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Lucidone from *Lindera erythrocarpa* Makino fruits suppresses adipogenesis in 3T3-L1 cells and attenuates obesity and consequent metabolic disorders in high-fat diet C57BL/6 mice

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ABSTRACT

Obesity is associated with an increased risk of development of numerous diseases including type 2 diabetes, hypertension, hyperlipidemia, and cardiovascular disease. In this study, we investigated the effects of lucidone *in vitro* on gene expression during adipogenesis in 3T3-L1 cells and *in vivo* on high-fat diet induced obesity in C57BL/6 mice. Lucidone at 40 μmol/L suppressed adipogenesis in 3T3-L1 cells by reducing transcription levels of adipogenic genes, including PPARγ, C/EBPα, LXR-α, LPL, aP2, GLUT4 and adiponectin. Five-week-old male C57BL/6 mice fed a high fat diet (60% energy from fat) supplemented with lucidone at a dosage of 1250 mg/kg of diet for 12 weeks had reduced body and liver weight, reduced epididymal and perirenal adipose tissue, decreased food efficiency (percentage of weight gain divided by food intake), and lowered plasma cholesterol, triglyceride, glucose, and insulin levels. Dissection of adipose tissue from lucidone-treated mice showed a reduction in the average fat-cell size and percentage of large adipocytes. These results provide evidence that dietary intake of lucidone alleviates high fat diet-induced obesity in C57BL/6 mice and reveals the potential of lucidone as a nutraceutical to prevent obesity and consequent metabolic disorders.

Introduction

Incidence of obesity worldwide has expanded dramatically over the last two decades due to diet and lifestyle changes. Obesity is associated with an increased risk for the development of numerous diseases including type 2 diabetes, hypertension, hyperlipidemia, and cardiovascular disease (Nawrocki and Scherer 2005). The development of obesity involves extensive adipose tissue remodeling by adipocyte hypertrophy, adipocyte hyperplasia and angiogenesis (Avram et al. 2007). Adipocyte hypertrophy results from an excessive accumulation of lipids from energy intake such as a high fat (HF) diet. Changes in adipocyte number result from a complex interplay between proliferation and differentiation of preadipocytes (Gregoire 2001).

Green and colleagues established the 3T3-L1 preadipocyte cell line, which has accelerated our knowledge of the mechanism of preadipocyte differentiation, or adipogenesis, at the molecular level (Green and Kehinde 1975). Several studies have demonstrated natural compounds with a potential to suppress adipogenesis in 3T3-L1 cells and, further, prevent obesity in animal models. For example, berberine inhibits adipocyte differentiation through the PPARγ pathway and reduces the expression of adipogenic enzymes (Choi et al. 2006; Huang et al. 2006). In addition, berberine reduces body weight gain and blood glucose in HF diet induced obesity mice (Xie et al. 2011) and decreased blood lipid level both in obese individuals and in SD rats (Hu et al. 2012). Curcumin suppresses preadipocyte differentiation and retards body weight gain in diet induced obesity (DIO) mice (Ejaz et al. 2009) *via* attenuating lipogenesis in the liver (Shao et al. 2012). Epigallocatechin gallate (EGCG) inhibits the adipocyte differentiation process *via* activating AMP-activated protein kinase (Hwang et al. 2005) and inhibiting the PPARγ pathway (Lin et al. 2005), further alleviating fatty liver disease in DIO mice (Bose et al. 2008). These studies

Abbreviations: PPARγ, peroxisome proliferator-activated receptor γ; C/EBP, CCAAT-enhancer-binding protein; LXR-α, liver X receptor α; LPL, lipoprotein lipase; aP2, adipocyte protein 2; GLUT4, glucose transporter type 4; ND, normal diet; HFD, high fat diet; LSH/L, lucidone-supplemented HFD at the lower dosage, 0.025%; LSH/H, lucidone-supplemented HFD at the higher dosage, 0.125%; DIO, diet-induced obesity.

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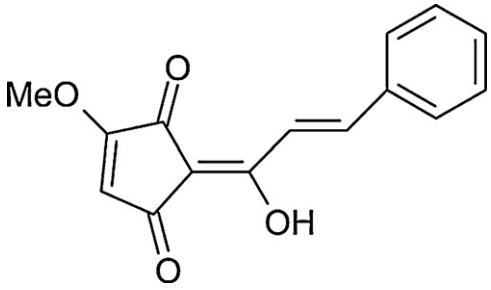

Fig. 1. Chemical structure of lucidone.

suggest that natural products that suppress adipogenesis in 3T3-L1 cells may have bioactivity *in vivo* that prevents body weight gain under unhealthy eating habits.

Lucidone (Fig. 1) is one of the major constituents of the fruit of *Lindera erythrocarpa* Makino (Wang et al. 2008). The fruit is used as a folk medicine for analgesic, digestive, diuretic, antidote and antibacterial purposes. Our previous studies have showed the anti-inflammatory effects of lucidone in RAW 264.7 cells (Kumar and Wang 2009) and ICR mice (Senthil Kumar et al. 2010). Hepatoportective effect against alcohol-induced oxidative stress in human hepatic cells (Senthil Kumar et al. 2012). The novel bioactivity of lucidone was investigated in this study; we determined the effects of lucidone on adipocyte differentiation process at the molecular level in 3T3-L1 cells, and the effects of dietary lucidone in high fat diet-fed C57BL/6 mice on body weight changes, physiological and metabolic variables and adipocyte size distribution.

Materials and methods

Phytocompound

Lucidone was prepared from *L. erythrocarpa* according to previously described protocols (Wang et al. 2008). The purity of the compound obtained was higher than 99.5% based on the results of HPLC and ^{1}H NMR analyzed.

Cell culture and stimulation

The 3T3-L1 cells were purchased from the Bioresource Collection and Research Center (Hsinchu, Taiwan). Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% bovine serum (BS), 100 units/ml penicillin and 100 μg/ml streptomycin (GIBCO, Invitrogen, Carlsbad, CA) at 37 °C in a 5% CO_2-humidified incubator. Cell differentiation was stimulated with a mixture of 1 μmol/l dexamethasone, 0.52 mmol/l isobutylmethylxanthine and 0.17 μmol/l insulin (Sigma–Aldrich) starting at 2 days postconfluence (designated as day 0) for 4 days (day 1 to day 4). The stimulation medium was then replaced with maintenance medium (DMEM supplemented with 10% FBS) for another 4 days (day 5 to day 8). To evaluate the effect of lucidone on adipogenesis, the indicated concentrations of lucidone were added at day 0 and day 4 when refreshing culture medium.

Oil Red O staining

The 3T3-L1 adipocytes were washed with PBS and then fixed with 4% paraformaldehyde for 1 h at room temperature. The supernatant was discarded and washed with deionized water. Cells were stained for 10 min at room temperature with freshly prepared 0.3% Oil Red O solution. To quantify the relative lipid content accumulated in cells, Oil Red O dye was eluted with 100% isopropanol and incubated for 10 min with gentle shaking and the absorbance at 510 nm was detected using an ELISA microplate reader (μQuant, Bio-Tek Instruments, Winooski, VT). The results were confirmed by three independent experiments.

Table 1
Sequences of primers used for qRT-PCR.

18S forward	5′-CGC CGC TAG AGG TGA AAT TCT-3′
18S reverse	5′-CAT TCT TGG CAA ATG CTT TCG-3′
PPARγ forward	5′-CAA GAA TAC CAA AGT GCG ATC AA-3′
PPARγ reverse	5′-GAG CTG GGT CTT TTC AGA ATA ATA AG-3′
C/EBPα forward	5′-AGC AAC GAG TAC CGG GTA CG-3′
C/EBPα reverse	5′-TGT TTG GCT TTA TCT CGG CTC-3′
LPL forward	5′-GGC CAG ATT CAT CAA CTG GAT-3′
LPL reverse	5′-GCT CCA AGG CTG TAC CCT AAG-3′
Adiponectin forward	5′-TCC TGG AGA GAA GGG AGA GAA AG-3′
Adiponectin reverse	5′-TCA GCT CCT GTC ATT CCA ACA T-3′
aP2 forward	5′-AGT GAA AAC TTC GAT GAT TAC ATG AA-3′
aP2 reverse	5′-GCC TGC CAC TTT CCT TGT G-3′
GLUT4 forward	5′-GAT TCT GCT GCC CTT CTG TC-3′
GLUT4 reverse	5′-ATT GGA CGC TCT CTC TCC AA-3′
LXR-α forward	5′-AGG AGT GTC GAC TTC GCA AA-3′
LXR-α reverse	5′-CTC TTC TTG CCG CTT CAG TTT-3′

Quantitative real-time PCR analysis

Cellular RNA was extracted from 3T3-L1 cells after MDI induction at different time points, 0.5 h, 1 day, 2 days, 4 days, 6 days and 8 days, using TRIzol reagent according to the manufacturer's instructions (Invitrogen). The complementary DNA was synthesized using high-capacity cDNA reverse transcription kits (Applied Biosystems, Carlsbad, CA) according to the manufacturer's instructions. Gene expression levels were analyzed using StepOne Real-Time PCR System (Applied Biosystems). The analyses for the genes including β-actin (Mm00607939_s1), C/EBPβ (Mm00843434_s1) and C/EBPδ (Mm00786711_s1) were conducted using Hot Start Fluorescent PCR ROX Core Kit (Tagman) (BIO Basic, Canada). The complementary DNA was denatured at 94 °C for 4 min, followed by 40 cycles of PCR (94 °C, 30 s; 60 °C, 30 s). The analyses for the genes described in Table 1 were conducted using Power SYBR Green PCR Master Mix (Applied Biosystems). The complementary DNA was denatured at 95 °C for 10 min, followed by 40 cycles of PCR (95 °C, 15 s; 60 °C, 60 s). β-Actin and 18S ribosomal RNA were used as the endogenous control independently in the comparative cycle-threshold method.

Animal maintenance and experimental setup

Three-week-old male C57BL/6 mice were obtained from Bio-LASCO (Taiwan). The mice were housed in groups of 4 per cage, under standard temperature-controlled conditions with a 12 h/12 h light-dark cycle and free access to food and water throughout the experiments. All animal experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals and Taiwan laws relating to the protection of animals, and were approved by the local ethics committee. Before the experiments, mice were fed with certified rodent diet provided energy 14.4 kJ/g which is 24.1% from protein, 13.2% from fat and 62.7% from carbohydrates (5002, LabDiet, St. Louis, Missouri, USA) for 2 weeks. The micronutrient and macronutrient compositions were previously described (Keenan et al. 1997). At the beginning of the experiment, mice were randomly divided into 4 groups of 8 mice each. Three experimental groups were fed with a high-fat diet (HFD) (DIO rodent purified diet w/60% energy from fat, 58Y1, TestDiet) which provided 21.4 kJ/g of energy that was 18.1% from protein, 61.6% from fat and 20.3% from carbohydrates (Table 2) with or without dietary lucidone supplementation at two dosages, 250 mg/kg of diet (LSH/L, lucidone-supplemented HFD at the lower dosage, 0.025%) and 1250 mg/kg of diet (LSH/H, lucidone-supplemented HFD at the higher dosage, 0.125%). The control group was fed with certified