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Original Article

Anti-adipogenesis mechanism of pterostilbene through the activation of heme oxygenase-1 in 3T3-L1 cells

Young-Jin Seo[1], Kui-Jin Kim[1], Eun-Jeong Koh, Jia Choi, Boo-Yong Lee*

Department of Food Science and Biotechnology, College of Life Science, CHA University, CHA Biocomplex, 335 Pangyo-ro, Bundang-gu, Seongnam, Kyonggi 463-400, Republic of Korea

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ABSTRACT

Background: Pterostilbene is a stilbenoid and major compound and has diverse biological activities, such as antioxidant, anti-cancer, and anti-inflammatory. However, it has not been shown whether pterostilbene affects the mitotic clonal expansion during adipogenesis in 3T3-L1 cells.

Purpose: In the present study, we aimed to demonstrate the detailed mechanism of pterostilbene on anti-adipogenesis in 3T3-L1 cells.

Methods: Preadipocytes were converted to adipocytes through treatment with MDI (IBMX; 3-isobutyl-1-methylxanthine, DEX; dexamethasone, insulin) in 3T3-L1 cells. Oil Red O staining was performed to measure intracellular lipid accumulation. Western blot analysis was conducted to analyze protein expressions.

Results: Our results showed that pterostilbene decreased the lipid accumulation compared to MDI-induced differentiation, using Oil Red O staining. Next, we found that pterostilbene suppressed the expression of C/EBPα, PPARγ, and aP2 as well as the mitotic clonal expansion-associated proteins CHOP10 and C/EBPβ, by western blot analysis. Our results indicated that pterostilbene may repress adipocyte differentiation through the activation of HO-1 expression prior to entering into the mitotic clonal expansion in 3T3-L1 cells. RNA interference was used to determine whether HO-1 acts as a regulator of CHOP10.

Conclusion: Our results revealed that pterostilbene induced HO-1 expression which acts as a regulator of CHOP10. Together, we demonstrated that pterostilbene suppresses the initiation of mitotic clonal expansion *via* up-regulation of HO-1 expression during adipocyte differentiation of 3T3-L1 cells.

Introduction

Obesity is characterized by an increase in adipose tissue mass and is becoming a public health problem that affects millions of people worldwide (Bastarrachea et al., 2004). It has been suggested that obesity is associated with an increased number (hyperplasia) and size (hypertrophy) of adipocytes (Drolet et al., 2008; Jo et al., 2009). In particular, both hyperplasia and hypertrophy might be developed through the expression of adipogenic-specific genes during adipogenesis that is a differentiation process of adipocyte precursor cells to mature adipocytes. Some studies have reported that hyperplasia and hypertrophy are associated with insulin resistance and sensitivity (Choe et al., 2016; Gustafson et al., 2015). Adipogenesis procedures are initiated by MDI mixture including IBMX; 3-isobutyl-1-methylxanthine, DEX; dexamethasone, and insulin *in vitro*, which induces the synchronized initiation of the cell cycle and allows to occur mitotic clonal expansion (MCE) (Bernlohr et al., 1985; Cornelius et al., 1994).

During MCE, MDI mixture decreases the expression of C/EBP homologous protein10 (CHOP10) and subsequently increases the level of CCAAT/enhancer-binding protein β (C/EBPβ) in 3T3-L1 adipocytes (Cao et al., 1991; Yeh et al., 1995). After that, the sufficient expression of C/EBPβ protein activates transcription factors of the C/EBPα and peroxisome proliferator-activated receptor γ (PPARγ) genes (Darlington et al., 1998; Tang et al., 2003). Moreover, both C/EBPα and PPARγ, late stage adipogenic markers, cause the expression of adipocyte protein 2 (aP2). It has been shown that intracellular lipid is accumulated through the modulation of triglyceride (TG) synthesis-associated enzymes such as Lipin1 and diacylglycerol acyltransferase 1 (DGAT1) in adipocyte differentiation (Zhang et al., 2008).

The endoplasmic reticulum (ER) is the main organelle of protein

Abbreviations: Ptero, Pterostilbene; MCE, Mitotic clonal expansion; CoPP, Cobalt protoporphyrin; TG, Triglyceride; DEX, Dexamethasone; IBMX, 3-isobutyl-1-methylxanthine; C/EBP, CCAAT-enhancer-binding proteins; PPARγ, Peroxisome proliferator-activated receptors-gamma; aP2, Adipocyte protein 2; CHOP, C/EBP homologous protein; HO-1, Heme oxygenase-1

* Corresponding author.
E-mail addresses: bylee@cha.ac.kr, kuijin.kim@gmail.com (B.-Y. Lee).
[1] These authors contributed equally to this work.

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and lipid metabolism and leads to lipid accumulation through the activation of TG synthesis-associated enzymes in adipocytes (Gregor and Hotamisligil, 2007). The loss of ER homeostasis is caused by ER stress-associated proteins such as eukaryotic initiation factor 2 (eIF2), activating transcription factor (ATF) family, and heme oxygenase-1 (HO-1) (Sano and Reed, 2013).

Recently, it has been suggested that HO-1 is a negative regulator of ER stress in obesity (Son et al., 2013). Moreover, the loss of HO-1 function by siRNA silencing of HO-1 resulted in an induction in the expression of C/EBPα, PPARγ, and aP2 proteins as well as an increase in the mesenchymal stem cell-committed adipocyte differentiation *in vitro*. CoPP-mediated HO-1 activation inhibited lipid accumulation *in vitro* (Vanella et al., 2013). HO-1 can bind a number of transcription factors *via* cAMP response elements binding protein (CREB) (Ozen et al., 2015). CHOP10 is a well-known ER stress response-associated protein and a crucial marker of MCE, which also has a CREB binding site (Hou et al., 2013). These data potentially suggest that activation of HO-1 might regulate CHOP10 expression and subsequently attenuate adipocyte differentiation.

Pterostilbene was found in 0.05% in *Vaccinium ashei* Reade (Ericaceae) (Rimando et al., 2004). It has diverse pharmacological activities such as antioxidant, anti-cancer, and anti-inflammatory (Briggs et al., 2016; Wang et al., 2015). Recently, a report showed partial evidence of the effects of pterostilbene on adipogenesis in 3T3-L1 cells (Hsu et al., 2012). However, the underlying mechanism of how pterostilbene suppresses adipocyte differentiation *via* the modulation of HO-1 protein in 3T3-L1 cells remains unclear. In the present study, we thus aimed to elucidate whether pterostilbene affects adipocyte differentiation through the regulation of the HO-1 protein in 3T3-L1 cells.

Materials and methods

Materials

Pterostilbene (HPLC purity, 99.8%) was purchased from Xian Yiyang Bio-Tech co. Ltd. (Shaanxi, China). The structure of pterostilbene is provided as Fig. 1(A). 3T3-L1 preadipocytes (CL-173) were purchased from ATCC (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM), bovine calf serum (BCS), foetal bovine serum (FBS), penicillin-streptomycin (P/S), insulin, and trypsin-EDTA were purchased from Gibco (Gaithersburg, MD, USA). DEX, IBMX, Oil Red O, and isopropanol were purchased from Sigma-Aldrich (St Louis, MO, USA). Antibodies specific for aP2 (#2509) were purchased from Cell Signaling Technology (Danvers, MA, USA). CHOP10 (sc-575), C/EBPβ (sc-150), C/EBPα (sc-61), HO-1 (sc-10789), GAPDH (sc-25778), PPARγ (sc-7196), Lipin1 (sc-98450), DGAT1 (sc-32861), Cyclin D1 (sc-753), Cyclin A (sc-751), p21 (sc-6246), and p27 (sc-1641) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell culture and differentiation

3T3-L1 preadipocytes were cultured in DMEM with 10% BCS, 3.7 g/l sodium bicarbonate, and 1% P/S. For the induction of adipocyte differentiation, the cells were treated with 10% FBS and MDI mixture (0.5 mM IBMX, 1.0 μM DEX, and 4 μg/ml insulin). The cells were maintained at 37 °C in 5% CO_2 incubator, and the medium was changed with 10% FBS and 4 μg/ml insulin at least once every two days.

Cell viability assay

3T3-L1 preadipocytes (1×10^4 cells/well) were incubated with DMEM with 10% BCS media overnight in 96-well plates. The 3T3-L1 cells were treated with pterostilbene (0, 1.5, 3, 6, and 12 μM) for 24 h. The growth was detected using the MTT assay. MTT reagent was added to the 96-well plate and incubated for 3 h at 37 °C. Then, the supernatant was gently eliminated, and 100 μl of DMSO was added to extract the intracellular formazan in the 3T3-L1 cells. The MTT-formazan product was measured by ELISA reader Wallac 140 victor (Perkin Elmer, Boston, MA, USA) at 570 nm.

3T3-L1 preadipocytes (2×10^4 cells/well) were incubated with DMEM with 10% BCS media overnight in 96-well plates. The 3T3-L1 cells were treated with pterostilbene (0, 1.5, 3, 6, and 12 μM) for 24 h. The growth was detected using the crystal violet (CV) assay. CV reagent was added to the 96-well plate and incubated for 30 min at 37 °C. Then, the supernatant was gently eliminated, and 100 μl of DMSO was added to extract the intracellular CV in the 3T3-L1 cells. The CV product was measured by ELISA reader Wallac 140 victor (Perkin Elmer) at 570 nm.

Oil Red O staining

Differentiated 3T3-L1 cells were fixed with 10% formaldehyde at 4 °C for 1 h. After washing with 60% isopropanol, the fixed cells were

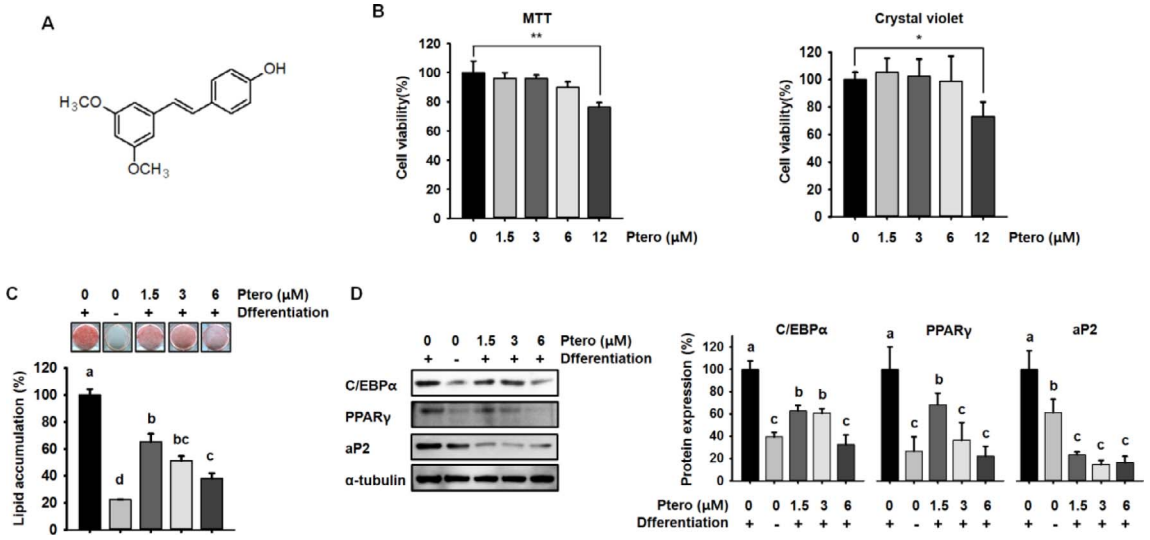

Fig. 1. Effect of pterostilbene on adipogenic differentiation through the regulation of adipogenic factors in 3T3-L1 cells. (A) Chemical structure of pterostilbene. (B) Effect of pterostlbene on cell viability in 3T3-L1 cells. (C) Lipid accumulation stained by Oil Red O treatment with the presence or absence of pterostilbene in 3T3-L1 adipocytes. (D) Representative western blot of adipogenic factors such as C/EBPα, PPARγ, and aP2 in 3T3-L1 cells. The experiment was performed in triplicate. $*p < 0.05$ and $**p < 0.01$ vs. vehicle-treated group. Values with different letters are significantly different, $p < 0.05$.