Tangeritin inhibits adipogenesis by down-regulating C/EBPα, C/EBPβ, and PPARγ expression in 3T3-L1 fat cells

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ABSTRACT. The treatment of obese patients is a topic investigated by an increasing number of researchers. This study aimed to elucidate the possible inhibitory effect of tangeritin on the development and function of fat cells. 3T3-L1 fat cells were grown to confluence and subjected to different concentrations of tangeritin. The most effective tangeritin inhibition concentration was determined by the MTT assay. The treated cells were subjected to real-time reverse transcriptase PCR and western blot analysis, to detect changes in the CCAAT/enhancer binding protein (C/EBP)α, C/EBPβ, and peroxisome proliferator activated receptor (PPAR)γ expression levels. The MTT assay revealed that the fat cell growth was inhibited at a 20 ng/mL concentration of tangeritin. The results of real-time PCR revealed a significant decrease in the expression of C/EBPα, C/EBPβ, and PPARγ mRNA, following the treatment with tangeritin. Western blot analysis also presented similar results at a protein level. Therefore, we concluded that tangeritin inhibits adipogenesis via the down-regulation of C/EBPα, C/EBPβ, and PPARγ mRNA and protein expression in 3T3-L1 cells.

Key words: Tangeritin; 3T3-L1; C/EBPα; C/EBPβ; PPARγ; Adipogenesis

INTRODUCTION

Citrus trees are the largest fruit trees in the world. The second most abundantly produced fruits in China are citrus fruits; in fact, China ranks highest in the production of citrus fruits in the world. Red orange citrus fruits have been produced in China over several years, and is mainly distributed through the Chongqing, Sichuan, Hubei, and Fujian regions (Cristancho and Lazar, 2011) (Cristancho and Lazar, 2011). Obesity is characterized by significantly excessive body weight, and a very thick fat layer. It denotes the excessive saving of fat tissues in the body, and not simple weight gain. The increase in accessibility of material comfort has led to an increase in obesity over a range of ages, especially in the youth. So far, a considerable amount of research regarding obesity has focused on inhibiting the formation of fat cells. However, there is a lack of studies investigating the role played by tangeritin in inhibiting the growth of fat cells.

CCAAT/enhancer binding protein (C/EBP) is a trans-acting factor that includes a leucine zipper structure, a DNA binding domain, and a protein function domain. The C/EBP family includes several proteins, such as C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPβζ (Floyd and Stephens, 2012) (Floyd and Stephens, 2012). Their C-terminals have a highly conserved DNA binding domain and a dimerization functional domain. These proteins play an important role in several processes, including cell proliferation, differentiation, tumorigenesis, apoptosis, and cell cycle, via the regulation of target cell transcription. Transcription is regulated by multiple pathways, including the protease degradation, phosphorylation, and protein interaction pathways. The nucleic acid hormone receptor superfamily, also called the peroxisome proliferator activated receptor (PPARs) family, includes PPARα, PPARβ, and PPARγ. PPARγ is considered to be the main gene regulating fat accumulation (Zuo et al., 2006; Paur et al., 2010; White and Stephens, 2010; Zhan et al., 2012). C/EBPβ promotes adipocyte differentiation, through the activation of C/EBPα and PPARγ. It has also been reported that exogenous PPARγ could affect the conversion of pre-adipocytes into mature fat cells (Huang et al., 2010; Paur et al; 2010; Yasmeen et al., 2012). Flavonoids are a type of plant polyphenols, with low molecular weight compositions. They exhibit a basic C6-C3-C6 (configuration) framework, and are divided into the following major groups: flavone, flavonol, dihydro flavonol, flavanone, isoflavone, flavanoids, dihydrogen isoflavones, flavonoids, anthocyanins, and ketone. Flavonoids are composed of polyphenolic base structures, and therefore play an important role in the removal of free radicals. Current research on flavonoids is mainly focused on its anti-tumor, anti-aging, anti-allergy, antiviral, and anti-inflammation activity, and its role in cardiovascular diseases and diabetes. However, the role of flavonoids in the regulation of adipocyte differentiation remains to be elucidated. This study aims to determine the effect of flavonoids extracted from red orange citrus fruits on 3T3-L1 fat cells.

MATERIAL AND METHODS

Materials

The 3T3-L1 preadipocyte cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin-streptomycin, in a humid atmosphere containing 5% CO_2 at 37°C. Cells in the logarithmic phase of growth were used for all experiments. The primers used for real-time

PCR were as follows: *GAPDH* (F: 5'-TGAGGACCAGGTTGTCTCCTGCG-3'; R: 5'-CACCACCCTG TTGCTGTAGCCA-3'), *C/EBPα* (F: 5'-AGGTGCTGGAGTTGACCAGT-3'; R: 5'-CAGCCTAGAGAT CCAGCGAC-3'), *C/EBPβ* (F: 5'-AACTCTCTGCTTCTCCCTCTG-3'; R: 5'-AAGCCCGTAGGAACA TCTTT-3'), *PPARγ* (F: 5'-TGCAAGGACCTCACAAGA-3'; R: 5'-ATGCTGGAGAAGTCAACG-3'). Primary antibodies against C/EBPβ (1:1000), C/EBPα (1:1000), and PPARγ (1:500) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Horseradish peroxidase (HRP)-tagged secondary antibodies were purchased from Zsbio (Beijing, China).

Methods

Real-time reverse transcriptase polymerase chain reaction (RT-PCR)

RNA was extracted from the cells using the TaKaRa RNA extraction kit (TaKaRa Bio Inc., Tokyo, Japan). cDNA was synthesized from the RNA using the Invitrogen reverse transcription kit (Invitrogen, Life Technologies), which included 1 µL (50 µM) oligo(dT)20, 2 µL (10 mM) dNTP, 4 µL synthesis buffer, 1 µL (0.1 M) DTT, 40 U RNase Out, and DEPC-H_2O, to which 2 µg RNA template was added. Each real-time RT-PCR reaction was performed in a reaction mixture of 25 µL, containing 2XRT²SYBR Green Master Mix (Superarray), 1 µL (10 µM) primers, and 1 µL of the template cDNA. The reaction conditions were set as follows: initial, single denaturation cycle at 95°C for 10 min, followed by 40 cycles at 95°C, 55°C, and 72°C for 30 s each.

MTT assay

Cells were seeded in 96-well plates at a density of 2 x 10^3 cells/well and incubated for 12h at 37°C. After addition of 20 µL of MTT solution to each well, plates were incubated for 4 h at 37°C and added with 150 µL DMSO. Absorbance of each well at 570 nm was read using a spectrophotometer.

Western blot analysis

Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (10% SDS-PAGE), and transferred to a polyvinyl difluoride (PVDF) membrane (BioRad, Hercules, CA, USA). The membrane was blocked with 5% non-fat milk, and incubated with rabbit anti-C/EBPβ (1:1000), anti-C/EBPα (1:1000), and anti-PPARγ (1:500) at 4°C overnight. After incubated in HRP-tagged secondary antibody (Abcam, San Francisco, CA) at 37°C for 1 h, the membranes were detected using chemiluminescence reagents (Univ, Shanghai, China).

Statistical analysis

All statistical analyses were performed using the SPSS v.11.0 software platform (SPSS Inc., Chicago, IL, USA). Differences between means were analyzed using Student's *t*-test; P values < 0.05 were considered to indicate a statistically significant result.

RESULTS

### Effect of tangeritin on 3T3-L1 cell growth.

Cells were seeded in 96-well plates at a density of 2×10^3 cells/well and incubated for 12 h at 37°C. The cells were incubated with different concentrations of tangeritin at 10umol/L, 20umol/L, 30umol/L, or 40umol/L. The MTT test revealed a decrease in 3T3-L1 cell survival with the increase in tangeritin concentration and the treatment time (Figure 1). The inhibitory effect of tangeritin was obvious at a concentration of 20 ng/mL.
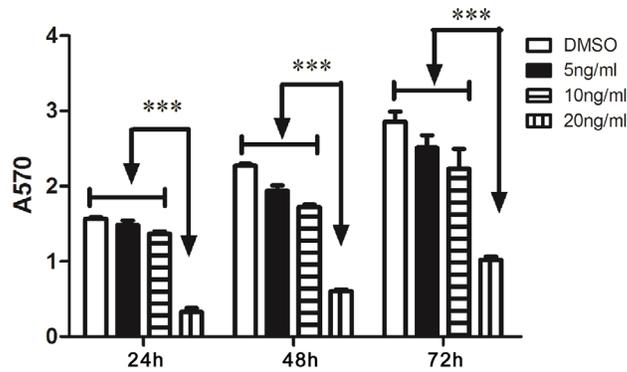
Figure 1. Inhibitory effect of tangeritin on 3T3-L1 fat cell growth at different time intervals.

### Tangeritin inhibits the formation of fat cells at an mRNA level.

The fat cells treated with 20 ng/mL of tangeritin led to a significant down-regulation in the expression of C/EBPα, C/EBPβ, and PPARγ mRNA, compared to the control (treated with dimethyl sulfoxide (DMSO); P = 0.007, 0.009, and 0.043, respectively) (Figure 2). This suggested that tangeritin inhibits fat cell formation at the mRNA level; however, this remains to be further validated.
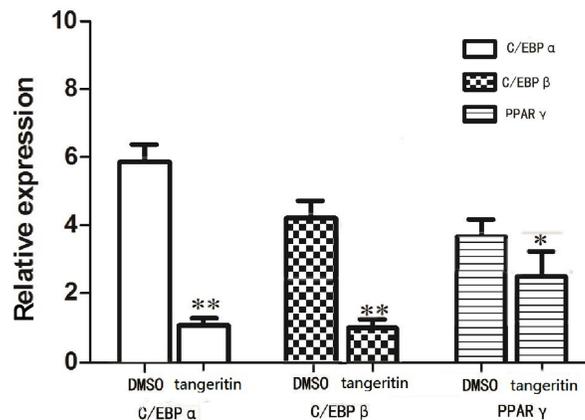
Figure 2. The expression of three important genes related to lipogenesis (C/EBPα, C/EBPβ, and PPARγ) was significantly down-regulated upon treatment with tangeritin.

Tangeritin inhibits lipogenesis by inhibiting the expression of essential proteins.

Western blot experiments confirmed the decrease in expression of C/EBPα, C/EBPβ, and PPARγ proteins following the treatment with tangeritin. This suggested that tangeritin inhibits lipogenesis by down-regulating C/EBPα, C/EBPβ, and PPARγ expression (Figure 3).
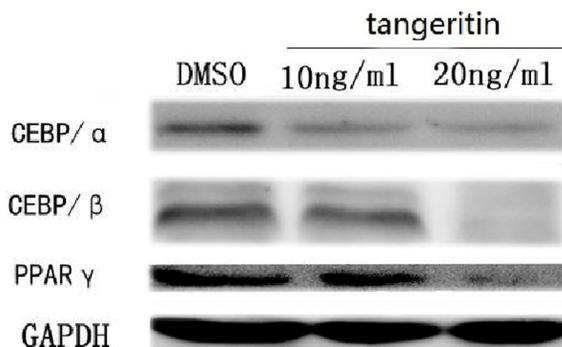
Figure 3. The expression of C/EBPα, C/EBPβ, and PPARγ proteins in 3T3-L1 cells was down-regulated after treatment with 10 and 20 ng/mL tangeritin; GAPDH was utilized as the control.

DISCUSSION

The recent advances in technology have led to a rapid increase in and wide availability of material comforts, resulting in increased obesity in people of all ages, especially the youth. Adipose tissue is distributed throughout the body; its distribution in each part of the body is important to maintain the steady state balance of energy (Staikos et al., 2013)(StaikosVerveridis et al., 2013). C/EBPs, including C/EBPα, C/EBPβ, and C/EBPγ, play an important role in the differentiation of pre-adipocytes to adipocytes, by regulating the transcription; transcription is regulated via the binding of proteins to the DNA in the form of a homologous or heterologous dimer (Rosati et al., 2001; Huang et al., 2012; Rahman et al., 2012; Esteves et al., 2014). The nucleic acid hormone receptor superfamily (PPARs) is composed of the PPARα, PPARβ, and PPARγ forms. PPARγ is considered to be the major gene regulating fat accumulation. Previous reports have suggested that the expression of even PPARγ in C/EBPα results in the loss of cell ability to differentiate into mature fat cells (Chen et al., 2011; Maier et al., 2014; Yang et al., 2014). PPARγ has several characteristics of nucleic acid receptors, and must bind to the appropriate receptor to regulate transcription. Previous studies have discovered that the PPARγ deleted mouse expresses little to no adipose tissue (Wegner et al., 1992; Buck et al., 1999; Kim et al., 2012). PPARγ is an important factor affecting fat formation, and the differentiation of fat cells are not completed when PPARγ activity is inhibited (Harmon et al., 2002; Yasmeen et al., 2012).

Flavonoids are polyphenolic plant components with low molecular weight. The current research on flavonoids has focused on its anti-tumor, anti-aging, anti-allergy, antiviral, and anti-inflammatory activities, as well as its effect on cardiovascular diseases and diabetes. However, the role of flavonoids in regulating adipocyte differentiation remains to be elucidated. The fat cells treated with tangeritin were subjected to the MTT assay to determine the best inhibitory concentration; the C/EBPα, C/EBPβ, and PPARγ mRNA and protein expression levels were then detected via real-time RT-PCR and western blot analyses. The results showed a significant downregulation in C/

EBPα, C/EBPβ, and PPARγ mRNA and protein expression following treatment with tangeritin. This revealed that tangeritin inhibits lipogenesis by reducing the C/EBPα, C/EBPβ, and PPARγ genes.

In conclusion, this study determined that tangeritin plays an important role in lipogenesis (for the first time), which provides a basis for future research. However, our study has a few shortcomings; for example, the mechanism of tangeritin regulation of C/EBPα, C/EBPβ, and PPARγ genes remains to be elucidated.

Conflicts of interest

The authors declare no conflict of interest.

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