflammatory effects on 3T3-L1 adipocytes by down-regulation of adipogenic enzymes (e.g., fatty acid synthase, acetyl-CoA carboxylase, acyl-CoA synthase, and lipoprotein lipase) and key adipogenic transcription factors.

It has been reported that the transcription factors GATA binding protein 1 and 3 (GATA-2 and GATA-3) are expressed in adipocyte precursors and control the pre-adipocyte to mature adipocyte transition [14]. Hu and Davies [14] found that berberine increases expression of these two transcription factors during inhibition of adipocyte differentiation of 3T3-L1. In this work, we found that PC treatment significantly inhibited gene expression of PPARγ and C/EBPα. Reduction of these two transcription genes also decreased expression of adipocyte specific genes, including FABP4.

We also found that PC treatment significantly inhibited gene expression of PPARγ and C/EBPα. Reduction of these two transcription genes also decreased expression of adipocyte specific genes,

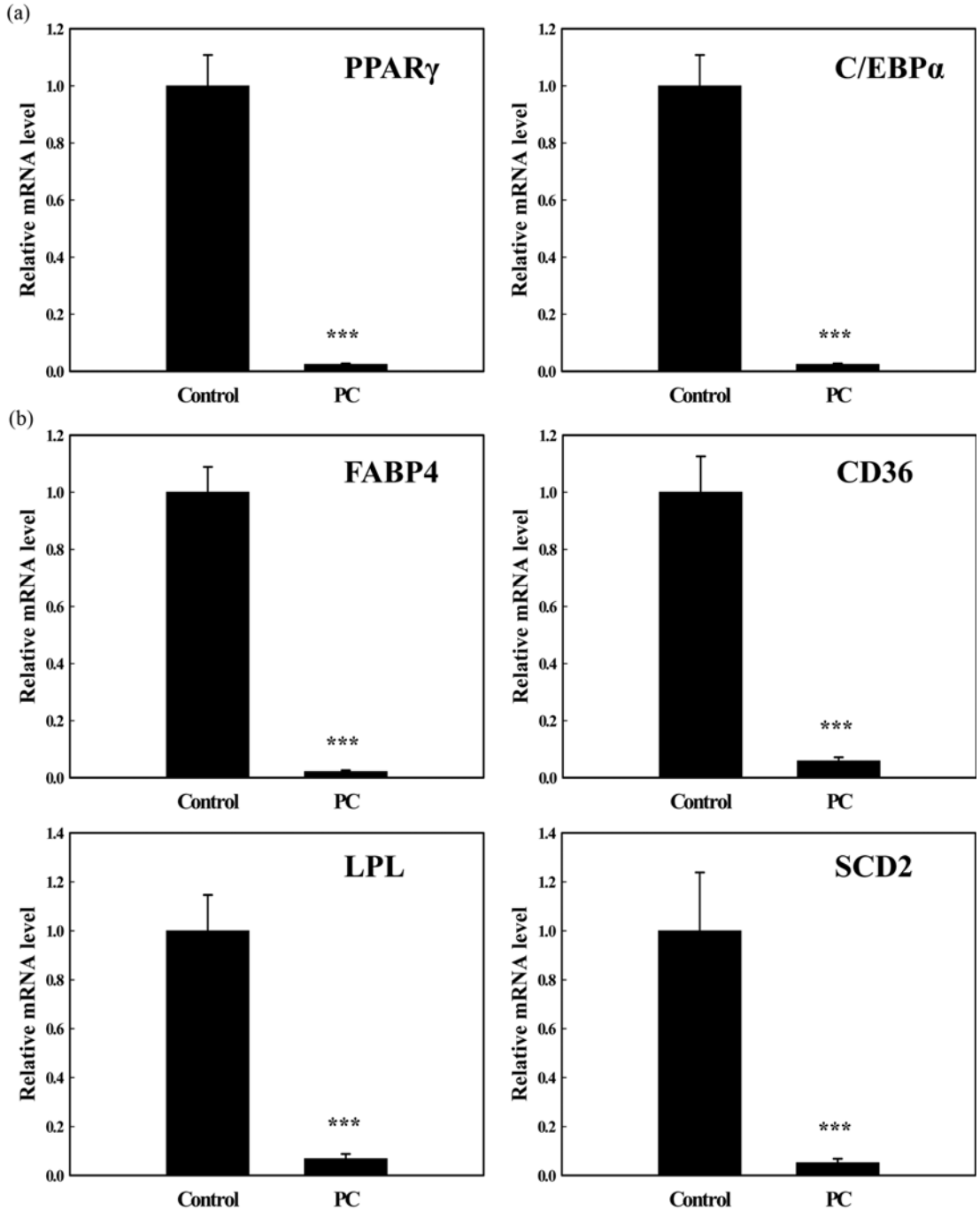

Fig. 4. Effect of PC on expression of adipogenic genes. Total RNA was extracted from cells treated with PC and without PC (75 μg/mL) on the 5th day of differentiation. mRNA expression of (a) PPARγ, C/EBPα, (b) FABP4, LPL, CD36, and SCD2 was determined using quantitative real-time RT-PCR. Relative mRNA levels of each gene were normalized to those of GAPDH. Data are presented as means±SD, and Student's t test was employed (*p<0.001).**

including FABP4, CD36, SCD2, and LPL, during adipocyte differentiation. GLUT4 and FABP4, which are expressed uniquely in adipose tissue and are widely known to induce insulin-regulated glucose translocation into the cell, play a critical role as the link between lipid metabolism, hormone action, and cellular function in adipocytes [7,17,24]. Therefore, we confirmed that PC reduced not only the mRNA levels of PPARγ and C/EBPα, but also the protein levels of PPARγ, FABP4, and GLUT4 in 3T3-L1 adipocytes in a dose-dependent manner.

In conclusion, our results demonstrated that PC could induce inhibition of 3T3-L1 adipocyte differentiation by reduction of PPARγ and C/EBPα mRNA levels involved in expression of FABP4, LPL, CD36, and SCD2, and marker protein levels of adipocyte differentiation, suggesting that PC has obvious potential as an anti-obesity material. These findings are the first to identify PPARγ and C/EBPα-related molecular mechanisms of the inhibitory effect on adipocyte

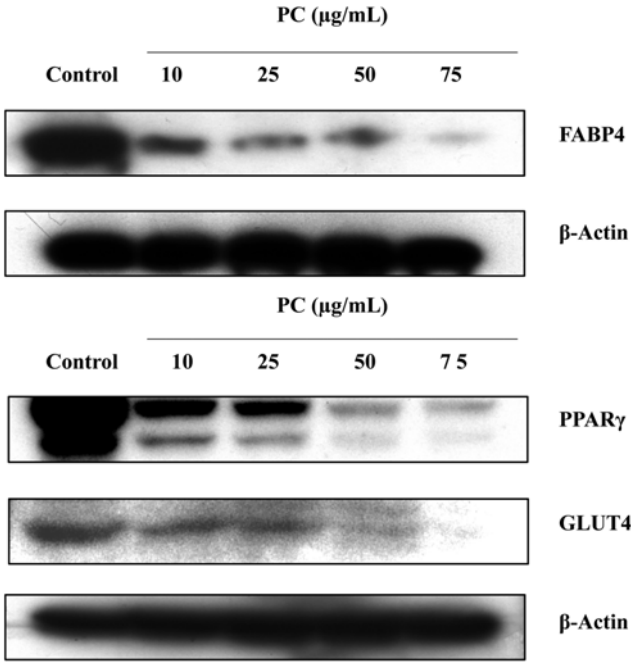

Fig. 5. Effect of PC on expression of adipogenic proteins. Adipogenic protein levels of PPARα, FABP4, and GLUT4 in response to PC treatment analyzed by Western blot analysis. Data are representative of two independent experiments.

differentiation by PC. To reinforce findings from the present *in vitro* study, further studies are required for determination of whether or not PC induces loss of body fat *in vivo*.

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