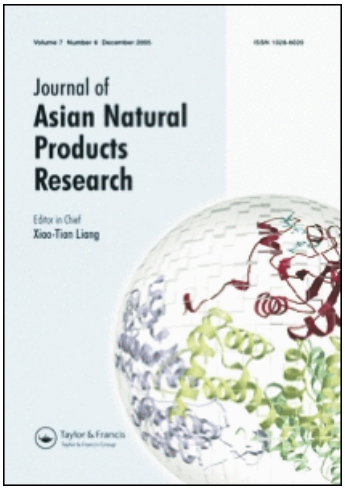

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Osthole regulates enzyme protein expression of CYP7A1 and DGAT2 via activation of PPARα/γ in fat milk-induced fatty liver rats

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Osthole (**1**), an active constituent isolated from *Cnidium monnieri* (L.) Cusson, has been used in the treatment of diseases for many years in clinical. The aim of this present study was to determine the effect of **1** on protein expression of PPARα/γ and related target molecules such as CYP7A and DGAT protein expression in the liver of hyperlipidemic fatty liver (HFL) rats, and to investigate the possible mechanism of treating HFL.

Keywords: osthole; hyperlipidemic fatty liver; PPARα/γ; DGAT2; CYP7A1

1. Introduction

Hepatic steatosis may be the result from the increase of lipogenesis, the decrease of mitochondrial and peroxisomal β-oxidation or microsomal cytochrome P450 ω-oxidation, and/or the ability of liver to export lipids [1]. The genes encoding peroxisomal, microsomal, and certain mitochondrial lipid metabolizing enzymes in liver are transcriptionally regulated by peroxisome-proliferator-activated receptors (PPARs). Its subtype, PPARα, is primarily involved in clearance of circulating and cellular lipids [2], whereas PPARγ mainly regulates lipid metabolism and action of insulin [3]. So, PPARs are important therapeutic targets of drugs which are either currently used or hold promises in the treatment of major metabolic disorders [4,5]. Fatty acids and eicosanoids have been identified as natural ligands for PPARs. More potent synthetic PPAR ligands, including fibrates and thiazolidinediones, have been proven to be effective in the treatment of dyslipidemia and type 2 diabetes by activation of PPARα and γ, respectively. In particular, lipanthyl, a ligand and activator of PPARα, is used in the treatment of hyperlipidemia and hyperlipidemic fatty liver (HFL). The drug increases the lipolysis and clearance of triglyceride (TG)-rich particles from plasma by activation of lipoprotein lipase and reduction of apolipoprotein CIII [6,7]. Therefore, it was acted as a positive drug in the experiment.

Osthole (**1**) is an active constituent isolated from *Cnidium monnieri* (L.) Cusson, one Chinese herbal medicine, which has been clinically used in many diseases. Modern pharmacological studies have proven that **1** has anti-inflammation, anti-oxidation, and anti-tumor functions. Our recent studies found that **1** could decrease the lipid levels in both serum and hepatic tissue in high fat-induced fatty liver quails and rats [8,9], but its possible mechanism is not completely clear. On the other hand, the chemical structure of **1** (Figure 1) is similarly to the agonists of PPARα, clofibrate analogs (**2**) (Figure 2). The aim of present study was to further investigate whether its mechanism was associated with PPARs and related target

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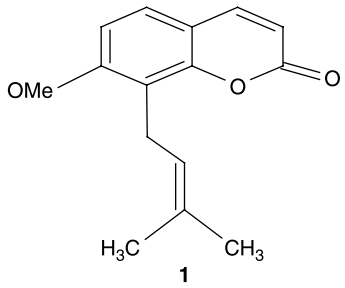

Figure 1. Structure of osthole.

proteins such as 7α-hydroxylase (CYP7A)1 and diacylgycerol acyltransferase (DGAT)2.

2. Results and discussion

In the present experiment, the results demonstrated that in fat milk-induced fatty liver rats, the levels of total cholesterol (TC) and TG in serum and hepatic tissue were significantly higher in model group than control group ($P < 0.05$ or $P < 0.01$). After administration of **1** 5–20 mg/kg for 6 weeks, the serum and hepatic tissue levels of TC and TG in **1** groups were significantly lower than those in model group ($P < 0.05$ or $P < 0.01$) (Tables 1 and 2). The results suggested that **1** was beneficial for the treatment of high fat-induced fatty liver.

Both animal data and human studies have showed that dual PPARα/γ agonists significantly decreased the high fat-induced hepatic TG accumulation, inhibited internal fatty synthesis, increased insulin sensitivity of liver and adipose tissues, and more, might reduce serum lipids and improve insulin resistance [10,11]. So we examined the PPARα/γ protein expression in rat liver. Expression of PPARα and γ was decreased and increased in model group as compared with control group, respectively ($P < 0.01$). After administration of **1** for 6 weeks, PPARα/γ expression was increased ($P < 0.01$ or $P < 0.05$) (Figures 3 and 4). The results suggested that **1** might be a novel PPARα/γ dual agonist. The combined activation of PPARα/γ could markedly counteract the high fat-induced fatty liver, its mechanism of lowering-lipid might be related to regulate the PPARα/γ-mediated lipogenic gene expression, and subsequently correct the imbalance of lipid metabolism toward lipid accumulation in the case of induced lipogenic transcription.

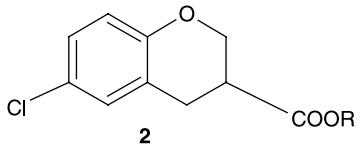

Figure 2. Structure of clofibrate analogs.

The conversion of cholesterol to bile acids in the liver is a major pathway for disposal of cholesterol from the body. CYP7A is the rate-limiting enzyme in bile acid biosynthetic pathway in the liver. This enzyme has an important role in controlling cholesterol homeostasis in the liver and fat absorption. It was reported that PPARα agonists such as lipanthyl down-regulated the CYP7A mRNA expression and activity, but the role of PPARα in CYP7A expression was not completely understood [12,13]. More recently, it was demonstrated that PPARa activators stimulated the murine CYP7A1 gene promoter through a PPARa:RXRa binding site of murine CYP7A1 gene promoter [13]. DGAT catalyzes the final and dedicated step in the synthesis of TG, and two subgroups of DGAT have been identified. DGAT2 is expressed primarily in the liver and responsible for the majority of TG synthesis, whereas DGAT1 is expressed in all tissues [14,15]. Recently, DGAT2-deficient mice have been reported to exhibit the obvious reduction of TG and fatty acids in the tissues and plasma, suggesting a critical role of DGAT2 in lipogenesis [16], Studies have also showed that PPARα agonist fibrates might lower DGAT2 (latent DGAT) activity [17]. The data of our studies demonstrated that after treatment with **1** for 6 weeks, the expression of CYP7A1 and DGAT2 was significantly increased and decreased in fat milk-induced fatty liver rats, respectively ($P < 0.01$) (Figures 5 and 6). The results suggested that **1** could increase the conversion of cholesterol to bile acids and decrease TG synthesis in the liver.

Table 1. Levels of TC and TG in serum after treatment with osthole for 6 weeks in hyperlipidemic fatty liver rats.

Group	TC (mM)	TG (mM)
Control	1.67 ± 0.17	0.70 ± 0.27
Model	2.23 ± 0.31$^{\Delta\Delta}$	1.08 ± 0.17$^{\Delta}$
Osthole 20 mg/kg	1.01 ± 0.27**	0.54 ± 0.26**
Osthole 10 mg/kg	1.24 ± 0.28**	0.69 ± 0.24**
Osthole 5 mg/kg	1.51 ± 0.45**	0.80 ± 0.22*
Lipanthyl 20 mg/kg	0.98 ± 0.18**	0.73 ± 0.19**

Data are expressed as means ± SD, with $n = 6$ per group. $^{\Delta}P < 0.05$, $^{\Delta\Delta}P < 0.01$ *vs* control; * $P < 0.05$, **$P < 0.01$ *vs* model.

Table 2. Levels of TC and TG in hepatic tissue after treatment with osthole for 6 weeks in hyperlipidemic fatty liver rats.

Group	TC (mg/g wet tissue)	TG (mg/g wet tissue)
Control	5.73 ± 0.85	24.12 ± 5.87
Model	9.04 ± 0.80$^{\Delta\Delta}$	42.88 ± 10.24$^{\Delta\Delta}$
Osthole 20 mg/kg	6.21 ± 1.32**	24.27 ± 7.38**
Osthole 10 mg/kg	6.80 ± 0.74**	26.31 ± 7.17**
Osthole 5 mg/kg	7.50 ± 1.46*	28.98 ± 9.34*
Lipanthyl 20 mg/kg	6.08 ± 0.81**	29.14 ± 4.88*

Data are expressed as means ± SD, with $n = 6$ per group. $^{\Delta\Delta}P < 0.01$ *vs* control; *$P < 0.05$, **$P < 0.01$ *vs* model.

Taken together, our studies first found that therapeutic mechanism of **1** might be due to inducing PPARα/γ protein expression and subsequently regulating the related target gene expression. Nevertheless, there are some deficiencies in the study, our further investigation will be need to clarify the exact effects of **1** on CYP7A1 and DGAT2 expression by using PPARα/γ antagonists or siRNA in cultured cells *in vitro*.

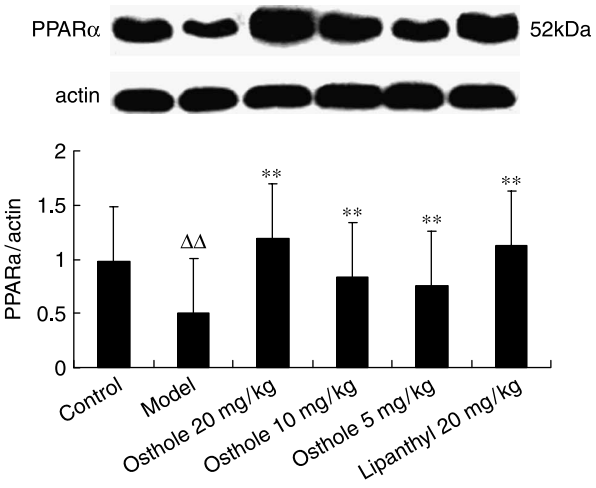

Figure 3. Representative Western blot of PPARα in hepatic tissue after treatment with osthole for 6 weeks in fat milk-induced fatty liver rats. Data are expressed as means ± SD, with $n = 6$ per group. $^{\Delta\Delta}P < 0.01$ *vs* control; ** $P < 0.01$ *vs* model.

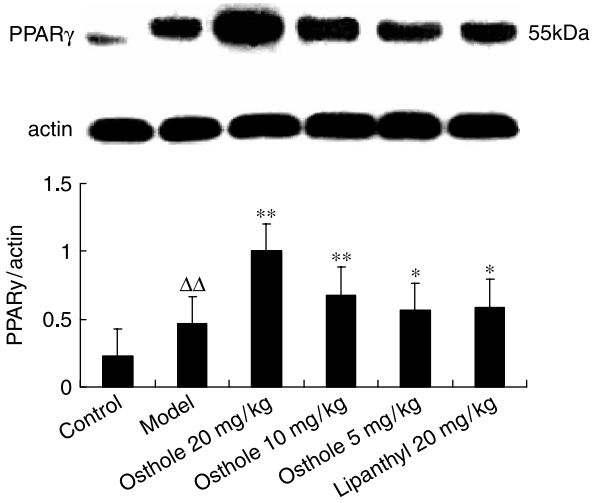

Figure 4. Representative Western blot of PPARγ in hepatic tissue after treatment with osthole for 6 weeks in fat milk-induced fatty liver rats. Data are expressed as means ± SD, with $n = 6$ per group. $^{\Delta\Delta}P < 0.01$ *vs* control; $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* model.

3. Materials and methods

3.1 Animals

Male Sprague–Dawley rats (200 ± 20 g) were obtained from the Animal Breeding Center of Soochow University, which were kept on a 12 h day/night rhythm with free access to food and water. The animals were allowed to acclimatize to the laboratory environment for 1 week prior to the study. All animal studies were conducted according to the regulations for the use and care of experimental animals in Soochow University.

3.2 Experimental design

Rat model was induced by feeding fat milk (containing 6% cholesterol, 15% lard, 0.2% propylthiouracil, 2% bile salt, 20% propylene

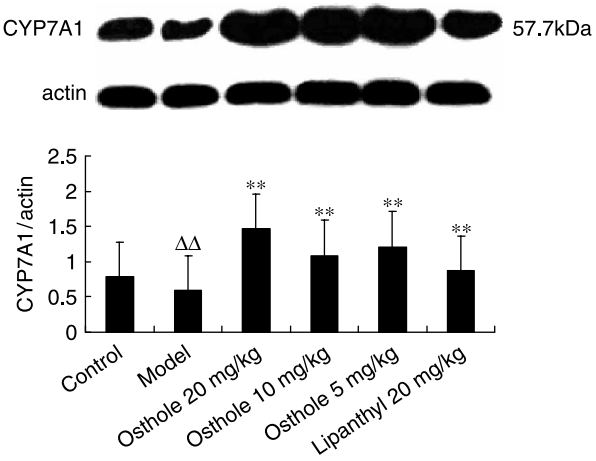

Figure 5. Representative Western blot of CYP7A1 in hepatic tissue after treatment with osthole for 6 weeks in fat milk-induced fatty liver rats. Data are expressed as means ± SD, with $n = 6$ per group. $^{\Delta\Delta}P < 0.01$ *vs* control; $^{**}P < 0.01$ *vs* model.

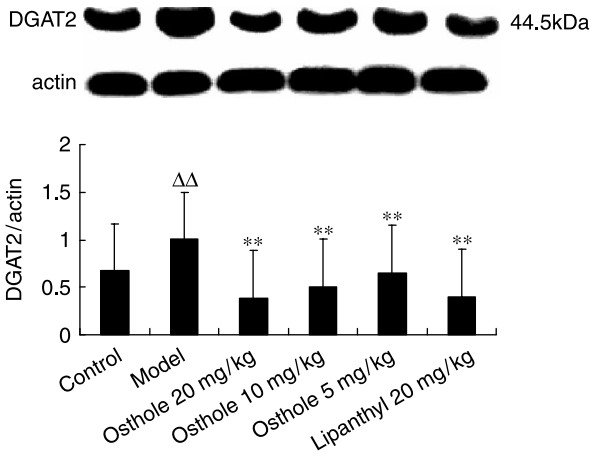

Figure 6. Representative Western blot of DGAT2 in hepatic tissue after treatment with osthole for 6 weeks in fat milk-induced fatty liver rats. Data are expressed as means ± SD, with $n = 6$ per group. $^{\Delta\Delta}P < 0.01$ *vs* control; $^{**}P < 0.01$ *vs* model.

glycol, and 20% Tween 80) at 1 ml/100 g (body weight per day) in the morning for 6 weeks [9,18], three rats were then killed and livers were taken for assessment of fatty hepatic development. After the model developed, the rats were randomly divided into five groups ($n = 6$): fatty hepatic model group, 5, 10, and 20 mg/kg **1** groups (**1** was provided by Dr Jia Zhou of Green Fount Natural Product Co. Ltd (Xi'an, China), the purity >98% by HPLC determination), 20 mg/kg lipanthyl group [lipanthyl was procured from Laboratories Fournier SA (Chenove, France)]. Drug-treated animals were administered by gavages in the afternoon for 6 weeks, control and model animals were treated with an equivalent volume of 0.5% sodium carboxymethyl cellulose solution. The high fat milk was terminated 2 weeks after administration. All rats were fed on a routine diet during the whole experiment, and then sacrificed at the sixth weekend of treatment, blood and partial hepatic tissues were collected for measurement of lipids, the rest of hepatic tissues were snap-frozen in liquid nitrogen and stored at − 80°C for Western blot.

3.3 Measurement of serum TC and TG

Rat blood was obtained after 12-h overnight fasting. Serum TC and TG were determined by colorimetric methods according to the procedure provided.

3.4 Measurement of TC and TG in rat liver tissue [19]

The hepatic lipid was extracted from liver tissue using a chloroform/methanol mixed solution (1/1, v/v). The prepared sample was then centrifuged at 1200*g* for 10 min and the obtained supernatant was used for measurement of TC and TG according to the colorimetric methods.

3.5 Western blotting analysis

Western blot analysis was performed as described by Qin *et al* [20]. Hepatic tissue samples were obtained and homogenized in a buffer consisting of 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 mM PMSF, 280 U/L aprotinin, 50 mg/L leupeptin, 1 mM benzamidine, and 7 mg/L pepstain A. Protein concentration was determined using a BCA Kit (Pierce, Rockford, IL, USA). Samples containing 50 μg of protein each were loaded on a 10% SDS-PAGE gel and subjected to

electrophoresis using constant applied current. After separation, proteins were transferred to a nitrocellulose membrane. The membrane was then rinsed and incubated with mouse monoclonal anti-PPARα (1:1000 dilution) (MA1-822) (Affinity BioReagents, Golden Colorado, USA), rabbit polyclonal anti-PPARγ (1:1000 dilution) (#07-466) (Cell Signaling, Lake Placid, NY, USA), goat polyclonal anti-DGAT2 (1:1000 dilution) (A-18) and goat polyclonal anti-CYP7A1 (1:1000 dilution) (N-17) (Santa Cruz Biotechnology, California, USA), or anti β-actin (1:5000 dilution) (Sigma, Missouri, USA) antibodies in Tris-buffered saline containing 0.2% Tween 20 (TBST) and 3% nonfat dry milk for 3 h, respectively. Membranes were then washed and incubated with horseradish-peroxidase-conjugated donkey anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG used at 1:5000 in TBST containing 3% nonfat dry milk for 1 h. The ECL™ Western blotting detection reagent (Amersham Biosciences, New Jersey, USA) was used for visualization and the results were analyzed quantitatively using SigmaScan Pro 5.0. The data were normalized with respect to ratios of β-actin detected on the same blot to control for variation in protein loading across samples.

3.6 Statistical analysis

All data are expressed as means ± SD. The significance of difference among groups was determined by using one-way ANOVA. $P < 0.05$ was considered statistically significant.

Acknowledgments

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