

Chlorogenic Acid Decreases Lipid Accumulation in 3T3-L1 Adipocytes by Modulating the Transcription Factors

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Abstract The aim of this study is to investigate the effect of chlorogenic acid (CA) on 3T3-L1 adipocytes and its mechanism action. CA at 1-25 μ M showed no cytotoxicity to 3T3-L1 pre-adipocytes and 3T3-L1 adipocytes. CA significantly inhibited oil red O-stained material (OROSM) and intracellular triglyceride levels in 3T3-L1 adipocytes in a concentration-dependent manner. In addition, CA down-regulated the glycerol-3-phosphate dehydrogenase (GPDH) and peroxisome proliferator-activated receptor γ (PPAR γ) activity. A real-time polymerase chain reaction revealed that CA inhibited the PPAR γ , CCAAT/enhancer-binding protein alpha (C/EBP α), sterol regulatory element binding protein-1c (SREBP-1c), and fatty acid synthase (FAS) gene expression, which may in part account for anti-adipogenesis of CA. Thus, CA could act as a potential lipid lowering functional resource.

Keywords: chlorogenic acid, adipocytes, anti-adipogenesis, lipid accumulation, transcription factor

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1. Introduction

A WHO survey indicates that in 2020, worldwide obesity has nearly triple since 1975. Most of the world's population live in countries where overweight and obesity kills more people than underweight [1]. Obesity is a civilization-related medical problem, which increases the risk of a number of diseases, such as hypertension, cardiovascular diseases, diabetes, high blood cholesterol, malignant neoplasms, osteoporosis, cancers and sleep disorders [2]. The fundamental cause of obesity or overweight is an energy imbalance between calories intake and expenditure. Overweight and obesity are preventable by choosing healthier foods and regular physical activity (WHO, 2020). Therefore, preventing, controlling and treating overweight and obesity have become important goals. Many studies have reported that some natural products and food components are able to prevent overweight and obesity. For example, djulis has been reported to be effective in the inhibition of adipogenesis in 3T3-L1 cell [3], and an in vivo study has shown that oral administration of djulis has beneficial

effects on hyperlipidemia in obese mice [4]. The phytochemicals such as betanin, rutin and kaempferol suppressed lipid accumulation due to reduced gene expression of PPAR γ , C/EBP α and SREBP-1c [3]. Also, some reports have noted that bioactive compounds, such as flavonoids, may cause an inhibition of adipogenesis and lipogenesis, and induction of lipolysis as well as apoptotic death of adipose cells [5]. These findings imply that complementary and alternative therapies with functional biomaterials can regulate lipid metabolism and prevent overweight and obesity. Consequently, the investigations of natural products or bioactive compounds for preventing and treating overweightness and obesity have received a great deal of attention.

Chlorogenic acid (CA) is a family of ester formed between quinic acid and 1-4 trans-cinnamic acids, residues of certain trans-cinnamic acids, most commonly caffeic, ferulic, and *p*-coumaric acids [6]. CA is found in the seeds and leaves of many dicotyledonous plants. Therefore, it has been extensively investigated and recognized to have numerous biological activities, such as antioxidant, antidiabetic, anti-inflammatory, anticancer, antineurodegenerative, antihypertensive, and antilipidemic activities [7]. In addition, 5-caffeoylquinic acid decreasing obesity in

high-fat-diet induced rats may be associated with up-regulation of PPAR α expression and down-regulation of LXR α expression. Beside these, there has been little research on the effectiveness of CA in regulating lipid accumulation. Thus, the aim of this study is to investigate the inhibitory effect of chlorogenic acid on lipogenesis of 3T3-L1 adipocytes, and its action mechanism is also elucidated.

2. Material and Methods

2.1. Materials

3T3-L1 cell (BCRC Number: 60159) was obtained from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan). Chlorogenic acid was obtained from the Alfa Aesar (Heysham, Lancashire United Kingdom). Oil red O solution, 3-isobutyl-1-methylxanthine, dexamethasone, and insulin were obtained from Sigma (St. Louis, MO, USA). The dye MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) was purchased from PanReac AppliChem (Darmstadt, Germany). Dulbecco's modified Eagle's medium (DMEM), Trypsin-EDTA, penicillin-streptomycin, bovine calf serum, and fetal bovine serum were purchased from Gibco (Grand Island, NY, USA). Anti-PPAR and anti- α -actin were purchased from Cell Signaling Technology (Danvers, MA, USA). All other chemicals were reagent grade.

2.2. Cell Culture

3T3-L1 preadipocytes were planted into 6-well plates and maintained in DMEM supplemented with 10% bovine calf serum at 37°C in a humidified 5% CO₂ incubator. Adipocytic differentiation was induced by the differentiation medium A (0.5 mM 3-isobutyl-1-methylxanthine, 1 μ M dexamethasone, 1 μ M insulin, 1.5 g/L sodium bicarbonate, 10% fetal bovine serum and 1% antibiotic dissolved in high glucose DMEM) that were added to culture medium in every 48 h for 4 days. Afterwards, the medium was changed to differentiation medium B (1 μ M insulin, 1.5 g/L sodium bicarbonate, 10% fetal bovine serum and 1% antibiotic dissolved in high glucose DMEM) and was freshly replaced every day. The cells were harvested 8 days after the initiation of differentiation. [3]

2.3. Cell Viability Assay

Cell viability was assessed by the MTT viability assay [8]. The culture media containing test samples (1-25 μ M) was added to each well and the cells were incubated for 24 h, with untreated cells served as control. Then, culture media was replaced with MTT (0.5 mg/mL) and incubated in the dark for 1 h. Formazan crystals were solubilized (100% DMSO), and absorbance was measured using an ELISA reader (Thermo Multiskan GO, Waltham, MA, USA) at 550 nm.

2.4. Oil Red O-Staining of 3T3-L1 Adipocytes

Intracellular lipid accumulation was measured using Oil

red O [9]. Freshly 60 mL Oil red O solution (0.5% in isopropanol) and 40 mL deionized water was prepared for the Oil red O working solution. Cells were incubated with test samples for 24 h at 37°C in a humidified 5% CO₂ incubator. Cells were washed twice with phosphate buffered saline (PBS, pH 7.4) and then fixed with 10% neutral buffered formalin for at least 20 min at room temperature. After fixation, cells were washed twice with PBS and stained with the Oil red O working solution for 60 min. The image of stained cells was photographed, and then eluted stained Oil red O by adding 100% isopropanol, finally, the optical density was measured at 510 nm in ELISA reader (Thermo Multiskan GO).

2.5. Quantification of Intracellular Triglyceride

Cells were incubated with test samples for 24 h at 37°C in a humidified 5% CO₂ incubator. Cells were collected and lysed in lysis buffer (1% Triton X-100 in PBS) for 30 min on ice box. The total triglyceride content in cells was determined using a commercial triglyceride assay kit (Cayman Chemical, Ann Arbor, MI, USA). The optical density of each well was determined at 550 nm using an ELISA reader (Thermo Multiskan GO). The protein concentration was estimated with the Pierce™ BCA protein assay kit (Thermo Scientific, Rockford, IL, USA) using bovine serum albumin as a standard. Inhibition (%) was expressed as percent decrease in triglyceride content against control (0%).

2.6. Determination of Glycerol-3-Phosphate Dehydrogenase Activity

3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells were incubated with 10-25 μ M of test samples for 24 h at 37°C in a humidified 5% CO₂ incubator. Cells were washed twice carefully with ice-cold PBS on 3T3-L1 adipocytes, and lysed in 25 mM Tris/1 mM EDTA, pH 7.5 for the measurement of glycerol-3-phosphate dehydrogenase (GPDH) specific activity. GPDH activity was determined by Glycerol-3-Phosphate Dehydrogenase Activity Colorimetric Assay Kit, (Biovision, Milpitas, CA, USA). The optical density of each well was determined at 450 nm using an ELISA reader (Thermo Multiskan GO). Protein concentration was determined by the Pierce™ BCA protein assay kit (Thermo Scientific) using bovine serum albumin as a standard. Enzyme activity was expressed as units of activity/mg protein. Inhibition (%) was expressed as percent decrease in GPDH activity against control (0%).

2.7. RNA Extraction and Real-Time PCR

Total RNA was extracted using Total RNA Isolation Kit (NA017-0100, GeneDireX, Las Vegas, NV, USA) according to the manufacturer's instructions. RNA quantity and purity were checked by spectrophotometric analysis at 260 and 280 nm. One microgram of total RNA was reverse transcribed into cDNA using GScript First-Strand Synthesis Kit (MB305-0050, GeneDireX). The lists of specific primers (Invitrogen, Carlsbad, CA, USA) were: GAPDH, Forward: 5'GTATGACTCCACTC

ACGGCAA-3' (N3541G11), Reverse: 5'-GGTCTC GCTCCTGGAAGATG-3' (N3541H01); PPAR γ , Forward: 5'-TTTTCAAGGGTGCCAGTTTCGATCC-3' (N3541H02), Reverse: 5'-AATCCTTGCCCTCTGAGAT-3' (N3541H03); C/EBP α , Forward: 5'-CGCAAGAGCCGA GATAAAGC-3' (N3541G05), Reverse: 5'-CACGGCTCA GCTGTTCCA-3' (N3541G06); Sterol regulatory element binding protein 1c (SREBP/1c), Forward: 5'-GGAG CCATGGATTGCACAT-3' (N3541H06), Reverse: 5'-GCTTCCAGAGAGGAGGCCAG-3' (N3541H07); FAS, Forward: 5'-TGGGTTCTAGCCAGCAGAGT-3' (N3541G09), Reverse: 5'-TACCACCAGAGACCGTTATGC-3' (N3541G10).

The primers were added at final concentration of 250 nM to a 20 μ L reaction mixture containing 4 μ L of 5 \times OmicsGreen qPCR Master Mix and 1 μ L of DNA template (stock conc. 1 μ g/mL). The PCR (StepOnePlus™ Real-Time PCR System, Applied Biosystems™, Foster, CA, USA) conditions were denaturation at 90°C for 15 s, annealing at 60–65°C for 20 s, and elongation at 72°C for 20 s in a cycle of 40 (OmicsGreen qPCR 5 \times Master Mix with ROX Dye, QE3931, Omics Bio, Taipei, Taiwan). The relative levels of gene expression were quantified using the $\Delta\Delta$ Ct method, which results in a ratio of target gene expression relative to equally expressed housekeeping genes (GAPDH) [10].

2.8. Western Blot Analysis

3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. Cells were incubated with 10-25 μ M of test samples for 4 h at 37°C in a humidified 5% CO₂ incubator. Cells were collected and lysed in ice-cold lysis buffer (20 mM Tris-HCl (pH 7.4), 2 mM EDTA, 500 μ M sodium orthovanadate, 1% Triton X-100, 0.1% SDS, 10 mM NaF, 10 μ g/mL leupeptin and 1 mM PMSF). The protein concentration was estimated with the Pierce™ BCA protein assay kit (Thermo Scientific) using bovine serum albumin as a standard. Proteins were fractionated on a SDS-PAGE gel and blotted for 2.5 h at 100 V. The proteins in the gel were transferred to a PVDF membrane. The membrane was blocked with 5% BSA in PBST (0.1% v/v Tween-20 in PBS, pH 7.2) for 1 h. α -actin (1:1000 dilution) and PPAR γ (1:1000 dilution) of primary antibodies were used in this study. Goat

anti-rabbit IgG-HRP and rabbit anti-rabbit IgG-HRP (Jackson, West Grove, Chester, PA, USA) were used as secondary antibodies, and ECL Prime (Advansta Inc., Menlo Park, CA, USA) reagent was used for developing. Membranes were incubated with primary antibody at 4°C overnight and then with secondary antibody for 1 h. Membranes were washed in PBST for 10 min three times between each step. Blots were developed using the Western Bright ECL kit (Advansta Inc.), exposed to Gel Electrophoresis Documentation-Multi-function Gel Image system (Topbio Co., Taipei, Taiwan). The relative expression of proteins was quantified densitometrically using the Image J software (Wayne Rasband, Madison, WI, USA) and calculated according to the reference bands of α -actin [3].

2.9. Statistical Analysis

Data are expressed as mean \pm SD, and ANOVA was conducted by using the SPSS software (SPSS Inc., Chicago, IL, USA). When a significant F ratio was obtained ($p < 0.05$) a post hoc analysis was conducted between groups by using a Duncan's multiple range tests or a Dunnett's test. p -Values of less than 0.05 were considered statistically significant.

3. Results and Discussion

3.1. Effect on 3T3-L1 Cell Growth

To study whether CA is able to induce cytotoxicity on 3T3-L1 cells, the cytotoxicity of CA was investigated using MTT assay. The effect of CA on cell growth in 3T3-L1 preadipocytes is shown in Figure 1A. The result indicated that CA at 1-25 μ M exhibited no cytotoxicity to 3T3-L1 preadipocytes after 24 h of incubation with CA. Moreover, the 3T3-L1 cells were allowed to differentiate into adipocytes for 8 days, and then the mature cells were incubated for 72 h in medium with different concentrations of CA (1-25 μ M). As shown in Figure 1B, none of CA in the range from 1-25 μ M showed any cytotoxic effects on the viability of 3T3-L1 adipocytes. These results indicate that CA at 1-25 μ M does not affect 3T3-L1 preadipocyte viability and is not cytotoxic to 3T3-L1 adipocytes.

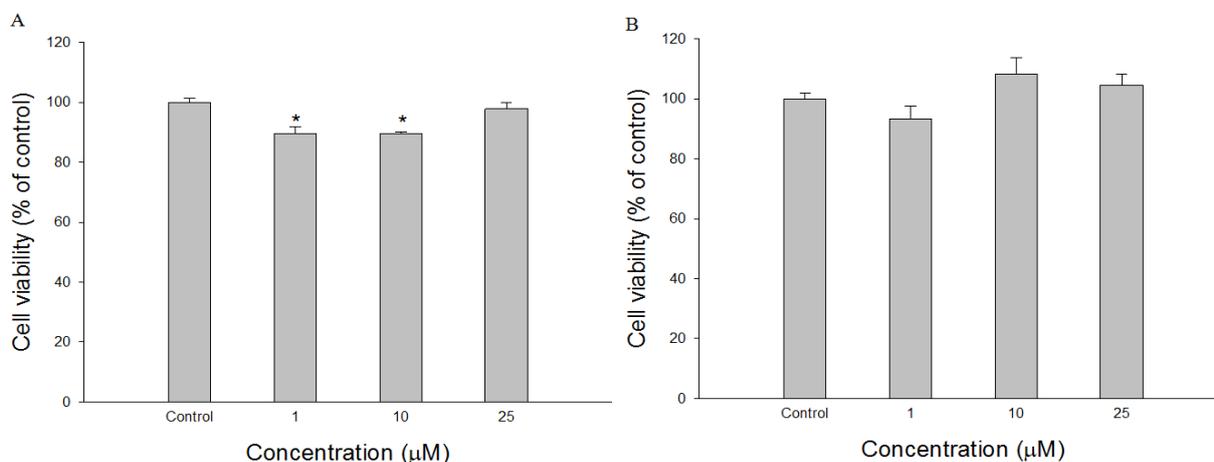


Figure 1. Effect of chlorogenic acid (CA) on cell proliferation in 3T3-L1 pre-adipocytes and adipocytes. (A) 3T3-L1 pre-adipocytes were treated with CA for 24 h. (B) 3T3-L1 adipocytes were harvested 8 days after the initiation of differentiation. The cells were treated with chlorogenic acid for 24 h. Data are expressed as the mean \pm SD of three independent experiments. * ($p < 0.05$), compared to the control

3.2. Inhibitory Effect on Lipid Accumulation in 3T3-L1 Adipocytes

In this study, 3T3-L1 adipocytes undergoing induced differentiation were incubated with different concentrations of CA (10 and 25 μM). The effect of CA on adipogenesis was determined in relation to control cells. The level of accumulated lipid was analyzed in the culture after completion of the differentiation process. Figure 2 shows the effects of CA on lipid accumulation in 3T3-L1 adipocytes. The microscopic images of oil red O-stained adipocytes and the number of lipid droplets in the differentiated cells are shown in Figure 2A and 2B. Staining of lipid droplets with oil red O showed that their accumulation in cells exposed to CA was lower (Figure 2A). Meanwhile, lipid content in adipocytes was dependent on the CA dose (Figure 2B). Quantitatively, 25 μM CA reduced lipid accumulation by 28.0% in 3T3-L1 adipocytes. In addition, further determination of the effect of CA on the triglyceride level was done. Figure 2C shows the effect of CA on the triglyceride level in 3T3-L1 adipocytes. The addition of CA in the maturation medium caused a decrease in the triglyceride level. This reduction occurred in a dose-dependent manner, reaching a reduction of 19.6 and 21.7% for 10 and 25 μM CA, respectively.

Glycerol-3-phosphate dehydrogenase (GDPH) catalyzes the formation glycerol-3-phosphate, and plays an essential role in glycerolipid metabolism [11]. Therefore, it is regarded as a marker of adipogenesis. The present study examined the effect of CA on GDPH activity. As shown in Figure 3A, the intracellular GDPH activity was concentration-dependently inhibited in cells treated with CA. The cells treated with 25 μM CA showed suppression of GDPH activity up to 18.0%, in comparison with control cells. In addition, PPAR γ is a master regulator of

adipocyte differential and metabolism. As shown in Figure 3B, in the CA-treated adipocytes, the PPAR γ protein expression was decreased in a concentration-dependent manner. The results obtained indicate that CA has inhibitory activity on adipogenesis.

3.3. CA inhibits Adipocyte-Specific Gene Expression

To understand whether the suppressed increase in lipid accumulation and GDPH activity in adipocytes results from a CA-regulated alternation in the differentiation process, the expression of adipogenic genes was determined by quantitative real time RT-PCR. Figure 4 shows the effect of CA on expression of adipogenic genes in 3T3-L1 adipocytes. In the cells treated with CA at 10 and 25 μM , the mRNA levels of PPAR γ , C/EBP α , SREBP-1c and FAS were determined after the induction of the differentiation process in 3T3-L1 adipocytes. The results show that CA inhibited the mRNA expression of PPAR γ (Figure 4A), C/EBP α (Figure 4B), SREBP-1c (Figure 4C), and FAS (Figure 4D) in a dose-dependent manner, compared to the control cells. The mRNA expression levels of PPAR γ , C/EBP α , SREBP-1c and FAS in 3T3-L1 adipocytes treated with 25 μM CA decreased to 35%, 11 %, 36%, and 45%, respectively, of that of control cells. Consistent with the mRNA results, Western blot analyses revealed that CA markedly suppressed the protein expression levels of PPAR γ (Figure 3B). That is to say, CA-associated attenuation of PPAR γ gene expression was accompanied by a decrease in the corresponding proteins. These results indicate that CA is able to down-regulate the expression of adipogenic genes through an adipogenic process, thereby leading to inhibition of lipid accumulation in 3T3-L1 adipocytes.

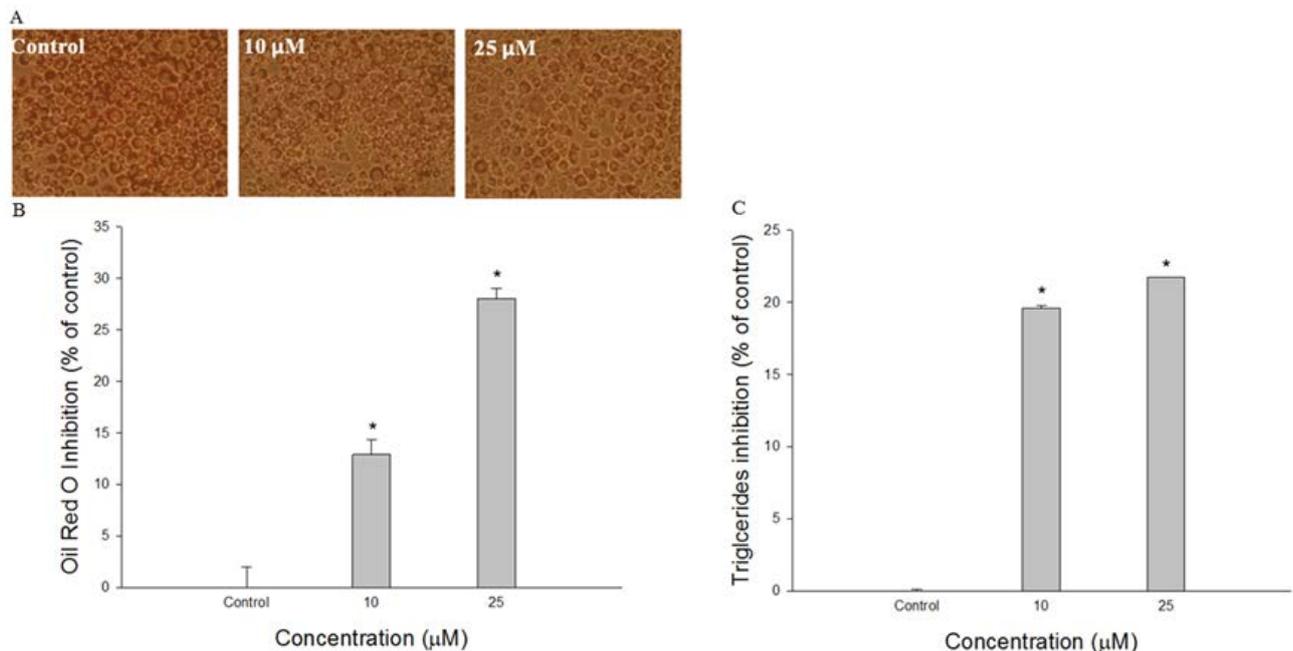


Figure 2. Effect of chlorogenic acid (CA) on adipogenesis. (A) The microscopic images of Oil red O-staining of CA-treated 3T3-L1 cells. (B) Oil red O staining of EECF-treated 3T3-L1 cells. 3T3-L1 cells were incubated with chlorogenic acid (CA) for 24 h at 37°C in 5 % CO₂ incubator. (C) CA-mediated suppression of intracellular triglyceride levels of 3T3-L1 cells. 3T3-L1 cells were incubated with CA for 24 h at 37°C in 5 % CO₂ incubator. Data are expressed as the mean \pm SD of three independent experiments. *(p < 0.05), compared to the control

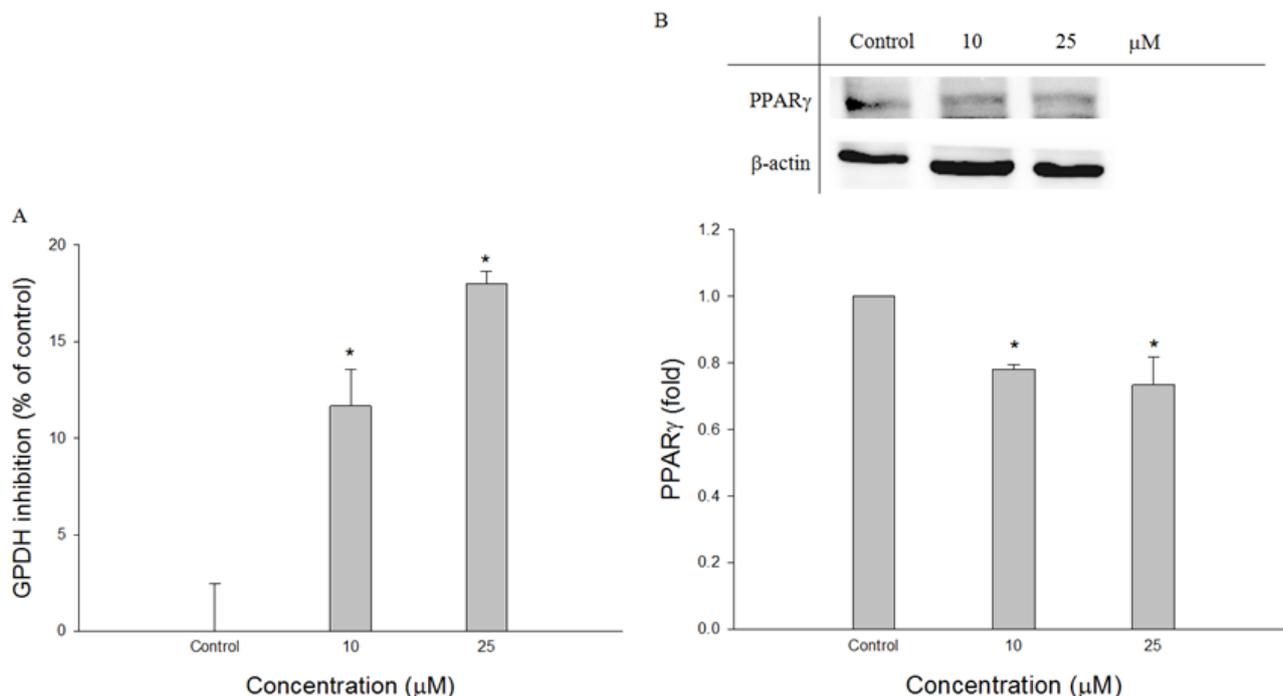


Figure 3. Effect of chlorogenic acid (CA) on GPDH activity (A) and PPAR γ protein expression (B) protein expression in 3T3-L1 cells. (A) The cells were incubated with CA for 24 h at 37°C in 5 % CO₂ incubator. (B) The cells were incubated with CA for 4 h at 37°C in 5 % CO₂ incubator. Data are expressed as the mean \pm SD of three independent experiments. *($p < 0.05$), compared to the control

It has been documented that at a molecular level, pre-adipocyte differentiation is regulated by PPAR γ and C/EBP α , the down-stream targets of SREBP-1c. PPAR γ , a ligand-activated transcriptional factor, is expressed predominantly in adipocytes and serves as the primary regulator of adipocyte differentiation [12]. Moreover, PPAR γ plays a pivotal role in inflammation and glucose metabolism [13]. C/EBP α is a key transcriptional factor in adipogenesis and its expression is activated by C/EBP β , C/EBP δ , and PPAR γ in adipocytes, [3,14]. In other words, preadipocyte differentiation is regulated by PPAR γ and C/EBP α . In addition, PPAR γ and C/EBP α activate the expression of genes involved in adipogenesis, such as adipocyte protein 2 (aP2), lipoprotein lipase (LPL), fatty acid transporter, GLUTA, SCD1, and CD36, which mediate fatty acid or glucose uptake into adipocytes, triglyceride hydrolysis and lipogenesis [15,16]. Thereafter, adipocytes incorporate glucose and free fatty acid to synthesize and accumulate lipids as energy, resulting in an increase in cell size [16]. SREBP-1c, a transcriptional factor, can stimulate genes related to de novo fatty-acid synthesis, such as FAS, ATP-citrate lyase (ACL), acetyl-CoA, carboxylase (ACC), stearoyl-CoA desaturase (SCP), and glyceraldehydes-3-phosphate acyltransferase (GPAT) [3,17]. As shown in Figure 4C, CA significantly reduced the mRNA levels of SREBP-1c, leading to prevention of SREBP-1c translocation to the nucleic acid, and finally inhibiting PPAR γ , C/EBP α , and FAS expression. Many natural products and bioactive compounds with anti-adipogenic effect such as rutin, kaempferol and betanin were reported to inhibit adipogenesis due to suppression of these transcriptional factors [3]. Figure 4 shows that CA down-regulated the expression of SREBP-1c and downstream target genes, PPAR γ ,

C/EBP α , and FAS in differentiated cells. Thus, CA effectively suppressed lipid formation and adipogenesis by blocking adipogenic transcriptional factors.

AMPK, playing an important role in regulating lipids metabolism, glycogen breakdown, glycolysis, and glucose uptake, is a key sensor of fuel and energy status in skeletal muscles [18]. The activation of AMPK leads to a decrease in the ACC activity, activation of malonyl CoA decarboxylase and reduction of the inhibitory effect of malonyl CoA to carnitine palmitoyl transferase-1, and further increases fatty acid oxidation [18]. The amino-imidazole carboxamide ribonucleotide (AICAR), an activator of AMPK, was proven to inhibit 3T3-L1 adipocyte differentiation by suppressing the expression of SREBP-1, PPAR γ , and C/EBP α [13]. In other words, activation of AMPK causes the inhibition of adipogenesis. Although the influence of CA on the up-regulation of AMPK activation was not explored, the findings of Chen et al. [13] may support our results that CA up-regulates AMPK activation, resulting in inhibition of SREBP-1c, PPAR γ , C/EBP α , and FAS expression during 3T3-L1 adipocyte differentiation.

CA, referred to an ester of caffeic acid and quinic acid, is present in a variety of edible plants. This phenolic phytochemical is known to have many biological activities. In addition, according to the results obtained, CA reduces the lipid accumulation by down-regulating the adipogenesis-related transcriptional factors, PPAR γ , C/EBP α , SREBP-1c, and FAS during the differentiation process. These novel biological findings are helpful in understanding the antiadipogenic activity of CA. Considering the multiple biofunctional effects of CA, it may contribute to an inhibition of adverse metabolic effects connected with excessive growth of the adipose tissue.

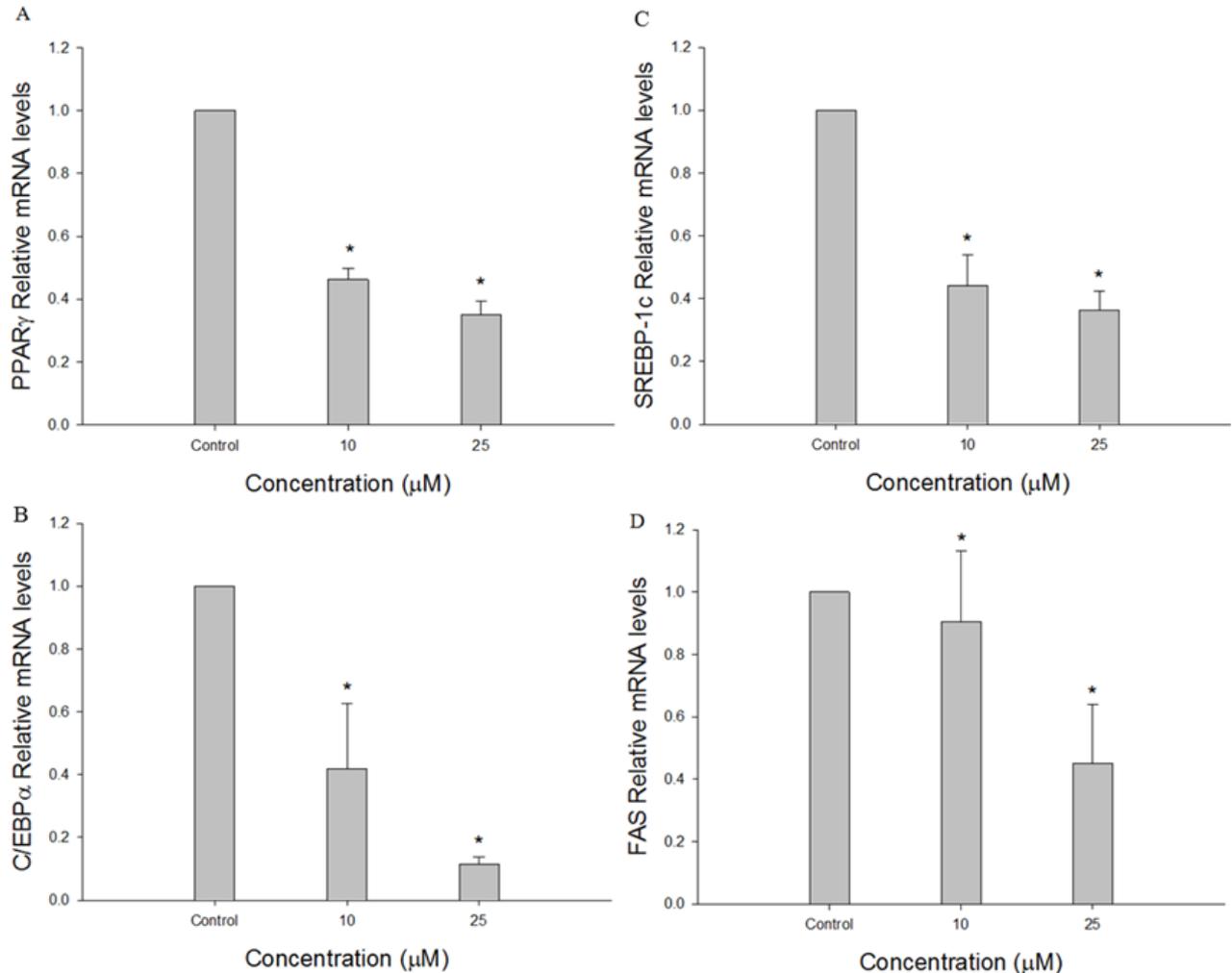


Figure 4. Effect of chlorogenic acid (CA) on expression of adipogenic genes in 3T3-L1 adipocytes. (A) The effect of CA on gene levels of PPAR γ in 3T3-L1 cells. The cells were incubated with CA for 2 h. (B) The effect of CA on gene levels of C/EBP α in 3T3-L1 cells. The cells were incubated with CA for 2 h. (C) The effect of CA on gene levels of SREBP-1c in 3T3-L1 cells. The cells were incubated with CA for 2 h. (D) The effect of CA on gene levels of FAS in 3T3-L1 cells. The cells were incubated with CA for 2 h. Data are expressed as the mean \pm SD of three independent experiments. * ($p < 0.05$), compared to the control

4. Conclusion

In this study, the results show that CA inhibits adipogenesis by down-regulating the expression of PPAR γ , C/EBP α , SREBP-1c and FAS. Consequently, the number of viable fully differentiated adipocytes is decreased, and lipid accumulation is limited. Therefore, CA with its anti-adipogenic effect, shows potential for development as an anti-obesity agent. However, further *in vivo* study is necessary to verify the anti-obesity activity of CA.

Acknowledgements

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Conflicts of Interest

The authors declare no conflict of interest.

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