Prevention Mechanism of 2,3,5,4′-Tetrahydroxy-stilbene-2-O-β-D-glucoside on Lipid Accumulation in Steatosis Hepatic L-02 Cell

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ABSTRACT

Aim: 2,3,5,4′-Tetrahydroxy-stilbene-2-O-β-D-glucoside (TSG), a natural stilbene, shows great activities in hepatic lipid regulation, especially for hepatic triglyceride lowering. However, information about its mechanisms on biosynthesis and degradation of triglyceride is still limited. This research pays close attention to clarify the mechanism of TSG on prevention of hepatic lipid accumulation. Materials and Methods: TSG was given to steatosis hepatocyte L-02 cell induced by fat emulsion incubation. The contents of free fatty acid, triglyceride, rate-controlling enzymes, and transcriptional regulatory factors, which play key role in biosynthesis and decomposition of triglyceride, were determined with or without TSG exposure. Results: TSG could reduce the free fatty acid material supply for the synthesis of endogenous triglyceride and it did so by reducing the expression of liver type fatty acid binding protein and fatty acid transport protein 4. TSG inhibited the expression of sterol regulatory element-binding protein 1c, and then reduce the contents of acetyl-CoA carboxylase 1 and fatty acid synthase. Therefore, TSG prevented biosynthesis of triglyceride. Mean while, TSG also promoted the decomposition of triglyceride by the activation of peroxisome proliferators activator receptors alpha. Conclusion: TSG could effective intervene the accumulation of triglyceride in hepatic cell. Thus, TSG could be considered as a promising drug candidate in prevention and treatment of lipid metabolic disorders, especially nonalcoholic fatty liver disease.

Key words: Decomposition, lipid accumulation, Polygonum multiflorum, 2,3,5,4′-tetrahydroxy-stilbene-2-O-β-D-glucoside, triglyceride biosynthesis

INTRODUCTION

With the rapid improvement of living standards and huge changes of lifestyle, lipid metabolism abnormality and related diseases have extremely high prevalence all around the world. Nonalcoholic fatty liver disease (NAFLD) is a major disease endangering human health, which is used to describe a condition of lipid accumulation in the liver.1-3 NAFLD is usually accompanied by diabetes, hyperlipidemia, and insulin resistance, a cluster of diseases collectively known as metabolic syndrome.4,5 Almost 20–30% of adults in developed countries are suffering from NAFLD.6 NAFLD used to be considered a disease mainly affecting middle-aged people; however, the diagnosis of NAFLD is increasingly more frequent during adolescence, and it has been shown to affect both sexes equally after adolescence.7,8 More disappointing is that one in five of those with NAFLD will not survive more than 15 years.9

In the development of NAFLD, the accumulation of lipid within hepatic cells, especially triglycerides, has been confirmed by numerous researches.1,2,4-7 In healthy human liver, the mean contents of total cholesterol and triglyceride are 3.9 and 19.5 mg/g wet weight, respectively. However, liver fat content increased to more than 50 mg/g (5% by wet weight) in fatty liver.9,10 This hepatic lipid accumulation results from an imbalance between lipid availability and lipid disposal. In general, the dietary triglyceride and carbohydrates will be oxidatively decomposed into free fatty acid (FFA), and then transported to the liver for triglyceride bio-synthetization or stored in adipose tissues.10-13 Lipid accumulation in hepatic cells is usually occurring under these following conditions: FFA absorption from peripheral adipose tissue or nutrient is increased; the endogenous synthesis of FFA is increased; decomposition of fatty acid is reduced; the function of very low density lipoprotein is inhibited, and so on.11

Therefore, regulations of triglyceride and FFA (raw material for triglyceride bio-synthetization) in hepatic cells will be an effective method to prevent the accumulation of triglyceride. Various methods have been applied to treat NAFLD, such as dietary modification, exercise, lifestyle, and pharmacological approaches.14,15 However, information about its mechanisms on biosynthesis and degradation of triglyceride is still limited. This hepatic lipid accumulation regulation and treatment research pays close attention to clarify the mechanism of TSG on prevention of hepatic lipid accumulation.
prevention and treatment of NAFLD. Drug treatment is still an important part of addressing these issues. However, there is still no specific drug in prevention and treatment of NAFLD. Most patients were treated with no-specific drugs, such as insulin sensitizers (metformin\textsuperscript{[12]} and thiazolidinediones\textsuperscript{[13]}), vitamin E\textsuperscript{[14]} and statins.\textsuperscript{[15]} Finding natural products in the traditional medicines seems to be a promising way:

The naturally occurring stilbenoid, 2,3,5, 4’-tetrahydroxy-stilbene-2-O-β-D-glucoside (TSG) is mainly distributed in Polygonum multiflorum Thunb. TSG and extractions of P. multiflorum show great lipid regulation effects in our previous studies.\textsuperscript{[16-18]} TSG also interacts with multiple targets in a variety of disease models to exert protective effects on human health.\textsuperscript{[19-21]} However, information about its mechanisms of regulation in biosynthesis and degradation of triglycerides is still limited. In this study, prevention mechanisms of TSG on hepatic lipid accumulation were evaluated by \textit{in vitro} steatosis hepatic L-02 cell.

**MATERIALS AND METHODS**

Experimental design

The whole research was carried out by L-02 cells, which were purchased from Cell Bank, Kunming Institute of Zoology, Chinese Academy of Science. Cells were grown in RPMI-1640 medium (Gibco Invitrogen Corporation, USA) supplemented with 10% fetal bovine serum (FBS; Hyclone, USA). Cultures were maintained in a humidified incubator (Series II water jacketed CO\textsubscript{2} Incubator Model 3111; Thermo Electron Corporation, USA) with 5% carbon dioxide 95% air at 37°C.

Five groups were involved: normal group (CON), model group (MOD), the TSG group (TSG powder, purity higher than 98%, was purchased from Nanjing Jingzhu Bio-technology Co., Ltd., and stored in a dry and cool dark place), Simvastatin (SIM; Hangzhou MSD Pharmaceutical Co., Ltd., China), and Fenofibrate (FEN; Laboratories Fournier S.A., France). The concentration of TSG (150 μmol/L) was optimized and selected from our previous research.\textsuperscript{[16,22]} Both SIM and FEN were considered as positive control.

L-02 cells were seeded in six-well plates (Corning, USA) at a cell density of 3x10\textsuperscript{5} in 2 mL/well and incubated till 80–90% confluence with 10% FBS-RPMI-1640 medium (0.2% FBS-RPIMI 1640 medium) for 12 h before test.

Subsequently, all cells, except normal group, were exposed to 1% fat emulsion–10% FBS-RPMI-1640 medium for steatosis. Fat emulsion was purchased from Sichuan Guorui Pharmaceutical Co., Ltd., China. This fat emulsion (each 250 mL) contained 50 g of refined soybean oil, 5.5 g of glycerol, and 3 g of refined lecinthin. In the same time, TSG, SIM, and FEN were synchronously given to these steatosis hepatic cells to intervene the steatosis procedure.

**Oil-red staining**

After treated with TSG, SIM, or FEN for 4, 8, 12, 18, 26, 36, 48, and 60 h, respectively, the cells were washed with 2 mL phosphate buffer saline (PBS) for two times. Then cells were fixed with 70% ethanol and further dehydrated with 100% propylene glycol. The cells were stained with Oil-Red O dye, and fat droplets in hepatocytes were stained red.

**Assessment of free fatty acid, triglyceride, protein, and transcriptional regulatory factors levels**

After treated with TSG, SIM, or FEN, the cells were washed with 2 mL PBS per well for two times. Cells were collected with cell scraper with 1 mL PBS and centrifuged for the removal of PBS at 6900 g for 10 min in 4°C. Then, the mixed suspension was blown with 50 μL iced PBS and lysed at 37°C after refrigerated for 30 min in 20°C. This freeze–thawing procedure was repeated for three times. Finally, cell lysis was collected after centrifugation at 6900 g for 20 min in 4°C.

Contents of FFA and triglyceride were measured by assay kits purchased from Bio Sino Bio-technology and Science Inc. and Nanjing Jiancheng Bioengineering Co., Ltd., China. The liver type fatty acid binding protein (L-FABP), fatty acid transport protein 4 (FATP4), microsomal triglyceride transfer protein (MTTP), Apo lipoprotein B100, acetyl-CoA carboxylase 1 (ACACA), fatty acid synthase (FASN), peroxisome proliferators activator receptors alpha (PPAR-α), sterol regulatory element-binding protein 1c (SREBP-1c), and lipoprotein lipase (LPL) contents were tested by ELISA assay kits purchased from Cusabio Biotech Co., Ltd., China.

**Statistical analysis**

All data in this study were expressed in the form of mean ± SD. The data were evaluated by one-way analysis of variance with a significance level of 0.05. Relationships between FFA, triglyceride, and key enzymes and transcriptional regulatory factors were assessed by Pearson’s correlation coefficient. Results were classified into two significance levels using the P values of 0.05 and 0.01.

**Table 1:** Contents of TG and FFA in steatosis L-02 cells at each time point (x ± s, n=3).

|        | 4 h     | 8 h     | 12 h    | 18 h    | 26 h    | 36 h    | 48 h    | 60 h    |
|--------|---------|---------|---------|---------|---------|---------|---------|---------|
| TG (mmol·L\textsuperscript{-1}) |        |         |         |         |         |         |         |         |
| CON    | 1.12 ± 0.02\* | 1.18 ± 0.01\* | 1.23 ± 0.00* | 1.31 ± 0.02** | 1.34 ± 0.04*** | 1.49 ± 0.05**** | 1.61 ± 0.02***** | 1.76 ± 0.04****** |
| MOD    | 1.39 ± 0.04\* | 1.73 ± 0.10* | 1.84 ± 0.10* | 2.23 ± 0.07** | 2.66 ± 0.02* | 2.99 ± 0.01** | 3.06 ± 0.01*** | 3.02 ± 0.01**** |
| TSG    | 1.35 ± 0.05* | 1.44 ± 0.02** | 1.58 ± 0.10* | 1.87 ± 0.05* | 2.10 ± 0.02** | 2.27 ± 0.04** | 2.62 ± 0.09** | 2.46 ± 0.04*** |
| SIM    | 1.39 ± 0.04* | 1.46 ± 0.01** | 1.51 ± 0.02** | 1.77 ± 0.05* | 1.89 ± 0.02** | 2.11 ± 0.04** | 2.46 ± 0.11** | 2.46 ± 0.04*** |
| FEN    | 1.30 ± 0.02* | 1.52 ± 0.04** | 1.61 ± 0.02* | 1.91 ± 0.05* | 2.09 ± 0.04** | 2.12 ± 0.02** | 2.45 ± 0.02** | 2.65 ± 0.05*** |
| CON    | 70.60 ± 0.16** | 75.64 ± 0.42** | 76.73 ± 0.48** | 77.79 ± 0.15* | 70.02 ± 1.16 | 73.79 ± 0.06* | 72.93 ± 0.39 | 71.46 ± 0.23* |

| FFA (ng·mL\textsuperscript{-1}) |        |         |         |         |         |         |         |         |
| MOD    | 72.57 ± 0.13** | 69.54 ± 0.37** | 69.30 ± 0.40** | 62.82 ± 1.08** | 68.36 ± 0.66 | 72.65 ± 0.24** | 72.36 ± 0.27 | 78.43 ± 0.36** |
| TSG    | 71.93 ± 0.27** | 69.78 ± 0.15** | 73.79 ± 1.59 | 75.50 ± 2.55 | 70.92 ± 0.04** | 74.72 ± 0.74 | 82.02 ± 4.04 | 85.00 ± 4.44** |
| SIM    | 71.64 ± 1.25 | 71.64 ± 1.25 | 72.33 ± 0.53** | 72.33 ± 0.53** | 70.62 ± 0.66 | 70.62 ± 0.66* | 71.28 ± 0.06** | 71.28 ± 0.06** |
| FEN    | 72.43 ± 0.01** | 72.43 ± 0.01** | 72.67 ± 0.25** | 72.67 ± 0.25** | 73.26 ± 0.31* | 73.26 ± 0.31 | 76.26 ± 0.40** | 76.26 ± 0.40** |

Intracellular TG and FFA contents were tested by assay kits from 4 to 60 h, mean ± SD were calculated and presented. For the statistical analysis, the * indicates a significant difference compared with control group, \* P < 0.05, ** P < 0.01, *** P < 0.001; the # indicates a significant difference compared with model group, # P < 0.05, ## P < 0.01, ### P < 0.001.
RESULTS

TSG inhibited the formation of lipid droplets and suppressed free fatty acids supply

Oil-Red O staining [Figure 1] showed that the amounts of stained lipid droplets in model group were increased in a time-dependent manner. Lipid droplets merged with each other inside the cell obviously after induced for 48 h by 1% fat emulsion–10% FBS-RPMI-1640 medium. Compared with normal cells [Table 1], the intracellular content of triglyceride in model group (3.06 ± 0.01 mmol/L) increased almost two times than the control group (1.61 ± 0.02 mmol/L).

However, intracellular FFA content remained at about 70 ng/mL in steatosis hepatic cells. We speculated that more FFA was absorbed from 1% fat emulsion for the synthesis of endogenous triglyceride. Further investigation on L-FABP and FATP4 expressions were conducted to confirm our speculation. As shown in [Figure 2], the expressions of L-FABP and FATP4 in model cells were raising continuously. L-FABP content increased to about four times in the normal cells after induction for 36–48 h.
**Figure 2**: Contents of L-FABP and FATP4 in treated steatosis L-02 cells (x ± s, n = 3). Intracellular L-FABP and FATP4 contents were tested by ELISA assay kits from 4 to 60 h, mean ± SD were calculated and presented as bar graphs. For the statistical analysis, the * indicates a significant difference compared with control group, * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \); the # indicates a significant difference compared with model group, # \( P < 0.05 \), ## \( P < 0.01 \), ### \( P < 0.001 \).
Figure 3: Contents of SREBF-1c and PPAR-α in treated steatosis L-02 cells (x ± s, n = 3). Intracellular SREBF-1c and PPAR-α contents were tested by ELISA assay kits from 4 to 60 h, mean ± SD were calculated and presented as bar graphs. For the statistical analysis, the * indicates a significant difference compared with control group, * P < 0.05, ** P < 0.01, *** P < 0.001; the # indicates a significant difference compared with model group, # P < 0.05, ## P < 0.01, ### P < 0.001.
h. L-FABP and FATP4 were responsible for the transport and absorption of fatty acids.\textsuperscript{23,24} Therefore, high level of extracellular FFA content promoted hepatic L-FABP and FATP4 expressions, which provided more FFA material supply for the synthesis of endogenous triglyceride. On the one hand, TSG partly suppressed the expressions of L-FABP and FATP4 in steatosis hepatic cells [Figure 2], and TSG stored similar contents intracellular FFA as normal group; however, FFA content in TSG group had a slight increase compared with model group [Table 1]. On the other hand, TSG relieved a certain degree of lipid droplets formation [Figure 1] and the elevation of triglyceride was also alleviated by TSG after 18 hinduction ($P < 0.01$). These indicated TSG may also prevent triglyceride biosynthesis from intracellular FFA.

TSG prevented triglyceride biosynthesis

To clearly illustrate how TSG prevent triglyceride biosynthesis, we measured the influence of TSG on several triglyceride synthases. ACACA and FASN genes coded two rate-controlling enzymes in the triglyceride synthesis pathway. ACACA was in charge of transform Acetyl-CoA to Malonyl-CoA, where as FASN was in charge of transform Malonyl-CoA to Fatty Acyl-CoA.\textsuperscript{25}

After 8 h induction by fat emulsions [Table 3], the content of ACACA in model group raised about 50% than the normal group ($P < 0.001$). TSG effectively prevented ACACA content from increasing; however, the regulation activity of TSG was still weaker than FEN and SIM. Both FEN and SIM could keep the ACACA content similar with that in normal group. The content of FASN also showed significantly increasing during the process of steatosis [Table 3]. TSG intervention could also decrease FASN content significantly.

As TSG effectively inhibited ACACA and FASN expressions, both of which were rate control enzymes of triglyceride synthesis, we considered that triglyceride accumulation prevention effect of TSG was not only contributed by reducing of external FFA absorbing but also by inhibition of triglyceride biosynthesis from FFA.

TSG-regulated triglyceride transportation

MTTP, an important lipid transfer protein in endoplasmic reticulum,\textsuperscript{26} took part in triglyceride transportation and very low-density lipoprotein assembly. Fat emulsion induction suppressed the MTTP expression in model group, whereas TSG and positive drugs could enhance the expression of MTTP within 48 and 60 h. TSG gave out better up regulation effect on MTTP expression than FEN and SIM [Table 2]. MTTP expression in TSG group (133.3 ± 0.84 pg/mL) returned to about 83% of normal group (159.8 ± 0.01 pg/mL). However, we noticed that neither steatosis process nor TSG treatment altered Apo lipoprotein B100 expression significantly. Therefore, TSG regulated triglyceride transportation mainly through MTTP expression adjustment.

Figure 4: Contents of LPL in treated steatosis L-02 cells (x ± s, n = 3). Intracellular LPL contents were tested by ELISA assay kits from 4 to 60 h, mean ± SD were calculated and presented as bar graphs. For the statistical analysis, the * indicates a significant difference compared with control group, ** $P < 0.05$, *** $P < 0.01$, **** $P < 0.001$; the # indicates a significant difference compared with model group, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. 

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In our research, LPL activated almost all genes in the synthesis of fatty acid and triglyceride acted as a critical connection point in the network of lipid regulation and alleviated triglyceride accumulation in liver cells.

TSG promoted triglyceride decomposition

LPL was in charge of hydrolyzing triglycerides in lipoproteins into FFAs and monoacyl glycerol molecule.[29] In our research, LPL expression decreased significantly from 26 h after fat emulsion induction. We found TSG could obviously reverse this decreasing [Figure 4] and increase the content of LPL from 260.32 ± 9.07 pg/mL to 476.03 ± 35.07 pg/mL at 48 h. TSG exhibited even better LPL regulation activity than SIM and FEN at the end of the research. Promotion of triglyceride decomposition by TSG also helped to alleviate triglyceride accumulation in liver cells.

TSG suppressed activation factors in lipid metabolism

Transcriptional regulatory factors, such as SREBP-1c and PPAR-α, also play key role in triglyceride synthesis and transportation. SREBP-1c acted as a critical connection point in the network of lipid regulation and activated almost all genes in the synthesis of fatty acid and triglyceride in liver.[29] Activation of PPAR-α would promote oxidation of fatty acid and triglyceride decomposition, reduce lipid accumulation, and increase LPL content.[29] The expression of LPL regulated by PPAR-α, was critical to lipid metabolism. Activation of PPAR-α would result in increased LPL activity in the liver and skeletal muscle leading to higher triglyceride clearance.[30]

Our results [Figure 3] showed that fat emulsion induction reduced PPAR-α expression where as increased the content of SREBP-1c. Fortunately, these alternations induced by steatosis process were prevention by TSG. SREBP-1c increasing was effectively inhibited after treatment with TSG for 36 h. The expression of PPAR-α returned to normal level after treated with TSG for 2 days.

DISCUSSION

Regulation of lipid accumulation was a complicated biological process involving regulation of membrane transport proteins and cellular enzymes. Finding natural products in the traditional medicine system seemed to be a promising way in the prevention and treatment of NALFD. In this research, activities and mechanisms of TSG on biosynthesis and decomposition were investigated on this steatosis hepatic cell. Judging from Pearson's correlation coefficient between FFA, triglyceride, and key enzymes and transcriptional regulatory factors [Table 4], the intracellular FFA content was mainly negatively correlated with SREBP-1c expression from 8 to 18 h. In the beginning of the steatosis process, excess intracellular FFA was synthesis to triglyceride mainly involving regulation of membrane transport proteins and cellular enzymes. Therefore, high SREBP-1c expression was negatively correlated with intracellular FFA content.

The intracellular triglyceride content was correlated with several factors, such as FABP, FATP4, FASN, MTTP, and SREBP-1c. Expression of SREBP-1c was inhibited after TSG treatment. SREBP-1c increasing was effectively inhibited after treatment with TSG for 36 h. The expression of PPAR-α returned to normal level after treated with TSG for 2 days.
Figure 5: The hypothetical mechanisms of TSG on the triglyceride accumulation in hepatic cells. Dotted lines indicate normal biochemical pathways; green lines and + indicate the acceleration effects of TSG; red lines and – indicate the inhibition effects of TSG. Schematic representation of the mechanism has shown that TSG prevents triglyceride accumulation in hepatic cells through the multi targets regulation in triglyceride biosynthesis and decomposition. TSG inhibited SREBP-1c, promoted PPAR-α, and regulated rate control enzymes in triglyceride metabolism, thereby causing pronounced suppression on triglyceride accumulation in hepatic cells.

Table 4: Pearson correlation analysis between FFA, TG and key enzymes or proteins.

| Time  | L-FABP | FATP4 | ACACA | FASN | MTTP | Apo-B100 | LPL  | SREBF1c | PPAR-α |
|-------|--------|-------|-------|------|------|----------|------|---------|---------|
| 4 h   | -0.977*| -     | -     | -    | -    | -        | -    | 0.884*  | -       |
| 8 h   | -      | -     | -     | -    | -    | -        | -    | -0.911* | -       |
| 12 h  | -      | -     | -     | -0.942*| -    | -        | -    | -0.905* | -       |
| 18 h  | -      | -0.893*| -     | -    | -    | -        | -    | -0.932* | -       |
| 26 h  | -      | -     | -     | -    | -    | 0.903*   | -    | -       | -       |
| 36 h  | -      | -     | -     | -    | -    | -        | -    | -       | -       |
| 48 h  | -      | -     | -     | -    | -    | -        | -    | -       | -       |
| 60 h  | -      | -     | -     | -    | -    | -        | -    | -       | -       |
| 4 h   | -0.985**| -     | 0.925*| -    | 0.899*| 0.888*   | -    | 0.965**| -0.885* |
| 8 h   | 0.983**| -     | -     | -    | 0.930*| -        | -    | 0.901*  | -       |
| 12 h  | 18 h   | 18 h  | 18 h  | 0.997**| -    | -        | -    | 0.977*  | -       |
| 18 h  | 0.983**| -     | -     | -    | -    | -        | -    | -       | -       |
| 26 h  | 0.978**| -     | -     | -    | 0.896*| 0.903*   | -    | 0.974** | -       |
| 36 h  | 0.845  | -     | -     | -    | -    | 0.937*   | -    | -       | -       |
| 48 h  | 0.976**| 0.923*| -     | -    | -    | -        | -    | -       | -       |

- data was not listed when significance (p) was higher than 0.05; *: p < 0.05, significance (p) was less than 0.05; **: p < 0.01, significance (p) was less than 0.01.

PPAR-α. These agonists of the nuclear receptor PPAR were therapeutically used to combat hyperglycemia associated with the metabolic syndrome and type II diabetes and may be also beneficial for the NAFLD patients.

Our latest in-vivo research has confirmed these benefits. TSG as a natural stilbene significantly inhibited the accumulation of FFA in liver, which cuts off the supply of raw triglyceride materials for endogenous synthesis, possibly mediated by the decrease in L-FABP and FATP4 expression. From these results we concluded that TSG displayed multi targets regulation effects on triglyceride accumulation in hepatic cell [Figure 5]. It could not only limit the raw materials supply of triglyceride synthesis by reducing L-FABP and FATP4 expressions, but also inhibit SREBP-1c expression, and then reduce the contents of ACACA and FASN. Therefore, TSG could prevent the biosynthesis of triglyceride. Mean while, TSG also activated PPAR-α and increased β-oxidation of fatty acid. Thus, the decomposition of triglyceride was promoted by treatment of TSG.
CONCLUSION

TSG obviously blocked the first hit of NAFLD in the process of the lipid accumulation and could be considered as a promising drug candidate in prevention and treatment of NAFLD. Further researches of TSG on the second hit of NAFLD are still under investigation.

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Conflicts of interest

There are no conflicts of interest

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