

Cryptotanshinone Inhibits Lipid Accumulation in Differentiating 3T3-L1 Preadipocytes by Down-regulating C/EBP- α , PPAR- γ , FAS, Perilipin A, and STAT-3

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Differentiation of preadipocyte, also named adipogenesis, leads to the phenotype of mature adipocyte that is filled with many lipid droplets. Excessive lipid accumulation in adipocytes leads to the development of obesity. In this study, we investigated the effect of 11 different natural compounds on lipid accumulation during the differentiation of 3T3-L1 preadipocytes into 3T3-L1 adipocytes. Strikingly, among the natural compounds, cryptotanshinone at 10 μ M most strongly reduced triglyceride (TG) contents in 3T3-L1 cells after 8 days of the differentiation. Furthermore, cryptotanshinone at 10 μ M significantly suppressed lipid accumulation in 3T3-L1 cells after 8 days of the differentiation. Cryptotanshinone at 1 to 10 μ M tested did not affect the survival of 3T3-L1 cells after 8 days of the differentiation. On mechanistic levels, cryptotanshinone time-differentially decreased the expression levels of CCAAT/enhancer-binding protein- α (C/EBP- α), peroxisome proliferator-activated receptor- γ (PPAR- γ), fatty acid synthase (FAS), and perilipin A but also the phosphorylation levels of signal transducer and activator of transcription-3 (STAT-3) during the 3T3-L1 cell differentiation. Taken together, these findings demonstrate that cryptotanshinone inhibits lipid accumulation in differentiating 3T3-L1 cells, which appears to be mediated through the reduced expression and/or phosphorylation levels of C/EBP- α , PPAR- γ , FAS, Perilipin A, and STAT-3.

Keywords: Cryptotanshinone, CCAAT/enhancer-binding protein- α , Perilipin A, Perilipin A transcription-3

Introduction

Obesity is a serious public health epidemic with about 2 billion adults overweight or obese. The association between obesity and adverse health consequences, including hyperlipidemia, cardiovascular disease, type 2 diabetes, and cancer, are well established [1]. As a result, obesity is now among the leading factors for global morbidity and mortality [2]. Increasing evidence also indicates that obesity is induced as a result of excessive adipocyte differentiation, also known as adipogenesis, in the adipose tissues and adipocytes [3,4]. Any compound that inhibits excessive lipid accumulation in adipocytes could therefore be a potential preventive and therapeutic option for obesity.

Adipogenesis is a biological process characterized by morphological, cellular, and biochemical changes [4]. The differentiation process requires fibroblast-like preadipocytes to develop into mature adipocytes [3] under the control of adipogenesis-related transcription factors, including CCAAT/enhancer-binding proteins (C/EBPs), peroxisome proliferator-activated receptors (PPARs), and signal transducer and activator of transcription (STAT) proteins [5-8], fatty acid synthase (FAS) and lipid droplet (LD) associated proteins like perilipin A [9,10]. Central to the early stage of 3T3-L1 adipocyte differentiation, is the expression and phosphorylation of STAT-3 which then induces C/EBP- β transcription [6,11].

The potential application of natural products as traditional herbal medicines for the treatment of obesity by reducing lipid accumulation, adipocyte cell size and/or stimulating lipid metabolism are well documented [12]. In this study, we investigated the effect of 12 different natural compounds with anti-oxidative, anti-inflammatory,

and/or anti-cancerous activities, including azomycin, amygdalin, hesperetin, bergenin, cryptotanshinone, astragaloside A, sodium danshensu, tetrandrine, rotundine, indirubin, and chrysophanic acid, on lipid accumulation in 3T3-L1 adipocytes. Here we demonstrate that among the 12 natural compounds tested, cryptotanshinone most strongly inhibits lipid accumulation in differentiating 3T3-L1 preadipocytes and the inhibition is mediated through control of the expression and/or phosphorylation levels of C/EBP- α , PPAR- γ , FAS, perilipin A, and STAT-3.

Materials and Methods

Materials

Antibodies of C/EBP- α , C/EBP- β , PPAR- β , PPAR- γ , STAT-3, phospho (p)-STAT-3, STAT-5, and p-STAT-5 were purchased from Santa Cruz Biotechnology (Delaware, CA). FAS antibody was purchased from BD Bioscience (San Jose, CA). Cryptotanshinone was purchased from Selleckchem (Houston, TX). Antibody of β -actin was purchased from Sigma (St. Louis, MO). Perilipin A antibody was purchased from Bio Vision (Milpitas, CA).

3T3-L1 Cell culture and differentiation

3T3-L1 murine white preadipocytes (ATCC, Manassas, VA) were grown up to the contact inhibition stage and remained in the post-confluent stage for 2 days in DMEM supplemented with 10% calf bovine serum (Gibco, Waltham) and penicillin-streptomycin (Welgene, Daegu). Differentiation was induced by changing the medium to DMEM supplemented with 10% FBS (Welgene, Daegu) plus a cocktail of hormones (MDI) that include 0.5 mM IBMX (M) (Sigma, St. Louis), 0.5 μ M

dexamethasone (D) (Sigma, St. Louis), and 5 µg/ml insulin (I) (Sigma, St. Louis) in the absence or presence of individual natural substance at 10 µM concentration or cryptotanshinone at the indicated concentrations. After 48 h MDI-induction, the differentiation medium was replaced with DMEM supplemented with 10% FBS and 5 µg/ml insulin in the absence or presence of individual natural substance at 10 µM or cryptotanshinone at the indicated concentrations for additional 3 days. The cells were then fed every other day with DMEM containing 10% FBS in the absence or presence of individual natural substance at 10 µM or cryptotanshinone at the indicated concentrations until day 8.

Oil red O staining

On day 8 of differentiation, control or cryptotanshinone-treated 3T3-L1 cells were washed twice with PBS, fixed with 10% formaldehyde for 2 h at room temperature (RT), washed with 60% isopropanol and dried completely. The fixed cells were then stained with Oil Red O working solution for 1 h at RT and then washed twice with distilled water. Lipid droplets were observed by light microscopy (Nikon).

Cell count analysis

3T3-L1 preadipocytes were seeded in 24-well plates and incubated overnight. Cells were similarly grown under the above-mentioned differentiation conditions. On day 8 of differentiation, control or cryptotanshinone-treated 3T3-L1 cells, which cannot be stained with trypan blue dye, was counted under microscope. The cell count assay was done in triplicates. Data are mean ± standard error (SE) of three independent experiments.

Quantification of intracellular TG content by AdipoRed assay

On day 8 of differentiation, intracellular TG contents in control or cryptotanshinone-treated 3T3-L1 cells was measured using a commercially available AdipoRed Assay Reagent kit according to the manufacturer's instructions (Lonza, Basel). After a 10 min incubation, fluorescence was measured on Victor³ (Perkin Elmer, Waltham) with an excitation at 485 nm and an emission at 572 nm.

Preparation of whole cell lysates

At the designated time point, 3T3-L1 cells were washed twice with PBS and exposed to a modified RIPA buffer [50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 0.1% sodium dodecyl sulfate, 0.25% sodium deoxycholate, 1% Triton X-100, 1% Nonidet P-40, 1 mM EDTA, 1 mM EGTA, proteinase inhibitor cocktail (1x)]. The cell lysates were collected and centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was saved and protein concentrations were determined with Bradford reagent (Bio-Rad, Hercules).

Western blot analysis

Proteins (50 µg) were separated by SDS-PAGE (10%) and transferred onto nitrocellulose membranes (Millipore, Burlington). The membranes were washed with TBS (10 mM Tris, 150 mM NaCl) supplemented with 0.05% (vol/vol) Tween 20 (TBST) followed by blocking with TBST containing 5% (w/v) non-fat dried milk. The membranes were incubated overnight with antibodies specific for C/EBP-α (1:1,000), C/EBP-β (1:1,000), PPAR-β (1:1,000), PPAR-γ (1:1,000), p-STAT-3 (1:2,000), STAT-3 (1:2,000), p-STAT-5 (1:2,000), STAT-5 (1:2,000), FAS (1:1,000), perilipin

A (1:2,000) or β -actin (1:10,000) at 4°C. The membranes were then exposed to secondary antibodies coupled to horseradish peroxidase for 2 h at RT. The membranes were washed three times with TBST at RT. Immunoreactivities were detected by ECL reagents. Equal protein loading was assessed by the expression level of actin protein.

Statistical analyses

Cell count analysis was done in triplicates and repeated three times. Data were expressed as mean \pm SE. The significance of difference was determined by One-Way ANOVA. All significance testing was based upon a P value of <0.05 .

Results

Cryptotanshinone at 10 μ M strongly reduces triglyceride (TG) contents in differentiating 3T3-L1 preadipocytes with no cytotoxicity

The goal in this study was to identify natural compounds that inhibits lipid accumulation during the differentiation of 3T3-L1 preadipocytes into adipocytes. Fig. 1A is a scheme of 3T3-L1 preadipocyte differentiation. Initially, we measured the effect of each of 12 natural compounds at a final concentration of 10 μ M on intracellular triglyceride (TG) contents in 3T3-L1 cells on D8 of differentiation. As shown in Fig. 1B, high triglyceride (TG) contents were observed in control 3T3-L1 cells incubated with MDI, insulin, and FBS on D8 of differentiation. Strikingly, among the natural compounds tested, cryptotanshinone most strongly reduced intracellular TG contents in 3T3-L1 cells. Notably, tetrandrine or indirubin also substantially reduced TG contents in 3T3-L1 cells. Next, we investigated the effect of each of 12

natural compounds at 10 μ M on the survival of 3T3-L1 cells on D8 of differentiation using cell count analysis. As shown in Fig. 1C, no cytotoxicity to 3T3-L1 cells was noted for individual of 12 natural compounds tested. Because of the strongest lipid-lowering effect with no cytotoxicity, we chose cryptotanshinone in further studies.

Cryptotanshinone at 10 μ M largely inhibits lipid accumulation in differentiating 3T3-L1 preadipocytes

We next investigated the treatment effect of various concentrations of cryptotanshinone on lipid accumulation in differentiating 3T3-L1 preadipocytes by an Oil Red O staining. As shown in Fig. 2A (upper panels), many lipids were formed in control 3T3-L1 cells on D8 of differentiation, compared with undifferentiated cells on D0. However, treatment of the preadipocytes with cryptotanshinone for 8 days blocked lipid accumulation in differentiating 3T3-L1 preadipocytes in a dose-dependent manner. The cryptotanshinone's inhibitory effect on lipid accumulation in differentiating 3T3-L1 preadipocytes was also confirmed by light microscopic measurement (Fig. 2A, lower panels). As shown in Fig. 2B, cryptotanshinone also dose-dependently reduced intracellular TG contents in differentiating 3T3-L1 preadipocytes on D8. Apparently, 10 μ M of cryptotanshinone maximally inhibited lipid accumulation and reduced TG contents in these cells. Results of cell count analysis further revealed that cryptotanshinone at 1 to 10 μ M tested did not affect the survival of 3T3-L1 cells on D8 (Fig. 2C). Due to the strong repressive effects on lipid accumulation and TG contents with no cytotoxicity, we chose this 10 μ M of cryptotanshinone in further studies.

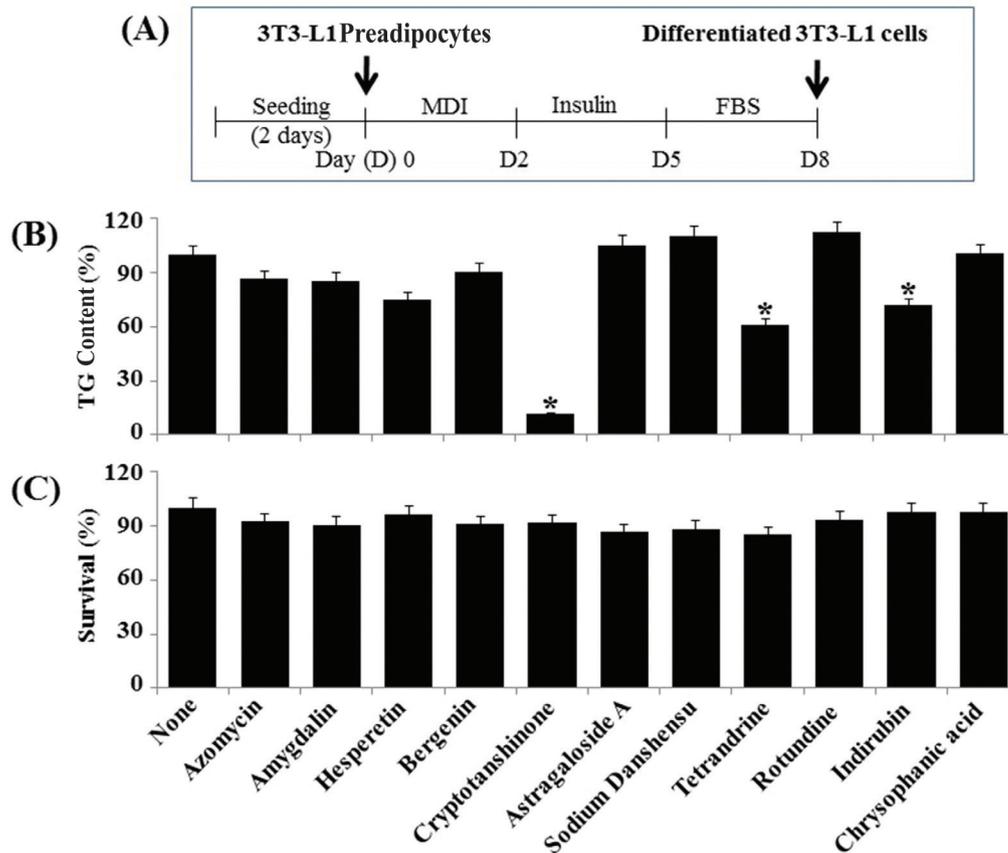


Fig. 1. Effects of 12 natural compounds on the triglyceride (TG) contents and survival of differentiating 3T3-L1 preadipocytes. (A) Experimental scheme for 3T3-L1 preadipocyte differentiation. (B) Quantification of the intracellular TG contents in vehicle control (none) (DMSO, 0.1%) or individual natural compounds (10 μ M)-treated 3T3-L1 preadipocytes on day 8 (D8) of differentiation using AdipoRed assay. (C) 3T3-L1 preadipocytes were grown under the above-mentioned 3T3-L1 preadipocyte differentiation condition in Figure 1B. On D8, the viability of vehicle control (none) or individual natural compounds (10 μ M)-treated 3T3-L1 preadipocytes was measured by cell proliferation assay. Data are mean \pm SE of three independent experiments, each done in triplicate. * $p < 0.05$ vs. control (no natural compounds).

Cryptotanshinone at 10 μ M decreases the expression and/or phosphorylation levels of C/EBP- α , PPAR- γ , and STAT-3 in differentiating 3T3-L1 preadipocytes

We next examined the effect of cryptotanshinone at 10 μ M on the protein expression and/or activity (phosphorylation) of adipogenic transcription factors (C/EBPs, PPARs, and STATs) by Western blotting analysis. Notably, cryptotanshinone

strongly reduced the expression levels of C/EBP- α in differentiating 3T3-L1 cells on D5 and D8. However, cryptotanshinone did not alter the expression levels of C/EBP- β in differentiating 3T3-L1 cells on D2 and D5; rather this small molecule enhanced the expression levels of C/EBP- β in these cells. Cryptotanshinone also had no effects on the expression levels of PPAR- β in differentiating 3T3-L1 cells on the time periods tested. On the other hand, the expression levels of PPAR- γ were

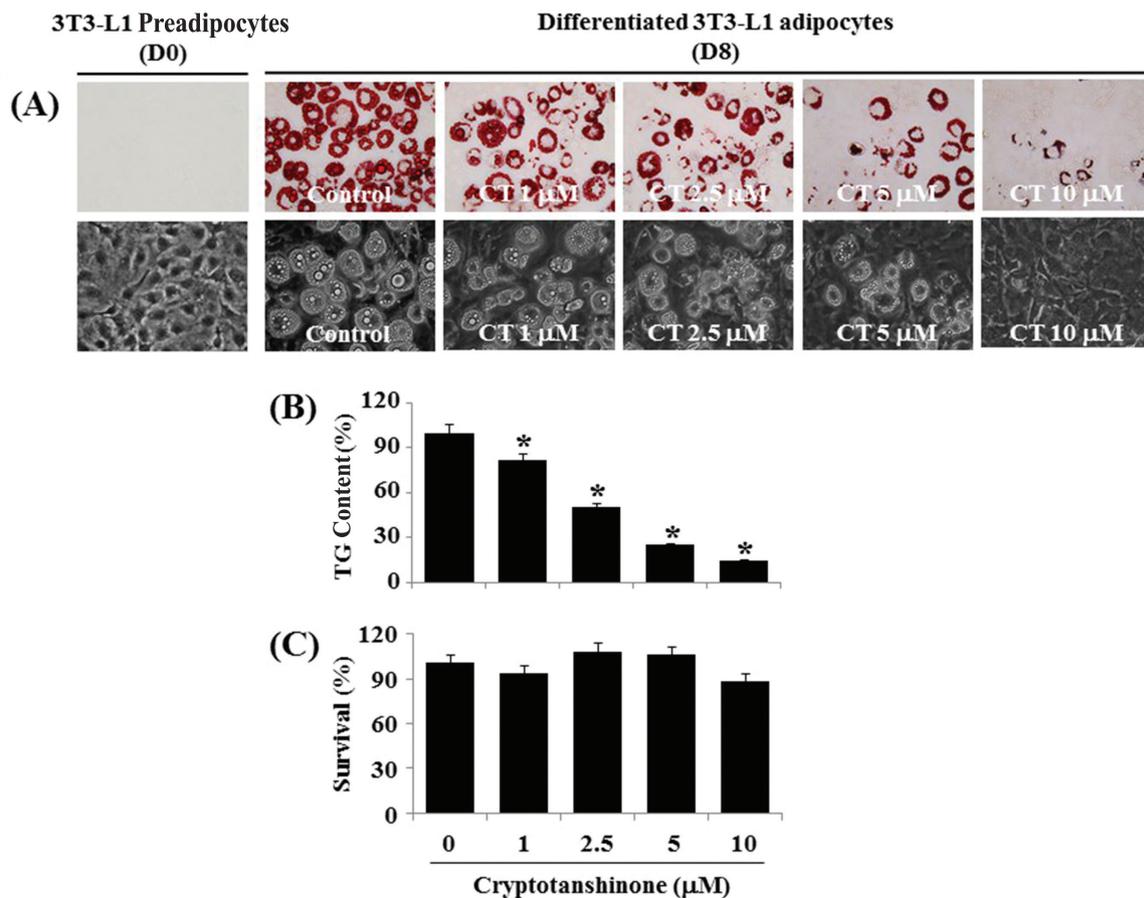


Fig. 2. Effects of cryptotanshinone on lipid accumulation, triglyceride (TG) contents, and survival of differentiating 3T3-L1 preadipocytes. (A) Measurement of the cellular lipid (lipid droplets) accumulation in 3T3-L1 preadipocytes (undifferentiated, D0) or differentiated adipocytes on day 8 (D8) of differentiation in the absence (vehicle control; DMSO, 0.1%) or presence of cryptotanshinone (CT) at the indicated concentrations by Oil Red O staining. Phase-contrast images were also taken after the treatment (lower panels in A). (B) Quantification of the cellular TG contents in vehicle control or cryptotanshinone-treated 3T3-L1 preadipocytes on D8 by AdipoRed assay. (C) 3T3-L1 preadipocytes were grown under the above-mentioned 3T3-L1 preadipocyte differentiation condition in Figure 2A. On D8, the number of surviving cells in vehicle control or cryptotanshinone-treated 3T3-L1 preadipocytes was measured by trypan blue dye exclusion. Data are mean \pm SE of three independent experiments, each done in triplicate. * p <0.05 vs. control (no cryptotanshinone).

substantially reduced in differentiating 3T3-L1 cells on D2, D5, and D8. In addition, although cryptotanshinone did not affect the phosphorylation levels of STAT-3 in differentiating 3T3-L1 cells on D2, it largely reduced the protein phosphorylation levels in differentiating 3T3-L1 cells on D5 and D8. Distinctly, while the

expression levels of total STAT-3 proteins remained constant in differentiating 3T3-L1 cells on D2 and D5, there was a large reduction of total STAT-3 expression on D8. Cryptotanshinone did not influence STAT-5 phosphorylation and total expression levels in differentiating 3T3-L1 cells on D2, D5 and D8.

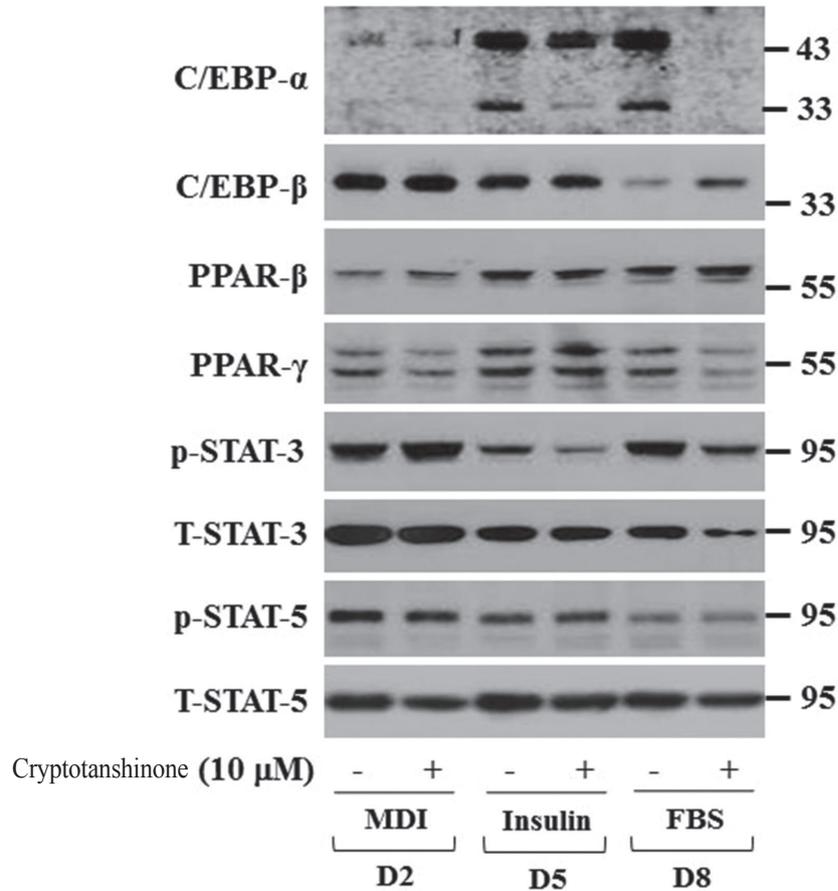


Fig. 3. Effect of cryptotanshinone on the expression and/or phosphorylation levels of C/EBP- α , C/EBP- β , PPAR- β , PPAR- γ , STAT-3, and STAT-5 in differentiating 3T3-L1 cells. 3T3-L1 preadipocytes were induced to differentiate with induction medium in the absence (vehicle control, 0.1%) or presence of cryptotanshinone (10 μ M), and harvested at day (D) 2, 5, and 8, respectively. Cellular protein at the indicated time period was extracted and analyzed by Western blotting analysis using respective antibody of C/EBP- α , C/EBP- β , PPAR- β , PPAR- γ , STAT-3, and STAT-5. p-STAT-3: phosphorylated STAT-3, T-STAT-3: total STAT-3, p-STAT-5: phosphorylated STAT-5, T-STAT-5: total STAT-5.

Cryptotanshinone at 10 μ M also down-regulates the expression levels of FAS and perilipin A in differentiating 3T3-L1 preadipocytes

We next investigated the effect of cryptotanshinone at 10 μ M on the expression levels of FAS, a lipogenesis-related protein, and perilipin A, a LD-interacting protein during 3T3-L1 preadipocyte differentiation. As shown in Fig. 4,

cryptotanshinone reduced the expression levels of both FAS and perilipin A in differentiating 3T3-L1 preadipocytes on D2, D5, and D8.

Discussion

There is a wealth of information that excessive preadipocyte differentiation (adipogenesis) and the resultant high fat accumulation leads to the

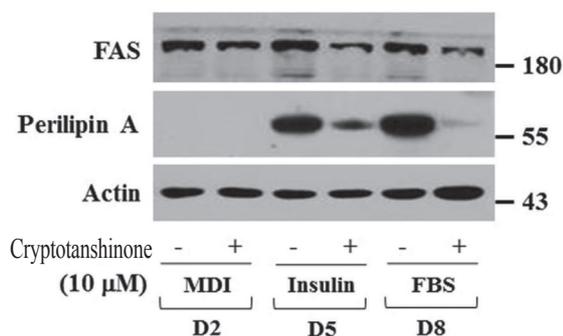


Fig. 4. Effects of cryptotanshinone on the expression of FAS and perilipin A in differentiating 3T3-L1 preadipocytes. 3T3-L1 preadipocytes were induced to differentiate with induction medium in the absence (vehicle control, 0.1%) or presence of cryptotanshinone (10 μM), and harvested at day (D) 2, 5, and 8, respectively. Cellular protein at the indicated time period was extracted and analyzed by Western blotting analysis using respective antibody of FAS and perilipin A.

development of obesity [2,3]. In this study, we report that cryptotanshinone strongly inhibits lipid accumulation and reduces TG contents in differentiating 3T3-L1 preadipocytes and the effects are mediated through control of the expression and phosphorylation levels of C/EBP- α , PPAR- γ , FAS, perilipin A, and STAT-3.

It is documented that adipocyte differentiation is controlled by the expression and activity of several adipogenic transcription factors, including C/EBP- α , C/EBP- β , PPAR- β , PPAR- γ , and STAT-3/5 [3-6, 13-15]. It has been previously reported that cryptotanshinone, one of tanshinones found in *Salvia miltiorrhiza Bunge* (Lamiaceae), reduces lipid accumulation in 3T3-L1 cells and the reduction is mediated through down-regulation of C/EBP- α , C/EBP- β , and PPAR- γ along with inhibition of STAT-3 phosphorylation in the early phase of 3T3-L1 preadipocyte differentiation [16]. We here also observed that cryptotanshinone strongly inhibits

lipid accumulation and reduces TG contents in differentiating 3T3-L1 preadipocytes, further supporting its lipid-lowering effect in culture. However, in this study, we further found that cryptotanshinone reduces the expression levels of C/EBP- α , but not C/EBP- β , in differentiating 3T3-L1 preadipocytes. Furthermore, the present study showed that while cryptotanshinone does not inhibit STAT-3 phosphorylation in the early (D2) phase of 3T3-L1 preadipocyte differentiation, it markedly inhibits STAT-3 phosphorylation in the middle (D5) and late (D8) phase of the cell differentiation. Difference in cryptotanshinone regulation of C/EBP- α , C/EBP- β , PPAR- γ , and STAT-3 between the present study and previous one may be due to use of different passage of 3T3-L1 cells, use of different duration of 3T3-L1 cell differentiation, and use of different cell lysis buffers to prepare whole cell lysates (proteins). Nevertheless, these results suggest that cryptotanshinone's inhibitory effect on lipid accumulation in differentiating 3T3-L1 cells is closely attributable to down-regulation of the expression and phosphorylation levels of C/EBP- α , PPAR- γ , and STAT-3.

We and others have demonstrated that the expressions of FAS and perilipin A proteins are also increased in 3T3-L1 preadipocyte differentiation [17-20]. At present, little is known about cryptotanshinone regulation of FAS and perilipin A in 3T3-L1 cells. Notably, we found that cryptotanshinone reduces the expression levels of both FAS and perilipin A in differentiating 3T3-L1 cells. Given that FAS is a lipogenic enzyme responsible for the synthesis of fatty acid [21], and perilipin A interacts with and stabilizes the newly formed LDs during 3T3-L1 preadipocyte differentiation [9,22], it is likely that down-regulation of FAS and perilipin A may further contribute to the cryptotanshinone's anti-lipogenic (lipid-lowering) effects.

Although it is obvious that cryptotanshinone has abilities to inhibit lipid accumulation and reduce TG contents in 3T3-L1 cells, it should be noted that 3T3-L1 cells used herein are cultured cell-lines and that the effective doses of cryptotanshinone may be different in in vivo white (pre)adipocyte cells and tissues. Therefore, future studies are warranted to evaluate the anti-adipogenesis (anti-obesity) effects of cryptotanshinone on obese animal models.

In summary, our study shows that cryptotanshinone has strong anti-adipogenic and anti-lipogenic effects in differentiating 3T3-L1 preadipocytes and the effects are linked to the reduced expression and phosphorylation levels of C/EBP- α , PPAR- γ , FAS, perilipin A, and STAT-3. These findings advocate that this natural phytochemical cryptotanshinone may have preventive and/or therapeutic potential against obesity.

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