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

[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...
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, 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); Dubelcco’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 bovine serum (Gibco, Massachusetts, American; LPDS (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-C₁₈ (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-C₁₈ (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% CO₂ 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'-AAAAATCAC CCCACTGCAAACCTC TCCCCCCTGC-3' were inserted into
pGL4.26 by primer annealing to generate SREBP reporter plasmids, termed as pSRE-Luc. HL-7702 cells were transfected with pSRE-Luc with lipofectamine 2000. 24 h later, the cells were switched to culture medium with 200 μg·mL⁻¹ of hygromycin B. Fresh medium was exchanged every 3 days until colonies formed after about 2 weeks. Individual colonies were visually identified and isolated with cloning cylinders and named as HL-7702/SRE-Luc cells. 20 000 HL-7702/SRE-Luc cells were seeded into 96-well plates using normal culture medium. After 24 h, HL-7702/SRE-Luc cells were incubated with low steroid medium with drugs or DMSO for additional 18 h. The luciferase activity was measured using luciferase assay kit and normalized by the concentration of total proteins using Enhanced BCA Protein Assay Reagent (Beyotime, Shanghai, China).

**Assays for cell viability, triglycerides, and total cholesterol**

After 18-h treatment, the cell viability was assessed by MTT. Triglycerides (TG) and total cholesterol (TC) were measured using commercial kit (Applygen, Beijing, China). 20 000 cells/well were seeded into 6-well plates. After 18 h, the cells were starvation in serum free medium for 6 h, and then colonies formed after about 2 weeks. Individual colonies were visually identified and isolated with cloning cylinders and named as HL-7702/SRE-Luc cells. 20 000 HL-7702/SRE-Luc cells were seeded into 96-well plates using normal culture medium. After 24 h, HL-7702/SRE-Luc cells were incubated with low steroid medium with drugs or DMSO for additional 18 h. The luciferase activity was measured using luciferase assay kit and normalized by the concentration of total proteins using Enhanced BCA Protein Assay Reagent (Beyotime, Shanghai, China).

**Immunofluorescent staining of SREBP*s**

HepG2 cells were plated on glass coverslips, after 24-h treatment of DMSO, 25-hydroxycholesterol (25-HC) or different concentrations of PER, the cells were fixed with 4% PFA and permeabilized with 0.2% TritonX-100/PBS (PBST). Primary antibodies and secondary antibody were applied to stain SREBP-1 and -2, respectively. Cell nuclei were counterstained with Hoechst 33342. Images were captured with Carl Zeiss LSM 700 system.

**Animals and treatments**

All the mice were housed in animal rooms with constant temperature in humidity with a 12 h/12 h light/dark cycle and were allowed access to water and food ad libitum. Six-week-old male C57BL/6 mice (weighing 18–20 g, purchased from Yangzhou University, China) were fed with Western type diet (WD) or normal chow diet (NCD) for 6 weeks. WD was the normal chow diet supplemented with 20% fat, 1.25% cholesterol, and 0.5% cholic acid W/W as described before [20]. Before the formal experiments, we performed preliminary experiments to select the dose. WD-fed mice were gavaged with vehicle, lovastatin (60 mg·kg⁻¹·d⁻¹), or different concentrations of PER (75 and 150 mg·kg⁻¹·d⁻¹) daily for 7 weeks. At the 6th week, oral glucose tolerance test (OGTT) and insulin tolerance test (ITT) were performed. At 7th week, the animals were sacrificed, the blood and tissue samples were collected. All experiments and animal care were conducted in accordance with the Provision and General Recommendation of Chinese Experimental Animals Administration Legislation and were approved by the Science and Technology Department of Jiangsu Province (license number: SYXK(Su) 2012-0005).

**Plasma, liver, and biochemical analysis**

After fasting overnight, all the animals were sacrificed. Plasma insulin levels were quantified with the Mouse Insulin Elisa kit (CUSABIO, Wuhan, China) according to the manufacturer’s instructions. TG, TC, high-density lipoprotein cholesterol (HDL-c), low-density lipoprotein cholesterol (LDL-c), alanine aminotransferase (ALT), and aspartate transaminase (AST) were quantified using commercial kits (Shanghai Kehua Bio-engineering Co., Ltd., Shanghai, China). Liver tissues were homogenized, and TG and TC were measured according to the manufacturer’s instructions respectively (Applygen, China).

**RNA extraction and real-time PCR**

Total RNA was extracted from the cells or liver tissues with trizol reagent (Invitrogen), and complementary DNA was synthesized from 0.5 μg of total RNA using reverse transcription kit (Vazyme, Nanjing, China). Quantitative real-time PCR was performed on the Roche light cycler 96 using SYBR Green I master mix (Roche). Mouse GAPDH Green I master mix (Roche). Mouse GAPDH was used as internal control. The procedures were as described previously [20]. All the assays were done in triplicate and normalized to GAPDH levels. Primer sets were designed from primer bank [21].

**Statistical analysis**

The results are expressed as means ± SEM. The data were compared by Student’s t-test. The differences were considered statistically significant when \( P < 0.05 \).

**Results**

**Petroleum ether fraction of rosemary exerts the best SREBP inhibitory effect**

We first evaluated the bioactivity of rosemary extract in liver cells. Indeed, we found that extracts of rosemary decreased SREBP reporter activity significantly, in dose- and time-dependent manner, without obvious cytotoxicity. Extracts of rosemary also decreased triglycerides and cholesterol contents dose-dependently in the liver cells. We next asked which sub-fraction contributed primarily to the SREBP inhibition. Using traditional extraction method, we separated rosemary extracts into various sub-fractions, in accordance with the polarity. The effects on SREBP activity of different sub-fractions were compared with original total extract. Total extract decreased SREBP...

reporter activity about 49%. EtOAc sub-fraction inhibited SREBPs reporter activity slightly, about 16% of reduction. The petroleum ether sub-fraction of rosemary (PER) had the strongest inhibition (about 55% of reduction), even significantly stronger than the total extract (Fig. 1a). Interestingly, n-BuOH sub-fraction and water sub-fraction increased 17% and 19% of SREBP reporter activity, respectively (Fig. 1a). Since PER gave the strongest inhibition of SREBP activity in the following experiments, we focused on this specific sub-fraction. We then confirmed the physiological effects of PER on lipid metabolism. It was shown that PER down-regulated SREBP reporter activity in dose- (Fig. 1b) and time- (Fig. 1c) dependent manner. As expected, PER reduced triglycerides (Fig. 1e) and cholesterol (Fig. 1f) contents dose-dependently. Collectively, PER contained the most active components which reduced lipid contents in liver cells and inhibited SREBPs activity.

![Fig. 1](image)

To further investigate the components in PER, we performed HPLC-ESI-Q-TOF/MS to identify active compounds. As a result, most of the compounds in PER were diterpenoids.

To investigate how PER affected SREBPs activity, we performed immunofluorescence staining to characterize the abundance and sub-cellular localization of SREBPs. As shown in Fig. 2, the liver cells displayed less SREBP-1 and 2 upon PER treatment, and the inhibitory effects appeared to be dose dependent. 25-HC blocks SREBPs processing in ER and Golgi, thus prevents the translocation from cytoplasm to nucleus [22-23]. Apparently, the function mechanism of PER was different from 25-HC. Since we observed that the majority of SREBPs were in nucleus, it was unlikely that PER affected SREBPs processing in ER or Golgi. The mRNA level of both SREBP-1c and SREBP-2 were dramatically reduced upon PER treatment (Figs. 3a and 3c), suggesting that the PER inhibited SREBPs, at least in part, by down-regulating their gene expression.

PER reduces the expressions of SREBPs and their downstream target genes in liver cells

To test whether PER affects lipid homeostasis related gene expression, we analyzed the mRNA levels of HL-7702 cells after PER treatment. As shown in Fig. 3a, 25-HC increased SREBP-1c expression. Notably, PER treatment decreased SREBP-1c mRNA to about 40% relative to solvent control, in a dose-dependent manner (Fig. 3a). The expression...
of FASN, one of the target genes of SREBP-1c, was also reduced similarly as SREBP-1c. Next, we quantified other SREBP-1c target genes involved in fatty acid synthesis. Quantitative RT-PCR results showed that the expressions of ACC2, SQLE, SCD-5, ACLY were reduced significantly upon PER treatment, consistent with SREBP-1c level (Fig. 3b). We further investigated cholesterol synthesis genes which are regulated by SREBP-2, and found PER down-regulated SREBP-2 and most of its target genes (Fig. 3c). These data indicated that not only SREBP-1c and SREBP-2 per se, but also their transcription activity decreased upon PER treatment. These data were in consistent with results showing that PER decreased cellular lipid levels (Figs. 1e and 1f).

PER ameliorates dyslipidemia in diet induced obese mice

We next investigated whether PER exhibited similar effects in vivo. Obesity was induced by feeding with Western-type diet for 7 weeks. Meanwhile, C57BL/6 mice were gavaged at the same time with vehicle, lovastatin (60 mg·kg⁻¹·d⁻¹), or different concentrations of PER (75 or 150 mg·kg⁻¹·d⁻¹) for 7 weeks. Both lovastatin and PER failed to affect food intake (data not shown). We also observed less body weight gain by either lovastatin or PER (data not shown). Lipids contents in plasma and tissues were measured. As shown in Fig. 4, Western-type diet dramatically increased total TC and TG levels. Either lovastatin or PER administration caused significant decreases in TC and TG. We further measured high density lipoprotein-cholesterol (HDL-c) and low density lipoprotein-cholesterol (LDL-c) in plasma and, similar to lovastatin, PER efficiently enhanced serum HDL-c and decreased LDL-c in WD-fed obese mice (Figs. 4c and 4d).

**Fig. 2** PER treatment reduces SREBPs expression and abundance in nucleus

HepG2 liver cells were treated with different concentration of PER for 18 h, cells were then fixed with 4% PFA. SREBP-1 and SREBP-2 were stained with corresponding primary antibodies. PER, petroleum ether sub-fraction of rosemary; scale bar, 20 μm

**Fig. 3** PER down-regulates mRNA of lipid synthesis genes in HL-7702 cells

(a) Effect of PER on mRNA of SREBP-1c and its target genes FASN and ACC1. (b) Effect of PER on expression of fatty acids synthesis genes in liver cells. (c) Effect of PER on expression of SREBP-2 and its target genes involved in cholesterol synthesis. PER, petroleum ether sub-fraction of rosemary; Data are expressed as mean ± SEM, n = 3, and significant differences compared with DMSO are indicated by *P < 0.05, **P < 0.01 and ***P < 0.001 (assessed by Student’s t-test)
Western-type diet induced intensive lipids deposition in various tissues, including liver, whiter adipose tissue, and brown adipose tissue. The most commonly used indicators for hepatic and extra hepatic tissue damage are the alanine aminotransferase (ALT) and aspartate aminotransferase (AST), after cells are injured; these enzymes leak into blood. Western type diet caused significant increases in ALT (Fig. 4e) and AST (Fig. 4f). Both lovastatin and PER blunted the elevated ALT and AST, indicative of reduced tissue damage of hyperlipidemia.

**Fig. 4** PER treatment improves blood hyperlipidemia in WD-fed mice
(a) Effect of PER on triglyceride in blood. (b) Effect of PER on total cholesterol in blood. (c) Effect of PER on high-density lipoprotein cholesterol in blood. (d) Effect of PER on low-density lipoprotein cholesterol in blood. (e) Effect of PER on ALT in blood. (f) Effect of PER on AST in blood. NCD, normal chow diet; WD, western type diet; LOV, lovastatin, 60 mg·kg\(^{-1}\)·d\(^{-1}\); PER-L, petroleum ether sub-fraction of rosemary 75 mg·kg\(^{-1}\)·d\(^{-1}\); PER-H, petroleum ether sub-fraction of rosemary 150 mg·kg\(^{-1}\)·d\(^{-1}\). Data are expressed as mean ± SEM, \(n = 10\), and significant differences compared with WD are indicated by * \(P < 0.05\), ** \(P < 0.01\) and *** \(P < 0.001\) (assessed by Student’s t-test).

In order to further quantitatively analyze the plasma lipid changes after PER treatment, we measured TC and TG contents by homogenizing same amount of 6 fresh liver tissues of each group. It was demonstrated that lovastatin and the PER effectively reduced the accumulation of lipids in the liver (Figs. 5a, 5c, and 5d). Histological analysis also confirmed the previous data. Both lovastatin and PER treatment reduced the accumulation of lipids in brown and white adipose tissues (BAT, WAT; Fig. 5a). The size of white adipocytes was also significantly reduced after lovastatin or PER treatment (Figs. 5a and 5e). Additionally, PER treatment dramatically increased glycogen content in liver, while lovastatin treatment did not lead to such changes (Fig. 5a). Taken together, these data demonstrated that PER decreased Western-type-diet-induced hyperlipidemia.

**PER ameliorates diet-induced insulin resistance**

Activated SREBP-1c is often observed in diabetic mouse models, and is responsible for muscle insulin resistance \cite{24-25}. SREBP inhibitors, such as betulin, alleviate insulin resistance in mice with diet-induced obesity (DIO) \cite{26}. We then investigated whether PER ameliorates diet-induced insulin resistance in mice.

The WD-fed mice exhibited elevated fasting blood glucose level, which was significantly reduced upon PER administration (Fig. 6a), comparable to lovastatin. Meanwhile, fasting plasma insulin was also elevated, indicative of insulin resistance in peripheral tissues. PER treatment significantly reduced plasma insulin level, while lovastatin did not exhibited such an effect (Fig. 6b), indicating that PER administration might have advantages compared to lovastatin in treating insulin resistance. To investigate whether PER indeed improves insulin resistance \textit{in vivo}, OGTT and ITT were performed. The WD-fed mice had higher blood glucose concentration than NCD at every time point after oral administration of glucose, indicative of obvious glucose intolerance. Lovastatin or high concentration of PER administration effectively improved glucose tolerance (Figs. 6c and 6d) in the WD-fed mice. In the WD-fed mice, blood glucose concentration responded much slower after insulin injection, compared to NCD control, indicating that the WD-fed mice
Fig. 5  PER treatment reduces lipid deposition in tissues of WD-fed mice
(a) Liver staining with H&E or PAS, white adipose tissue or brown adipose tissue with H&E, scale bar 50 μm. (b) Liver weight, (c) total liver triglycerides and (d) total liver cholesterol were measured accordingly after PER treatment for 7 weeks, n = 6. (e) Quantification of relative area WAT in (a), n = 17–49. NCD, normal chow diet; WD, western type diet; LOV, lovastatin, 60 mg·kg⁻¹·d⁻¹; PER-L, petroleum ether sub-fraction of rosemary 75 mg·kg⁻¹·d⁻¹; PER-H, petroleum ether sub-fraction of rosemary 150 mg·kg⁻¹·d⁻¹. Data are expressed as mean ± SEM, and significant differences compared with WD are indicated by *P < 0.05, **P < 0.01 and ***P < 0.001 (assessed by Student’s t-test)
Fig. 6 PER treatment improves insulin sensitivity in WD-fed mice

Six-weeks-old male C57 BL/6 mice were randomly grouped (n = 10) with free access to water and different types of food. To induce obese phenotype, mice were fed with western type diet (WD) for 6 weeks, and were gavaged with vehicle, lovastatin or different dose of petroleum ether fraction. Elevated fasting blood glucose (a) and fasting blood insulin (b) were significantly reduced by petroleum ether fraction treatment. (c) Effect of petroleum ether fraction on glucose tolerance in WD fed mice as determined by oral glucose tolerance test (OGTT). (d) Quantification of the area under the curve (AUC) from the OGTT in (c). (e) Effect of petroleum ether fraction on insulin resistance in WD-fed mice determined by insulin tolerance test (ITT). (f) Quantification of the AUC of the ITT in (e). NCD, normal chow diet; WD, western type diet; LOV, lovastatin, 60 mg·kg\(^{-1}\)·d\(^{-1}\); PER-L, petroleum ether sub-fraction of rosemary 75 mg·kg\(^{-1}\)·d\(^{-1}\); PER-H, petroleum ether sub-fraction of rosemary 150 mg·kg\(^{-1}\)·d\(^{-1}\). Data are expressed as mean ± SEM, and significant differences compared with WD are indicated by * \(P < 0.05\), ** \(P < 0.01\) and *** \(P < 0.001\) (assessed by Student’s t-test).

PER inhibited SREBP transcription activity in the livers of WD-fed mice

In order to further investigate whether PER regulate SREBPs activity in vivo, we measured the mRNA levels of SREBPs and their target genes in the liver tissues. Compared to NCD, SREBP-1c and SREBP-2 were increased significantly upon WD challenge (Figs. 7a and 7e). Lovastatin is a competitive antagonist of HMGCR, a transcription target of SREBP-2, thus it did not affect SREBP-2, and instead, it increased SREBP-1c significantly, which might bring risks of increased fatty acids over-production [27]. PER inhibited the SREBP-1c and SREBP-2, as well as their target genes in a dose-dependent manner (Fig. 7).

Discussion

In recent years, increasing trends of metabolic disorders such as diabetes, dyslipidemia, nonalcoholic fatty liver disease, and dysglycemia bring a global medical and public health threat [28-30]. Many drugs for metabolic diseases, such as sulfonylureas, statins, and thiazolidinedione, exhibited great concern regarding their side effects for their chronic usage. Rosemary has long been widely used as food seasoning, tea, and spices, and proves its safety for long-term usage. To find out the pharmacological active composition of rosemary, traditional extraction methods were used in the present study. In accordance with the polarity, the components in rosemary were divided into sub-fractions of petroleum ether, ethyl acetate, n-butanol, and water. Since SREBPs are master regulators of biosynthesis of fatty acids, triglycerides and cholesterol [31], there would be beneficial effects on lipid homeostasis after SREBPs inhibition, either by gene silencing [32] or small chemical inhibitors [20, 33]. Using liver cells stably carrying SREBP reporter, we evaluated the potential SREBP inhibitory effects of these fractions. Interestingly, besides the inhibitors of SREBPs, we also found n-BuOH and water fraction of rosemary exhibited agonist effects (Fig. 1a). According to the theory of solution, it could be predicted that the SREBPs agonists in n-butanol and water fractions were mainly polar compounds. During petroleum ether extraction procedure, these polar compounds were removed, thus the petroleum ether fraction exhibited the best inhibitory effects on SREBPs activity. These data indicated when using rosemary extracts for treating dyslipidemia, the extraction process is essential to guarantee the efficacy. Furthermore, PER-L had no significant effect on OGTT and ITT, but PER-H amount about 600 mg·kg\(^{-1}\) rosemary have, suggesting that appropriate dosage is essential for the efficacy of PER.

Some SREBP inhibitors, such as 25-HC, block SREBP maturation, at the same time, may also activate LXR to increase SREBP-1c mRNA, which is accompanied with elevated fatty acids synthesis and liver steatosis [27, 34]. In our study, we also found that 25-HC increased SREBP-1c expression, and, notably, PER further decreased SREBP-1c as well as its target genes PASN expression in vitro (Fig. 3a) and in vivo (Figs. 7a and 7b). We also observed much
Fig. 7  PER treatment inhibits SREBPs and their target genes in WD-fed mice liver

After 7 weeks of treatment, RNA from mice livers was prepared. For each group, equal amount of RNA from tissues of 6 mice were subjected to quantitative RT-PCR. Mouse GAPDH gene was used as internal control. (a–d) Changes of mRNA level of SREBP-1c (a) and its target genes FASN (b), ACC (c) and SCD-1 (d) after PER treatment. (e–g) Changes of mRNA level of SREBP-2 (e) and its target genes HMGCR (f) and LDLR (g) after PER treatment. NCD, normal chow diet; WD, western type diet; LOV, lovastatin, 60 mg·kg⁻¹·d⁻¹; PER-L, petroleum ether sub-fraction of rosemary 75 mg·kg⁻¹·d⁻¹; PER-H, petroleum ether sub-fraction of rosemary 150 mg·kg⁻¹·d⁻¹. Data are expressed as mean ± SEM. Equal amounts of RNA from 6 mice were pooled, n = 3, and significant differences compared with WD are indicated by *P < 0.05, **P < 0.01 and ***P < 0.001 (assessed by Student’s t-test)

lower content of TG in liver and plasma. These data indicated that PER exhibited specific SREBPs inhibition effects without affecting LXR activity. Betulin [20] and oxy-cholesterol [22] bind to SCAP and Insig respectively, inhibiting the cleavage of SREBPs and thereby abrogating their transcriptional activity. BF175 blocks the binding of MED15-KIX to SREBP, resulting in the inhibition of the SREBP transcriptional activity [33]. In the present study, PER treatment reduced SREBPs in both cytoplasm and nuclei (Fig. 2), suggesting different mechanisms. Further investigation is needed to unravel the molecular mechanism of PER in regulating SREBPs.

It is reported that the inhibition of SREBPs leads to enhanced secretion of FGF21 [19, 35], thereby stimulating brown adipose tissue (BAT) to burn lipids to produce heat. Activating BAT is a novel therapeutic approach to treating metabolic disorders [36-37]. Since PER inhibited SREBP and also reduced the lipids accumulated in BAT (Fig. 5a), it is very likely that PER could increase FGF21 level and the underlying mechanism needs to be investigated in the future.

In WD-induced obesity mice, PER treatment significantly increased glycogen storage, as indicated by PAS staining, compared to lovastatin (Fig. 5a). Since increasing liver glycogen synthesis either by GSK-3β knockout or GSK-3β inhibitors leads to coordinately reduced gluconeogenesis and subsequently increase insulin sensitivity [38], the effects on liver glycogen storage render advantage to PER over lovastatin [39].

According to the MS data, the PER of rosemary contained small polar compounds and mostly diterpenoids such as carnosic acid, rosmanol, and carnosol. Pharmacokinetic studies of rosemary have shown that these compounds have very similar metabolic processes in the body [16]. Carnosic acid (CA) is reported to be beneficial for lipids homeostasis. CA-enriched diet effectively ameliorates disorders of lipid and glucose metabolism in ob/ob mice [40]. Through regulating C/EBPα and PPAR-γ pathways, CA exerts its antiadipogenic effects in adipocytes differentiation [41-42]. CA also reported to increase glucose uptake in muscle cells via PME-1/PP2A/PKB signaling pathway [43]. CA reduces adipocyte inflammation by directly inhibiting TLR4-MyD88 [44]. These reports have demonstrated that CA is an important active component in rosemary that improves lipid and sugar metabolism [12]. Besides carnosic acid and carnosol, other chemicals are rarely reported. Whether these chemicals also contribute to the activity or even exhibit synergistic effects with CA needs further investigation.

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Conflict of interests

There is a pending patent application for the use of PER for the treatment of diet induced metabolic disturbance phenotypes.

References


