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Preventive and ameliorating effects of citrus D-limonene on dyslipidemia and hyperglycemia in mice with high-fat diet-induced obesity

Li Jing [a,b], Yu Zhang [c], Shengjie Fan [c], Ming Gu [c], Yu Guan [c], Xiong Lu [d], Cheng Huang [c,**], Zhiqin Zhou [a,b,*]

[a] College of Horticulture and Landscape Architecture, Southwest University, Chongqing 400716, China
[b] Key Laboratory of Horticulture Science for Southern Mountainous Regions, Ministry of Education, Chongqing 400715, China
[c] Drug Discovery Laboratory, School of Pharmacy, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China
[d] Scientific Experimental Center, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, China

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ABSTRACT

D-limonene is a major constituent in citrus essential oil, which is used in various foods as a flavoring agent. Recently, D-limonene has been reported to alleviate fatty liver induced by a high-fat diet. Here we determined the preventive and therapeutic effects of D-limonene on metabolic disorders in mice with high-fat diet-induced obesity. In the preventive treatment, D-limonene decreased the size of white and brown adipocytes, lowered serum triglyceride (TG) and fasting blood glucose levels, and prevented liver lipid accumulations in high-fat diet-fed C57BL/6 mice. In the therapeutic treatment, D-limonene reduced serum TG, low-density lipoprotein cholesterol (LDL-c) and fasting blood glucose levels and glucose tolerance, and increased serum high-density lipoprotein cholesterol (HDL-c) in obese mice. Using a reporter assay and gene expression analysis, we found that D-limonene activated peroxisome proliferator-activated receptor (PPAR)-α signaling, and inhibited liver X receptor (LXR)-β signaling. Our data suggest that the intake of D-limonene may benefit patients with dyslipidemia and hyperglycemia and is a potential dietary supplement for preventing and ameliorating metabolic disorders.

1. Introduction

The prevalence of obesity is increasing rapidly among adults, children and adolescents in areas in which high dietary fat intake is common (Canbakan et al., 2008). Obese individuals are at particular risk of type 2 diabetes, hyperlipidemia, cardiovascular problems and certain cancers (Wang et al., 2011; Yun, 2010). Recently, obesity has become a growing social problem (Ahima, 2011), and the treatment of obesity-related illness represents a significant expenditure in the national healthcare budgets in developed countries (Seidell, 1995). Given the low efficacy and side effects of drugs, much attention has been paid to dietary prevention and therapy for obesity and related disorders.

D-limonene (1-methyl-4-(1-methylethenyl) cyclohexane) is a monocyclic monoterpene that is mainly present in the citrus essential oils with lemon-like odors (Sun, 2007). D-limonene is listed in the Code of Federal Regulations as generally recognized as safe (GRAS) as a flavoring agent and is used in common foods such as fruit juices, soft drinks, baked foods, ice cream and desserts. In humans, D-limonene has demonstrated low toxicity after repeated dosing for up to 1 year (Sun, 2007). Previous studies have shown that D-limonene inhibits lipid peroxidation and prevents free radical-induced damage (Pandima Devi et al., 2004), physical and psychological stress (Fukumoto et al., 2008), and stress-induced hypertension (Kawakami et al., 2004; Shibata et al., 1991). D-limonene has also been reported to have important biological activities, such as antioxidant properties (Clegg et al., 1980; Clegg et al., 1982; Yang et al., 2010), anti-inflammatory activities (Bakkali et al., 2008) and chemopreventive or chemotherapeutic properties against several types of cancer (Miller et al., 2011; Sun, 2007).

It has recently been reported that D-limonene significantly reduced blood glucose in streptozotocin (STZ)-induced diabetic rats (Shu, 2010), and alleviated insulin resistance in rats with high-fat diet and N^{w}-nitro-L-arginine methyl ester (L-NAME)-induced nonalcoholic fatty liver disease (Santiago et al., 2012). However, no study has yet reported on the effect of D-limonene on obesity or obesity-related dyslipidemia and hyperglycemia.

* Corresponding author at: College of Horticulture and Landscape Architecture, Southwest University, Chongqing 400716, China. Tel.: +86 023 682 502 29; fax: +86 23 682 512 74.
** Corresponding author. Tel.: +86 21 513 22 182; fax: +86 21 513 221 93.
E-mail addresses: chuang.shutcm@gmail.com (C. Huang), zzqswu@yahoo.com (Z. Zhou).

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Recently, emerging evidences have shown that nuclear receptor transcription factors such as peroxisome proliferator-activated receptors (PPARs) and liver X receptors (LXRs) are the targets of many citrus-derived compounds for the treatment of metabolic diseases (Goldwasser et al., 2010; Kim et al., 2012a; Kumar Sharma et al., 2011). It has been reported that citrus naringin decreased serum lipid through the increase of PPARγ expression and inhibition of LXR expression in the liver of type 2 diabetic (Kumar Sharma et al., 2011). To our knowledge, there is no study reporting the role of D-limonene in PPAR or LXR signaling regulation.

The purpose of this study was to find out whether D-limonene is a potential dietary supplement for preventing and ameliorating metabolic disorders. We studied the effects of D-limonene on dyslipidemia and hyperglycemia in mice with high-fat diet-induced obesity. In addition, we investigated whether D-limonene is effective in regulating peroxisome proliferator-activated receptor (PPAR)-α signaling, and liver X receptor (LXR)-β signaling. For the first time, we reported on the effect of D-limonene in preventing and ameliorating metabolic disorders in high-fat diet-induced obese mice.

2. Materials and methods

2.1. Chemicals

D-limonene (98% purity) was purchased from Sigma (St. Louis, MO, USA) and dissolved in DMSO (0.1%). The other citrus-derived compounds (Table 1) were obtained from Sigma (St. Louis, MO, USA) or from Nanjing Traditional Chinese Medicine Institute (Nanjing, China), and were dissolved in DMSO (0.1%). Rosiglitazone, WY14643 and GW3965 were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Pre-adipocyte 3T3-L1 cells (obtained from ATCC) were cultured and kept in DMEM containing 10% fetal bovine serum (HyClone, Logan, UT). For adipocyte differentiation, the cells were grown in 24-well plates to full confluence for 2 days and then induced with differentiation medium (DM) containing 10 μg/ml insulin (Sigma, St. Louis, MO), 50 μM dexamethasone (Sigma, St. Louis, MO), and 0.8 mM isobutylmethylxanthine (IBMX, Sigma, St. Louis, MO). After 4 days of induction, the media was refreshed with DMEM containing 10% fetal bovine serum for differentiation at 37 °C under a 10% CO_2 atmosphere. DMSO-dissolved citrus-derived compounds were added separately to the medium at the indicated concentrations.

Table 1
Effects of 21 citrus-derived compounds on the differentiation of 3T3-L1 adipocyte.

No.	Name	Concentration (μM)	Effect
1	Naringenin	50	−
2	Naringin	50	±
3	Hesperidin	50	±
4	Diosmin	50	±
5	Neohesperidin	50	±
6	Poncirin	50	±
7	Quercetin	50	±
8	Hesperetin	50	+
9	Rutin	50	Death
10	Nobiletin	50	±
11	Limettin	50	+
12	Narirutin	50	±
13	Tangeritin	50	+
14	Neoeriocitrin	50	±
15	D-limonene	50	−
16	Nerol	50	Death
17	Geraniol	50	Death
18	Auraptene	50	−
19	Isobergapten	50	± ±
20	Sanguinarine citrate	50	Death
21	Neosperidindihydrochalcone	50	±

− inhibition, + promotion, ± ineffective.

2.3. Oil red O staining

The 3T3-L1 cells were fixed with 10% formalin at room temperature for 10 min and then stained with oil *red* O (Sigma-Aldrich) at 60 °C for 15 min after washing with PBS. Photographs were taken using an Olympus microscope (Tokyo, Japan).

2.4. Animals and diets

All animals received care according to the institutional guidelines approved by Shanghai University of Traditional Chinese Medicine. Female C57BL/6 mice (10–12 weeks old) were obtained from the SLAC Laboratory (Shanghai, China). For acclimatization, all mice were housed in a temperature-controlled (23 °C ± 2 °C) facility with relative humidity of 45% ± 5% under a 12-h light-dark cycle and had free access to standard diet (containing protein 21.1%, fat 5.1%, carbohydrate 60.0%, fiber 3.9%, minerals 7.9%, and vitamins 2.0%, w/w) and water.

After the acclimatization period, the female C57BL/6 mice were divided into three groups ($n=6$) for the prevention study, and were given a low-fat diet (LFD, 10% calories from fat, 20% calories from protein, 70% calories from carbohydrate; Research Diets, D12450B) or a high-fat diet (HFD, 60% calories from fat, 20% calories from protein, 20% calories from carbohydrate; Research Diets, D12492), or HFD enriched with D-limonene (0.5% w/w), ad libitum for 4 weeks. Food intake and body weight were measured every other day. In the therapeutic study, the female C57BL/6 mice were fed the HFD for 12 weeks to induce obesity, and then 12 of these obese mice ($n=6$/group) were randomized to receive the HFD, or HFD supplemented with 0.5% D-limonene for another 2 weeks, with another six normal mice fed low-fat diet as control group. Body weight and daily food intake were measured every day.

The dose of D-limonene used in this study was determined based on previous animal and human studies (Sun, 2007; Santiago et al., 2012). 0.5% D-limonene was used to provide the daily dose of about 0.6 g/kg body weight, a feasible daily dose for humans (Vigushin et al., 1998).

2.5. Intraperitoneal glucose tolerance test

C57BL/6 mice were fasted overnight (12 h), and blood samples were obtained from the tail vein of each mouse to determine the baseline glucose levels (0 min). Each mouse was then given an intraperitoneal injection of glucose (1 g/kg), and additional blood samples were collected at regular intervals (15, 30, 60, and 90 min) for glucose measurement. The glucose tolerance test area under the curve (AUC) was calculated according to the previous study (Pruessner et al., 2003), by using the formula termed 'area under the curve respect to ground'.

2.6. Serum chemistry analysis

Mice were fasted for 12 h prior to gathering blood samples to be used for the serum chemistry analysis. The heart blood serum (50 μl) was collected and serum triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-c) and low-density lipoprotein cholesterol (LDL-c) were measured using a Hitachi 7020 Automatic Analyzer (Hitachi, Ltd., Tokyo, Japan).