EFFECTS OF TOONA SINNENSIS ROEM LEAVES ETHANOL EXTRACT ON THE PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR ALPHA/ GAMMA EXPRESSION IN LIVER OF HIGH FAT DIET MICE

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Abstract - Toona sinensis Roem leaves (TSL) has been used as a traditional medicine for obesity and diabetes. TSL ethanol extract (TSL-E) acted as a peroxisome proliferator-activated receptor of gamma (PPARγ) ligand and revealed the hypoglycemic effect in high fat diet (HFD) mice. However, the effect of TSL-E on the steatohepatitis is not known. In this study, levels of PPARα, PPARγ, PCK2 and mHMGCs protein expression were investigated using western blot. Results showed that the expressions of PPARα and PPARγ were significantly increased by TSL-E in liver, indicating that TSL-E is not only the PPARγ ligand, but also the PPARα ligand. TSL-E was also found to activate the PPARα/γ in HepG2 cells transiently transfected with the (PPRE)-tk-luciferase vector; compared with the PPARα/γ specific full agonist, bezafibrate and PIO. In conclusion, TSL-E significantly elevates the expression of PPARα/γ *in* liver of HFD mice.

Key words - Toona sinensis; peroxisome proliferator-activated receptor; ethanol extracts; obese; liver; mice

1. Introduction

Toona sinensis Roem (TS) is a perennial tree widely grown in Asia. Its leaves have a special aroma and are often served as vegetable dishes in Taiwan. The leaves of TS (TSL) are also used in Chinese traditional medicine for the treatment of diarrhoea, chronic dysentery, bloody stools, seminal emissions, leucorrhoea, and metrorrhagia. Previous studies on TS have shown that TS contains various flavones including triterpenes, phenolic compounds, alkaloid, anthraquinone, tannins. Further investigations show that TS is also rich in epicatechin, scopoletin, quercetin and gallic acid (W. Y. Wang, Geng, Zhang, Shi, & Ye, 2007). TS could be considered as a natural antioxidant source (H. Y. Chen, Lin, & Hsieh, 2007) and potent antiglycative agent, which can be of great value in the preventive glycation-associated cardiovascular and neurodegenerative diseases (Hsieh, Lin, Ko, Peng, Huang, & Peng, 2005). Extracts of TSL also possess the hypoglycemia effect via increased insulin – induced glucose transporter 4 mechanism in adipose (Pei-Hwei, Tsai, Hsu, Wang, Hsu, & Weng, 2008). The improvements of GOT/GPT were shown in the studies that TSL extract was given to thioacetamide (TAA)-treated rats. These results imply that TSL possesses beneficial effects on liver injury through increments of detoxification and the metabolic pathway (Fan, Chen, Wang, Tseng, Hsu, & Weng, 2007.

PPAR are ligand-inducible transcription factors that belong to the nuclear hormone receptor superfamily. PPAR regulate gene expression by binding with RXR (Retinoid X Receptor) as a heterodimer partner to specific DNA sequence elements termed PPRE (Peroxisome Proliferator Response Element) (Gillespie, Tyagi, & Tyagi, 2011). PPAR have been implicated in many normal and disease-related biologic processes relevant to the heart and vasculature including lipid and energy metabolism, inflammation, embryo implantation, diabetes and cancer (Abranches, de Oliveira, & Bressan, 2011). There are 3 main iso-types of PPAR including PPARα, PPARβ (also called PPARδ) and PPARγ. PPARα is highly expressed in tissues with high rates of mitochondrial fatty acid oxidation, such as liver, heart, muscle, kidney and it is activated by fibrates, fatty acids (Garratt, Vickers, Gluckman, Hanson, Burdge, & Lillycrop, 2011). Recently, the PPARα gene regulatory pathway has been implicated in the hepatic metabolic response to diabetes mellitus and PPARα ligands such as fenofibrate and clofibrate have been implicated in peroxisome proliferation and liver tumors (McKeage & Keating, 2011). Fenofibrate also had a beneficial effect on atherogenic dyslipidemia in patients with the metabolic syndrome or type 2 diabetes mellitus, tending to increase HDL-C levels, and promoting a shift to larger low-density lipoprotein particles (Keating, 2011).

Fatty acid represents vital energy stores, but HFD are associated with the development of obesity and type 2 diabetes. Several evidences indicate the importance of both quantitative and qualitative changes in dietary FAs as relevant mechanisms for the development of nonalcoholic fatty liver disease (NAFLD) in both rodent and humans. Dietary lipid influences the rate of lipogenesis. Two key enzymes in the lipogenesis pathway, fatty acid synthetase and acetyl CoA carbonxylase, are reduced in animals receiving a HFD. In addition, the pentose pathway which provides reduced equivalents for de novo lipogenesis, decreases in HFD rats. In obese rats, both hepatic and adipose tissue lipogenic rates are decreased by HFD. Moreover, the animals still deposit more fat because of the increased uptake of fatty acids form the diet (Cong, Tao, Tian, Liu, & Ye, 2008).

2. Method

2.1. Cell culture

FL83B hepatocyte is a gift of Professor Lee-Yan-Sheen in National Taiwan University. The cells were incubated in F12K medium containing 10% FBS and 1% penicillin and streptomycin in 10cm petri dishes at 37 ^{0}C and 5 % CO_2. Experiments were performed on cells that were 80–90 % confluent.HepG2 hepatocytes were grown in DMEM plus 10% PBS and 1% P/S. Cells were subculture every two days, with cells were less than 90% confluences. Freezing and thawing of cells: 70-80% confluent cells were trypsinised, centrifuged at 1000 rpm,

for 5 min, at 4°C, washed with PBS and resuspended in freezing media – 10% (v/v) DMSO. In order to prevent the formation of intra cellular crystals, the aliquots were frozen immediately on ice then stored in -20^{0}C for 1 hour, - 80^{0}C for 1 day, and finally subsequently transferred to liquid nitrogen for long-term storage. Recovery involved rapid thawing of the vials and immediate resuspension in flasks holding pre-warmed culture media.

2.2. TSL-E extract

Toona sinensis crude extract (1g: 10ml) is soluble in 95% ethanol, shake at room temperature for 12 hours then concentrated by using vacuum freeze dryer. The powder was collected and stored in dark at room temperature. 100mg TSL-E extract were prepared in 1 ml DMSO to be 100mg/ml stock solution.

2.3. Protein extraction and qualification

Cells were washed by PBS and collected scraping in lysis buffer [(0,32M Sucrose, 10 mM KH_2PO_4, Na_3VO_4 1 mM; 2 mM PMSF; 1 mM EDTA; 1 mM NaF, pH 7.4]. After incubation on ice for 30 minutes, lysates were collected by centrifugation at 10,000 rpm for 30 minutes at 4^{0}C. Protein concentration was estimated by Bradford method. The protein assay dye reagent concentrate (BioRad) was diluted 5 fold with distilled water. BSA protein standard was diluted to 800, 400, 200, 100, 50, 25, 0 µg/mL with 50mM phosphate buffer saline (PBS). The concentrations PBS diluted protein samples were controlled in the standard curve range. 10 µl diluted protein samples and BSA protein standard were added to protein assay dye reagent, transferred to the ELISA plate. The OD 595 nm was read by Tecan Sunrise.

2.4. Electrophoresis and blotting

Equal amounts (20µg) of total proteins were electrophorylated on 10 % SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% nonfat dried milk in TBST buffer (20mM Tris-HCl, pH 7.4, 150mM NaCl, 0.1% Tween 20) for 1 hour then hybridized with specific primary antibody overnight at 4^{0}C. Subsequently, the membrane was washed with TBST buffer and incubated with secondary antibodies for 1 hour RT. Protein bands were visualized by enhanced chemiluminescence kits (ECL Plus, Amersham). The band quantification was performed using LAS-3000 (Fujifilm, Tokyo, Japan) and Multi Gauge software v 3.0 (Fujifilm)

2.5. Oil red O staining

HepG2 and Fl83B were cultured and treated with Oleic acid (OA) 200 µM for 24 hours, pretreated with TSL-E 100 µg/ml 12 hours before added OA or treated with 100 mg/ml TSL-E for 12 hours after added OA. To measure cellular neutral lipid droplet accumulation, cells were stained by the Oil red O method. After treatments, cells were washed three times with iced PBS and fixed with 10% formalin for 60 minutes. After fixation, cells were washed and stained with Oil red O solution (stock solution, 3 mg/ml in isopropanol; working solution, 60% Oil red O stock solution and 40 % distilled water) for 60 minutes at room temperature. After staining, cells were washed with water to remove unbound dye. To quantify Oil red O content levels, isopropanol was added to each sample shaken at room temperature for 5minutes, and samples were read by spectrophotometer at 500 nm using 100% isopropanol as blank.

3. Result

3.1. Effect of TSL-E on body weight, triglyceride content, serum cholesterol and serum glucose in HFD mice

C57BL/6 mice were fed with HFD for 8 weeks to induce obesity then treated with TSL-E for 6 weeks. The serum glucose (GLU), serum triglyceride (TG) and serum cholesterol (Chol) were determined each week. HFD mice have increased triglyceride, cholesterol and glucose level compared with control mice.

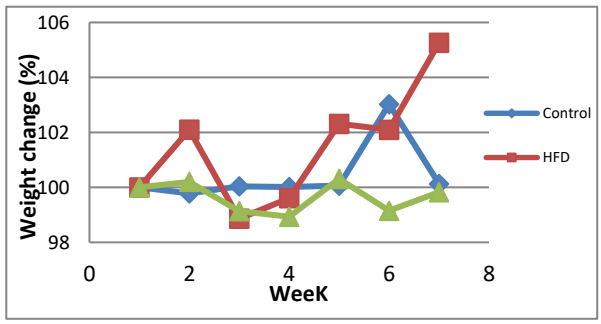

Figure 1. *Effect of TSL-E on body weight of HFD mice*

C57BL/6 mice were fed with HFD for 8 weeks to induce obesity then treated with TSL-E for 6 weeks. The body weight was determined each week. Body weight was set 100% as the body weight at the first week. At the end of treatment period, body weight of HFD mice rose significantly, up to 5% compared with the control. TSL-E feeding HFD mice restored the body weight to normal level of control mice

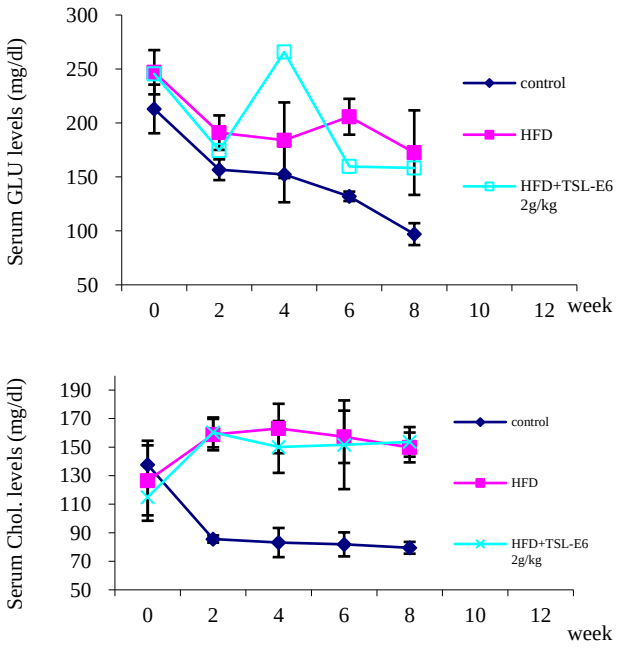

Figure 2. *Effect of TSL-E treatment on serum glucose, triglyceride and cholesterol level of HFD mice*

At the end of the treatment period, the HFD mice were overweight compared with the control. ones The

HFD+TSL-E mice, on the other hand, were lighter than the HFD animals, reaching a body weight that was not different from that of the control animals (Figure 1). Serum glucose level was higher in HFD mice than in control mice, whereas in HFD+TSL-E animals it was reduced at the end of the treatment period (Figure 2), respectively. However, HFD mice exhibited significantly elevated levels of the plasma triglyceride and serum cholesterol and HFD+TSL-E mice's levels were not significantly different from those of HFD mice (Figure 2).

People tend to develop fatty liver if they have certain other conditions, such as obesity, diabetes or high triglycerides. The results indicated that our model has successfully developed fatty liver or metabolic syndrome.

3.2. TSL-E exhibited both PPARα and PPARγ ligand binding activity

Using cell-based PPAR chimera transactivation assays, we investigated whether TSL-E acts as a dual agonist for both PPARα and PPARγ. HepG2 cells were transfected for 24 hours with the pBind–PPARα-LBD-plasmid and pG5L-TK–luc vector.

Twenty-four hours later, the transfected cells cultured for 24 hours in DMEM containing various concentration of TSL-E: 50 μg/ml, 100 μg/ml, 150 μg/ml. Bezafibrate 50μM was used as positive control. TSL-E 150 μg/ml significant increased PPARα-dependent luciferase activity and reached a level higher than bezafibrate, a full PPARα agonist. As shown in Figure 3, TSL-E increased PPARγ-dependent luciferase activity, however TSL-E is less PPARγ binding activity than troglitazone, a well-known PPARγ full agonist.

TSL-E was also found to activate the PPARα in HepG2 cells transiently transfected with the (PPRE)-tk-luciferase vector (Figure 3). TSL-E 50, 100 and 150 mg/ml all raised the PPARα-dependent luciferase activity. Interestingly, TSL-E 150 μg/ml showed higher PPARα-dependent luciferase activity compared with the PPARα-specific full agonist, bezafibrate.

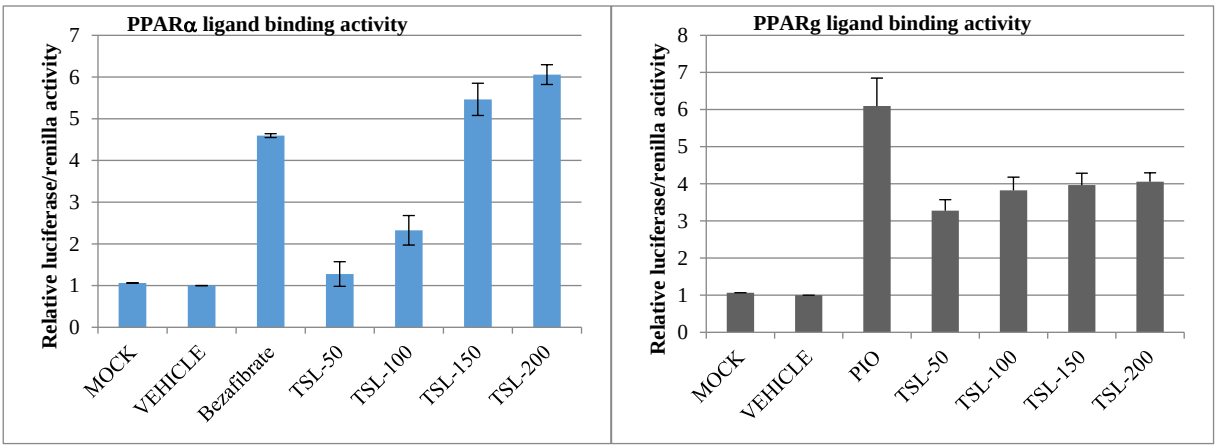

Figure 3. *The PPARα and PPARγ ligand bind activity of TSL-E by luciferase reporter assay*

HepG2 cells were transfected for 24 hours with the pBind–PPARα-LBD-plasmid and pG5L-TK–luc vector. Twenty-four hour later, the transfected cells cultured for 24 hours in DMEM containing various concentration of TSL-E: 50 μg/ml, 100 μg/ml, 150 μg/ml. Bezafibrate 50μM was use as positive control. TSL-E 150 μg/ml significant increased PPARα-dependent luciferase activity and reached to a level higher than bezafibrate, a full PPARα agonist

3.3. Effect of TSL-E on PPARα, PPARγ, PCK2 and HMG-CoA protein expression in liver of mice

PPARα and PPARγ are members of the PPAR family of nuclear transcription receptors and play a central role in glucose metabolism, lipid biosynthesis and insulin sensitivity. Phosphoenolpyruvate carboxykinase 2 (PCK2) is a key enzyme on gluconeogenesis pathway. 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-CS) contains an important catalytic cysteine residue that acts as a nucleophile in the first step of reaction: the acetylation of enzyme by acetyl-CoA to release the reduced coenzyme A. HMG-CS is an intermediate in both cholesterol synthesis and ketogenesis. To investigate the effects of TSL-E on glucose and lipid metabolism, the Western blot was used to identify the expression of PPARα, PPARα, PCK2 and HMG-CS in liver of mice.

As shown in Figure 4, PPARα and PPARγ protein expression were up regulated in HFD mice. Interestingly, the PPARα and PPARγ expression were higher in TSL-E treatment compared with that of HFD mice. PIO, a full PPARγ agonist significantly increased the PPARγ expression. The PPARα expression in TSL-E treatment was higher than that in PIO treatment while the PPARγ expression in TSL-E was lower than that in PIO treatment. Combined TSL-E and PIO did not change the PPARγ expression but significantly increased PPARα expression. HFD up regulated the HMG-CS expression (Figure 4). The expression of HMG-CS in HFD fed TSL-E were elevated and up to a level higher than that in HFD mice. TSL-E combined PIO decreased HMG-CS protein expression in liver of mice compared with TSL-E only. HFD and HFD+PIO elevated the PCK2 protein expression in liver (Figure 4). TSL-E treatment decreased the expression of PCK2 compared with HFD and HFD+PIO, respectively. TSL-E combined PIO also decreased PCK2 expression compared with PIO only.

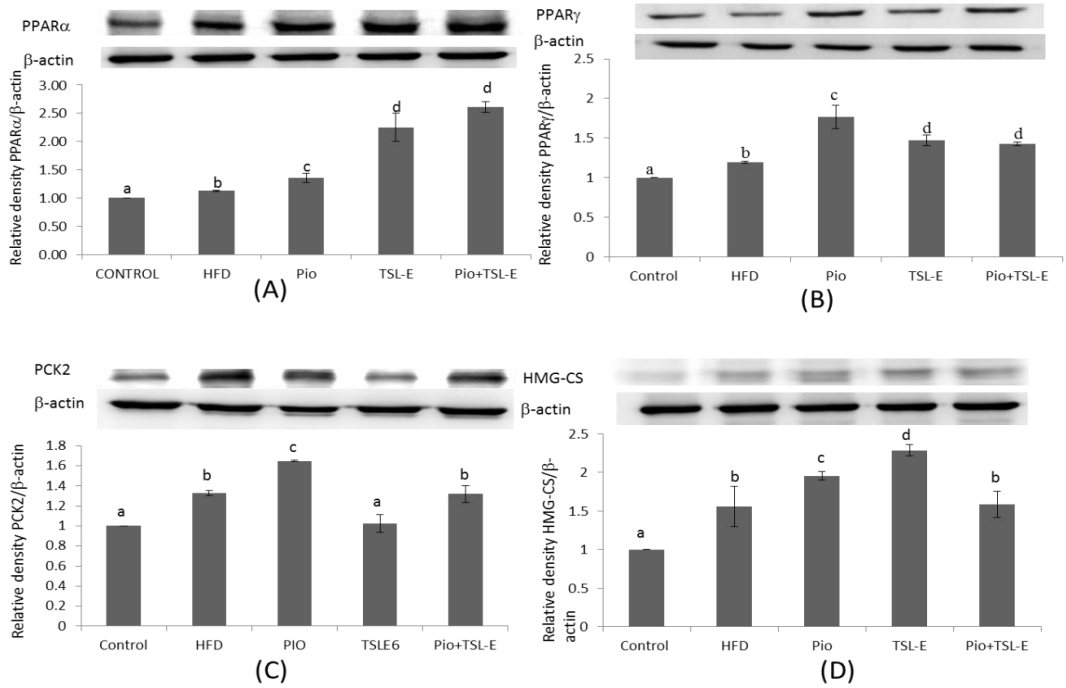

Figure 4. *The protein expression of PPARα, PPARγ, PCK2 and HMG-CoA in liver of mice*

The mice were then randomly divided into five groups (N=8) for the study. Control: mice were fed with normal diet; HFD: mice were fed with the HFD; PIO: mice were fed with the HFD plus pioglitazones (5mg/kg body weight); TSL-E: mice were fed with HFD plus TSL-E (0.5g/kg body weight) and PIO+TSL-E: mice were fed with the high-fat diet plus pioglitazone and TSL-E. TSL-E increased the protein expression of PPARα and PPARγ. The HFD mice treated with TSL-E showed decrease PCK2 and increased HMG-CoA protein expression in liver. Data are expressed as means plus standard deviations of three similar experiments. The data are given as means ± SE. a, b, c, d: bars with superscripts without a common letter differ significantly (student t test, P<0.05

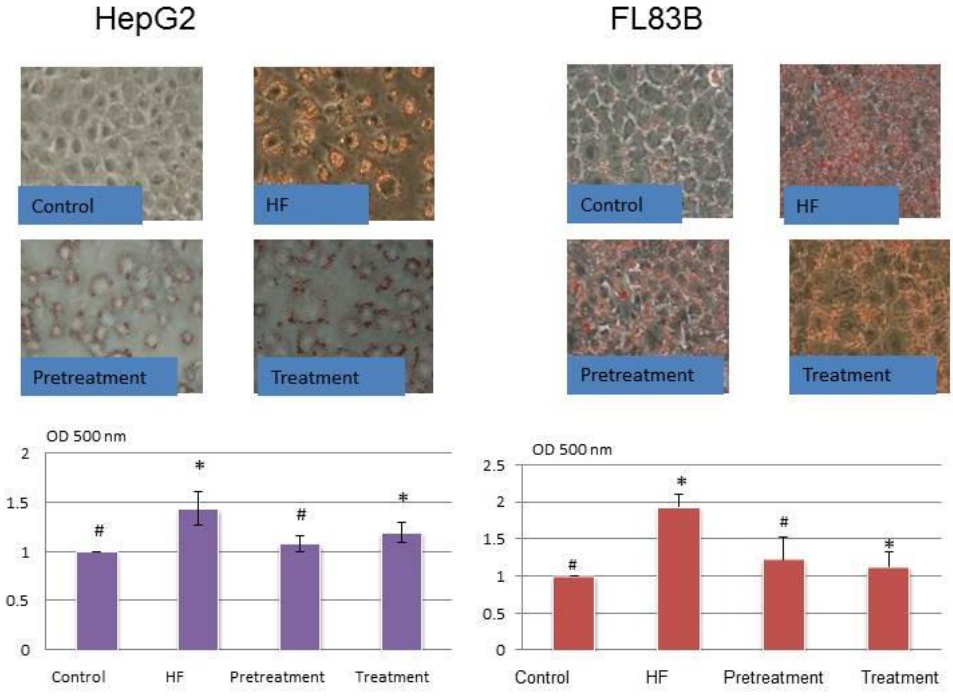

Figure 5. *Effect of TSL-E on lipid accumulation induce by OA in hepatocytes*

Determination of lipid accumulation by way of Oil Red O staining in HepG2 (A); Fl83B (B) and Fl83B in presence of insulin 5 μg/ml and extra-glucose 5mM (C). Cells treated for 24 hours 0leic acid (HF); pretreat with 100 mg/ml TSL-E for 12 hours before being exposed to OA (pretreatment) or added TSL-E 100 mg/ml for 12 hours after being exposed to OA (Treatment). Lipid accumulation was then visualized by way of Oil Red O staining, which allowed detection of TG and cholesterol esters. Cells were observed and photographed under a phase-contrast microscope (A-F, original magnification 40X).

3.4. Effect of TSL-E on lipid accumulation induced by Oleic acid

In order to further study the mechanism improving lipid metabolism i*n vitro*, HepG2 and Fl83B cells were treated with OA (200 μM) to simulate the condition of high plasma levels of FFA in animal model. As a result, 200 μM OA increased significantly the intracellular TG content in hepatocytes, a condition that is observed when plasma FFA is elevated in animal studies. We found 24 hours to be optimal incubation time. When the amount of TG was examined by Oil red O staining, the amount found in TSL-E-treated cells was also increased compared to untreated cells but was less than OA-treated cells. Our results showed that, although TSL-E has PPARγ agonist activity, it can decrease lipid accumulation in OA-treated HepG2 and FL83B. Pretreated cells with 100 μg/ml TSL-E for 12 hours before being added OA was more powerful to reduce lipid drop than treated TSL-E after being added OA (Figure 5). TSL-E decreased lipid accumulation in the normal condition and in the present of insulin and extra-glucose (Figure 5). However, when insulin and extra-glucose were added to medium, TSL-E also increased lipid accumulation in HepG2 compared with control and insulin groups.Moreover, to address the effect of TSL-E on lipid accumulation, HepG2 and Fl83B cells treated with OA (200 μM) to simulate the condition of high plasma levels of FFA. The combination of OA and TSL-E was tested at two conditions, pretreated for 12 hours before being added OA or treated for 12 hours after being exposed to OA. The results showed that TSL-E decreased lipid accumulation in both conditions, pretreatment and treatment, and confirmed that TSL-E promoted lipolysis in liver of HFD mice.

4. Conclusion

PPARα regulates genes involves in fatty acid uptake and oxidation, inflammation, and vascular function and plays a central role in fatty acid catabolism in liver and other tissues by up-regulating beta- and delta-oxidation (Abranches, de Oliveira, & Bressan, 2011). PPARγ ligands are particularly interesting for treating type 2 diabetes mellitus because they restore sensitivity to insulin, increasing glucose uptake into liver and skeletal muscle cells and reducing plasma glucose levels. By promoting β-oxidation via PPARα activity, TSL-E decreased gluconeogenesis which was elevated in HFD mice caused hypertension and IR. PPARα activation also controls hyperlipidemia in HFD mice by decreased lipogenesis. Moreover, TSL-E also showed the PPARγ ligand activity that increased the expression in liver of mice. Our results showed that, although TSL-E has PPARγ agonist activity, it can decrease lipid accumulation in OA-treated HepG2 and FL83B. While PCK2 protein expression is elevated in HFD mice, the TSL-E treatment decreased PCK2 compared with HFD groups. These results suggest that TSL-E is a more efficient activity on PPARα relative to PPARγ. The interaction between PPARα and PPARγ agonist activity of TSL-E can alleviate side effects such as increased body weight associated with PPARγ agonists. The classic full PPARγ agonists have a variety of side effects, chiefly weight gain due to edema and increased fat mass (Dunn, Higgins, Fredrickson, DePaoli, & Grp, 2011). However, the side effects associated with PPARγ activation may be circumvented through the combined activation of PPARα and PPARγ, which is known to result in a complementary and synergistic increase in lipid metabolism and insulin sensitivity (Huang, Lee, Yu, Chen, Tsai, & Chen, 2010). In summary, TSL-E might manipulate the lipolysis and energy homeostasis in liver through the regulation of PPARα/γ downstream signaling pathway.

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