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·Research articles·

Petroleum ether sub-fraction of rosemary extract improves hyperlipidemia and insulin resistance by inhibiting SREBPs

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[ABSTRACT] As a culinary and medicinal herb, rosemary is widely used. The present work aimed to investigate the effects of rosemary extracts on metabolic diseases and the underlying mechanisms of action. Liver cells stably expressing SREBP reporter were used to evaluate the inhibitory effects of different fractions of rosemary extracts on SREBP activity. The obese mice induced by Western-type diet were orally administered with rosemary extracts or vehicle for 7 weeks, the plasma and tissue lipids were analyzed. SREBPs and their target genes were measured by quantitative RT-PCR. We demonstrated that the petroleum ether sub-fraction of rosemary extracts (PER) exhibited the best activity in regulating lipid metabolism by inhibiting SREBPs, while water and *n*-BuOH sub-fraction showed the SREBPs agonist-effect. After PER treatment, there was a significant reduction of total SREBPs in liver cells. PER not only decreased SREBPs nuclear abundance, but also inhibited their activity, resulting in decreased expression of SREBP-1c and SREBP-2 target genes *in vitro* and *in vivo*. Inhibiting SREBPs by PER decreased the total triglycerides and cholesterol contents of the liver cells. In the mice fed with Western-type diet, PER treatment decreased TG, TC, ALT, glucose, and insulin in blood, and improved glucose tolerance and insulin sensitivity. Furthermore, PER treatment also decreased lipid contents in liver, brown adipose tissue, and white adipose tissue. Our results from the present study suggested that petroleum ether fraction of rosemary extracts exhibited the best potential of improving lipid metabolism by inhibiting SREBPs activity.

[KEY WORDS] Rosemary; SREBPs; Hyperlipidemia; Insulin resistance

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Introduction

Sterol regulatory element-binding proteins (SREBPs) are a family of transcription factors which regulate lipid metabolism [1]. There are three members, SREBP-1a, SREBP- 1c, and SREBP-2 [2]. SREBP-1a is only present in culture cells and not expressed in the liver *in vivo* [3]. SREBP-1c is the master regulator of fatty acid *de novo* synthesis and glucose metabolism mediated by insulin signaling [4], while SREBP-2 mainly regulates cholesterol synthesis. Due to the important role SREBPs play in regulating lipid metabolism, SREBPs become potent therapeutic targets for lipid metabolism disorders, such as hyperlipidemia, fatty liver, atherosclerosis, and diabetes [5].

Herbal medicines are important sources for anti-obesity drug discovery [6]. Edible food or herbs used as food additives are gaining intensive attention for the treatment of metabolic diseases, due to their safety profiles. Rosemary (*Rosmarinus officinalis* L. (Labiatae)) is a Mediterranean herb for both culinary and medicinal purposes. Multiple biological activities of rosemary have been reported [7-9]. Rosemary has been used for the treatment of diabetes [10-12], showing remarkable lipid lowering effects in rats [13]. Although many active components in rosemary have been identified, such as phenolic acids [14], flavonoids [15], terpenoids [16], and volatile components [17], the picture of active components contributing the hypoglycemia and hypolipidemia effects as well as their mechanisms, is still blurry. Carnosic acid-rich rosemary extract has been shown to reduce fasting glycaemia and plasma cholesterol levels by

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downregulating hepatic lipogenic genes [18]. As SREBPs play a central role in lipid metabolism, we wondered whether the hypoglycemia and hypolipidemia effects of rosemary may be through inhibition of SREBP pathways.

Many active components in rosemary have been identified; it seems that both water extracts and ethanol extracts exert significantly recovery of diabetic phenotypes [11]. Thus, a further comprehensive insight into components and activity of rosemary, as well as their molecular mechanism is required.

In the present study, we aimed to combine separation procedures with target based activity readout to quickly identify active components from rosemary. Using SREBP reporter cell lines, we were able to identify petroleum ether fraction of rosemary extracts with the best potential of lipogenic inhibition. Furthermore, we showed that petroleum ether fraction of rosemary exerted efficacy of treating lipid metabolic disorders *in vivo* by inhibiting SREBP activities.

Materials and Methods

Reagents

The following reagents were used in the present study: Primary antibodies of SREBP-1 (Santa Cruz, Dallas, American) and 2 (Abcam, San Francisco, American); Ethanol, petroleum ether, ethyl acetate, and n-butyl alcohol (Shanghai Ling Feng Chemical Reagent Co., Ltd., Shanghai, China); acetonitrile (High performance liquid chromatography (HPLC) grade, Mass spectrum grade, Merck, Darmstadt, Germany); dimethyl sulfoxide (DMSO, Biotech grade, 99.98%, Sigma-Aldrich, Saint Louis, American); HL-7702 and HepG2 liver cells (Keygen Biotech, Nanjing, China); Dulbecco's modified Eagle's medium (Cellgro-Corning, New York, American. Glucose (Sigma ; Fetal bovine serum (Gibco, Massachusetts, American; LPDS (Sigma- Aldrich)); Lovastatin (Aladdin, Beijing, China). 25-Hydroxycholesterol (Sigma-Aldrich); SYBR Green (Roche, Basel, Switzerland); Lipofectamine 2000 (Invitrogen, Massachusetts, American); Penicillin and Streptomycin sulfate (Gibco); Hygromycin B (Roche). Hoechst 33342 (Sigma-Aldrich); Luciferase assay kit (Promega, Wisconsin, American); Enhanced BCA Protein Assay Reagent (Beyotime, Shanghai, China); Triglycerides (TG) and total cholesterol (TC) assay kit (Applygen, Beijing, China).

Plant materials and extraction

Dried aerial parts of rosemary (*Rosmarinus officinalis* L. (Labiatae)) were purchased in Anhui province, China and identified by Professor Li Ping, and a voucher specimen (Xie 201401) was deposited at the Herbarium of Department of Pharmacognosy, China Pharmaceutical University, Nanjing, China. To prepare the extracts, dried aerial parts of rosemary (600 g) were reflux extracted by 6 L of 95% ethanol at 85 °C for 1 h, thrice. The extract was filtered and the filtrate was collected for evaporation to get crude ethanol extract, which . was then freeze-dried and dissolved in water. For further separation, petroleum ether and ethyl acetate were added in a 1 : 1 (*V*/*V*) ratio, after 3-times extraction, different sub- fractions of the plant were collected to get the petroleum ether sub-fraction (PER) and ethyl acetate fraction (EtOAc). The remaining aqueous layer was collected and further sub- fractionated by *n*-butyl alcohol to get water fraction (Water) and *n*-butyl alcohol fraction (*n*-BuOH). All the sub-fractions collected were then freeze-dried.

Chemical analysis of PER by HPLC-ESI-Q-TOF-MS

For HPLC analysis, 10 mg of rosemary powders were dissolved in 1 mL of 95% ethanol, and centrifuged at 12 000 r·min^{-1} for 10 min. The supernatants were collected and analyzed on GP-C_{18} (4.6 mm × 250 mm, 5 μm, Sepax) column in an Agilent 1260 HPLC system (American) with a VWD detector. The UV wavelength was set at 280 nm, and the sample injection volume was 10 μL. The mobile phase was consisted of (A) 0.1% formic acid and (B) acetonitrile. The linear gradient elution was optimized as follows: 10% B (0 min), 30% B (5 min), 50% B (10 min), 80% B (15 min), and 100% B (25-100 min). The flow rate was at 0.8 mL·min^{-1}.

For mass spectrum analysis, 10 mg of PER powders were dissolved in 1 mL of 95% ethanol, and centrifuged at 12 000 r·min^{-1} for 10 min. The supernatants were analyzed on Sepax GP-C_{18} (4.6 mm × 250 mm, 5 μm) column in Agilent 1290 UPLC-6520-Q-TOF-MS and the sample injection volume was 1 μL. The mobile phase was consisted of (A) 0.1% formic acid and (B) acetonitrile. The linear gradient elution was optimized as follows: 10% B (0 min), 30% B (5 min), 50% B (10 min), 80% B (15 min), and 100% B (25−30 min). The flow rate was at 0.8 mL·min^{-1}. The conditions of ESI sources were as follows: dry gas temperature 350 °C; flow rate 10 L·min^{-1}; nebulizer 35 psig; dual ESI, capillary 3 500 V, Chamber fragmentor 120 V, skimmer 65 V; and OCTRFV, 750 V; and the mass range was set at *m/z* 100−3 000. To optimize signals and obtain maximal structural information, the collision energy (CE) was adjusted from 20 V to 70 V for MS/MS experiments. All operations, acquisition, and analysis of date were monitored by Agilent LC-Q-TOF-MS Mass Hunter Acquisition Software Version A.01.05 (Agilent Technologies, Santa Clara, CA, USA) and operated under Mass Hunter Acquisition Software Version B.02.00 (Agilent Technologies).

Cell culture

HL-7702 and HepG2 cells were purchased from Keygen Biotech and ATCC. The cells were cultured at 37 °C in a 5% CO_2 incubator (Thermo). Under normal conditions, the cells were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum supplemented with 100 units/mL of penicillin and 100 g·mL^{-1} of streptomycin sulfate. Under the sterol-depleted condition, the cells were cultured with low sterols medium as described previously [19-20].

Generation of HL-7702/SRE-Luc reporter cell lines and luciferase assay

Three tandem SREBP binding sites 5′-AAAATCAC CCCACTGCAAACTC CTCCCCCTGC-3′ were inserted into pGL4.26 by primer annealing to generate SREBP reporter