Phytomedicine 23 (2016) 58–67

Contents lists available at ScienceDirect

Phytomedicine

journal homepage: www.elsevier.com/locate/phymed

Cryptotanshinone, a compound of *Salvia miltiorrhiza* inhibits pre-adipocytes differentiation by regulation of adipogenesis-related genes expression via STAT3 signaling

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ARTICLE INFO

Article history:
Received 16 April 2015
Revised 1 December 2015
Accepted 7 December 2015

Keywords:
Cryptotanshinone
Adipogenesis
C/EBPβ
PPARγ
STAT3

ABSTRACT

Background: Cryptotanshinone (CT), a major tanshinone found in *Salvia miltiorrhiza* Bunge (*Lamiaceae*), has various pharmacological effects such as antitumor, anti-inflammatory, and antioxidant properties. Despite its well-documented benefits in a wide range of diseases, the effect of CT on adipocyte differentiation has not been well characterized.
Purpose: The present study was designed to determine the *in vitro* anti-adipogenic effect and underlying molecular mechanisms of CT using 3T3-L1 murine pre-adipocytes.
Methods: We measured the levels of intracellular triglyceride accumulation and mRNA and protein expression of key adipogenic transcription factors and their target genes.
Results: Treatment with CT drastically reduced lipid accumulation in a dose- and time-dependent manner. Molecular assays showed that CT effectively suppressed the expression of C/EBPβ, C/EBPα, and PPARγ and of their target adipocyte-specific genes *aP2*, adiponectin, and *GLUT4* but activated the expression of anti-adipogenic genes such as *GATA2*, *CHOP10*, and *TNF-α*. CT treatment also inhibited the phosphorylation of STAT3 in the early phase of adipogenesis. A small-interfering-RNA-mediated knockdown of STAT3 potentiated the anti-adipogenic effect of CT.
Conclusion: Taken together, the results suggest that CT may be a good anti-adipogenic candidate because it regulates STAT3 during early adipogenesis.

Introduction

Obesity, i.e., increased storage of body fat, has attracted increasing research attention because of its association with a variety of diseases such as type 2 diabetes, cardiovascular diseases (e.g., hypertension), dyslipidemia, and certain type of cancers (Kopelman 2000). Adipocytes are the major constituents of fat tissue and play a pivotal role in energy homeostasis (Gregoire et al. 1998). Therefore, a clear identification of the molecular mechanisms that regulate the differentiation of pre-adipocytes to mature adipocytes should improve the understanding of this process and facilitate the development of anti-obesity drugs (Farmer 2006). A complex network of transcription factors, comprising CCAAT/enhancer-binding proteins (C/EBPδ, C/EBPβ, and C/EBPα) and the nuclear hormone receptor peroxisome proliferator-activated receptor γ (PPARγ), synchronize the expression of hundreds of proteins, thereby establishing the fat cell phenotype known as mature adipocytes (Rosen et al. 2000). C/EBPδ and C/EBPβ are the earliest transcription factors that respond to the adipogenic hormonal stimuli and then stimulate the expression of late transcription factors such as PPARγ, C/EBPα (Wu et al. 1995), adiponectin, adipocyte fatty acid-binding protein (aP2), leptin, lipoprotein lipase (LPL), and fatty acid synthase (FAS) required for the formation of mature adipocytes. Furthermore, several negative regulators, including C/EBP homologous protein 10 (CHOP10) and GATA-binding proteins 2 and 3 (GATA2/3) have been shown to attenuate adipogenesis (Batchvarova et al. 1995; Tong et al. 2005). In addition to these cellular transcription factors, many adipogenesis-regulatory signaling

Abbreviations: CT, cryptotanshinone; RA, retinoic acid; Tro, troglitazone; C/EBP, CCAAT/enhancer-binding protein; PPARγ, peroxisome proliferation-activity receptor γ; GAPDH, glycerol-3-phosphate dehydrogenase; IBMX, isobutyl-1-methylxanthine; MTT, 3-(4 5-dimethylthiazol-2-yl)-2 5-diphenyltetrazolium bromide; GLUT4, glucose transporter type 4; TNF-α, tumor necrosis factor α; CHOP10, C/EBP-homologous protein 10; GATA2, GATA-binding protein 2; STAT3, signal transducer and activator of transcription 3; aP2, adipocyte protein 2; MCE, mitotic clonal expansion.

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http://dx.doi.org/10.1016/j.phymed.2015.12.004
0944-7113/

pathways have been identified. Emerging evidence suggests that signal transducer and activator of transcription 3 (STAT3) plays an important role in adipogenesis by regulating PPARγ (Wang et al. 2010). Another report suggested that STAT3 could directly regulate C/EBPβ transcription at the early stage of differentiation (Zhang et al. 2011).

It has been reported that natural products from plants, e.g., flavonoids, stilbenoids, phenolic acids, and cyanidins, can reduce body fat accumulation, inhibit adipocyte differentiation, and induce lipolysis without any significant adverse effects on health (Rayalam et al. 2008; Wang et al. 2014b). Thus, natural products have been considered a source of safe and promising anti-obesity drugs (Yun 2010).

Dried roots of *Salvia miltiorrhiza* Bunge (*Lamiaceae*) have long been used in traditional Chinese medicine for the treatment of a broad range of pathologies including cerebrovascular diseases, liver diseases, Alzheimer's disease, acute cancer, chronic hepatitis, and diabetes mellitus (Zhou et al. 2005). Moreover, the extracts from the roots of this plant effectively prevent the development of bone loss and increase the blood estrogen level in a rat model (Chae et al. 2004). Several preparations that contain its major bioactive ingredients are also effective for microcirculation and coronary vasodilation and prevent inflammatory factors, and are therefore used against hypertension and inflammatory diseases (Xu et al. 2015). Also, a purified extract of this plant containing its major constituents, CT, tanshinone I, and tanshinone IIA can protect against liver toxicity *in vivo* and *in vitro* (Park et al. 2009). It has been reported that CT is one of the major bioactive lipophilic constituent along with tanshinones I and IIA, and salvianolic acid B is the major bioactive hydrophilic constituent present in roots of *S. miltiorrhiza* collected from different areas of China. Therefore these components are considered as appropriate markers for the quality control of this plant (Zhong et al. 2009). The content of CT in *S. miltiorrhiza* is variable depending on different extraction methods. A much higher yield of CT has been found in crude supercritical carbon dioxide extract and ethanol extract prepared from the roots of *S. miltiorrhiza* where concentration of CT was 106.83 $\pm$ 2.32 mg/g (w/w) and 35.45 $\pm$ 0.22 mg/g (w/w) respectively (Wu et al. 2012). CT has been found to possess various pharmacological properties including antioxidant, anti-inflammatory, anticancer, antitumor, antiangiogenic, and antidiabetic (Kim et al. 2007). In a previous *in vivo* study CT was found to reduce the concentration of body fat, serum cholesterol and triglyceride levels in mice (Kim et al. 2007). Nonetheless, the function of CT in adipogenic differentiation and the possible underlying molecular mechanism are still poorly understood. However, the other major components of *S. miltiorrhiza,* tanshinones IIA and salvianolic acid B have already been shown potential anti-adipogenic and anti-obesity effect *in vitro* and *in vivo*. Tanshinones IIA inhibits 3T3-L1 preadipocyte differentiation through antagonism of PPARγ transcriptional activities, reduce adipose mass and body weight, improve glucose tolerance, and lower the low-density lipoprotein to high-density lipoprotein *ratio* without changing the food intake in high-fat diet induced obese animal model (Gong et al. 2009). Salvianolic acid B has been shown to reduce body weight, white adipose tissue weight, adipocyte size, triglyceride and total cholesterol levels in obese mice as well as inhibit pre-adipocytes differentiation through repression of PPARγ and C/EBPα (Wang et al. 2014a). Therefore, we hypothesized that revealing the function and molecular mechanism of CT in pre-adipocytes differentiation might be an important background for the development of an anti-obesity herbal medicinal product using *S. miltiorrhiza* extracts or active components of this plant. We report here for the first time that CT can inhibit adipogenesis, and that STAT3 signaling may mediate this inhibition.

Materials and methods

Chemicals

CT (Lot number C5624, purity $\geq$ 98%, high-performance liquid chromatography; HPLC), retinoic acid (RA, purity $\geq$ 98%, HPLC), dexamethasone, isobutyl-1-methylxanthine (IBMX), insulin, Oil Red O dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), the Escort IV transfection reagent, RNase A, and propidium iodide were purchased from Sigma–Aldrich (St. Louis, MO, USA). DMEM (Dulbecco's modified Eagle's medium), phenol red free DMEM, the Opti-MEM serum-free medium, and newborn calf serum (NBCS) were purchased from Gibco (Grand island, NY, USA). Fetal bovine serum (FBS) was purchased from Atlas Biologicals (Fort Collins, CO, USA). A penicillin-streptomycin solution was purchased from Hyclone Laboratories, Inc. (South Logan, NY, USA). A 4% paraformaldehyde solution was purchased from Intron Biotechnology Inc. (Seongnam-si, South Korea). Antibodies against C/EBPβ, C/EBPα, PPARγ, adiponectin, STAT3, phospho-STAT3 (Tyr705), and horseradish peroxidase (HRP)-linked anti-rabbit IgG were purchased from Cell Signaling Technology (Danvers, MA, USA). The BCA Protein Assay Kit was purchased from Thermo Fisher Scientific (Rockford, IL, USA). Protein loading buffer was obtained from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). 2-Hydroxy-4-([(4-methylphenyl) sulfonyloxy] acetyl) amino-benzoic acid (S3I-201) was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

Cell culture and differentiation

3T3-L1 mouse embryonic fibroblasts were obtained from Korean Cell Line Bank (KCLB-10092.1) and cultured at 37 °C and 5% CO_2 in high-glucose DMEM containing 10% (v/v) heat-inactivated NBCS and 1% (v/v, final concentration) of 100 U/ml penicillin and 100 U/ml streptomycin until confluence and were then maintained in the same medium for 2 additional days. The medium was replenished every 2–3 days. The cells were induced to differentiate by replacement of the medium with DMEM containing 10% FBS, 0.5 mM IBMX, 1 μM dexamethasone, and 10 μg/ml insulin (MDI). After 2 days of incubation in this medium, the medium was replaced with DMEM supplemented with 10% FBS and 10 μg/ml insulin. After 5–7 days of induction of differentiation, we found that approximately 90% of the pre-adipocytes turned into round-shaped mature fat cells.

Cell viability assay

3T3-L1 pre-adipocytes were seeded in 96-well plates at the density of 1×10^4/well. Cells were grown and differentiated as described in the Cell culture and differentiation section. Both pre-adipocytes and adipocytes were treated with various concentrations of CT (2, 4, 8, and 10 μM) for 24 or 48 h and 6 days, respectively. Then, MTT solution was added to the phenol red free medium at the final concentration of 0.5 mg/ml, and the plates were incubated for 3 h at 37 °C. The resulting formazan crystals were dissolved in 150 μl of dimethyl sulfoxide (DMSO), and the absorbance was measured on a Victor™ X3 Multilabel reader (Perkin Elmer, Waltham, MA, USA) at a wavelength of 590 nm.

The lactate dehydrogenase (LDH) assay

To determine the cytotoxicity of CT, we performed an LDH activity assay. Briefly, 3T3-L1 cells were seeded in 96-well plates at a density of 1×10^4/well and grown to ~80% confluence. The cells were then treated with 0, 2, 4, 6, 8, and 10 μM of CT for 48 h.