Salvianolic acids improve liver lipid metabolism in ovariectomized rats via blocking STAT-3/SREBP1 signaling

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[ABSTRACT] Postmenopausal women, who have reduced circulating estrogen levels, are more prone to develop obesity and related metabolic diseases than premenopausal women. The absence of safe and effective treatments for postmenopausal obesity has changed the focus to natural products as alternative remedies. Total salvianolic acids (TSA) are the major water-soluble ingredients of Danshen. Salvianolic acid (SA) is the major constituent of the TSA. Salvianolic acids, including TSA and SA, are widely used in traditional Chinese medicine. In the present study, ovariectomized rats and LO2 cells were used to study the effects of salvianolic acids on body weight gain and hepatic steatosis. Salvianolic acids reduced ovariectomy (OVX)-induced body weight gain, attenuated the expressions of hepatic lipogenic genes, such as sterol regulatory element binding protein (SREBP)1, fatty acid synthase (FAS), and stearoyl-CoA desaturase (SCD)1, and decreased the liver triglyceride (TG) and total cholesterol (TC). For the molecular mechanisms, OVX and high glucose-induced phosphorylation of signal transducer and activator of transcription (STAT)-3 was inhibited by salvianolic acids treatment. In LO2 cells, inhibition of STAT-3 by siRNA attenuated the increased expression of SREBP1 and TG induced by high glucose. Salvianolic acids reduced the upregulation of SREBP1 and TG induced by high glucose in LO2 cells. In conclusion, these findings illustrated that salvianolic acids markedly alleviated the lipid metabolism disorders and protected against the postmenopausal obesity. The underlying mechanism was probably associated with the regulation of STAT-3 signaling.

[KEY WORDS] Salvianolic acids; Lipid metabolism; Weight gain; Signal transducer and activator of transcription 3
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Introduction

Salvia miltiorrhiza Bunge, also known as “Danshen”, is a shade-growing perennial flowering plant belonging to the

family Labiatae. It is an important and widely used medicinal plant in traditional Chinese medicine, which can exert protective effects on the liver, kidneys, and lungs, and used for prevention and treatment of vascular diseases \textsuperscript{[1-4]}. Salvianolic acid B is the major constituent of the salvianolic acids and the most active constituent among the phenolic acids, possessing a wide range of pharmacological effects, such as antioxidant, anti-ischemia-reperfusion and antitumor activities \textsuperscript{[5-8]}. However, knowledge about its role in postmenopausal obesity is still limited.

The menopause is an age-related loss of ovarian functions and a decline in circulating estrogen levels \textsuperscript{[9]}. Compared with premenopausal women, postmenopausal women are more prone to develop obesity and related metabolic diseases such as diabetes, cardiovascular disease, and non-alcoholic fatty liver disease \textsuperscript{[10-12]}. Hormone replacement therapy is the
most common and effective treatment for menopausal syndrome. However, the potential risks of breast cancer and endometrial cancer have resulted in much interest in investigating natural products to manage the increase in disease risk caused by the loss of ovarian hormones [13-14].

Signal transducer and activator of transcription (STAT)3 is a transcription factor closely associated with receptor kinases that mediate cellular signaling in response to ligand binding at the receptor. Studies reveal that STAT-3 has the ability to modulate many genes involved with cell growth, survival, and death [15]. Indeed, the STAT-3 signaling pathway is responsible for the regulation of gluconeogenesis and lipid metabolism in the liver in high-fat diet [16-17]. However, the potential molecular mechanisms of STAT-3 involvement in liver lipid metabolism need further study in an ovariectomy (OVX)-induced weight gain model.

Total salvianolic acids (TSA) are the water-soluble ingredients of Danshen. The composition has been revealed as salvianolic acid B, salvianolic acid A, caffeic acid, and other salvianolic acids [18]. TSA are widely used in orally administered herbal medicines and injection [19]. The aims of the present study were to evaluate the difference in effect on OVX-induced body weight gain between TSA and salvianolic acid (SA) and to explore the role of STAT-3 in liver lipid metabolism in OVX-induced weight gain model.

Materials and Methods

Reagents

Carboxymethyl cellulose sodium solution and estrogen (E2) were purchased from Sigma-Aldrich (St Louis, MO, USA). TSA and salvianolic acid (SA) were respectively purchased from Jiangsu Langze Medical Technology Co., Ltd. (Nanjing, China) and Shanghai Green Valley Pharmaceutical Co., Ltd. (Shanghai, China). TSA were water-soluble extract of Danshen, mainly contained two components, salvianolic acids B and A, in a ratio of 4 : 1. SA was the drug of salvianolate injection, mainly contains 80% pure salvianolic acid B.

Animals and treatments

This study was approved by Nanjing Medical University Institutional Animal Care and Use Committee (Permit Number: NJMU-IACUC-1403024), and the animals were treated humanely and with regard for alleviation of suffering. Fifty female Sprague-Dawley rats [20] weighing 220 ± 10 g were purchased from Beijing Vitalriver Experimental Animal Co., Ltd. [Certificate No: SCXK (Jing) 2012-0001]. All the animals were housed under controlled conditions (23 ± 2 °C, 55%–65% humidity, and 12 h/12 h light/dark cycle) with free access to food and tap water. The animals were acclimated for 1 week before experiments and then randomly divided into five groups of ten. All the other groups were subjected to bilateral OVX, except for the sham-operated (SHAM) group, which was subjected to the same surgical procedure, but the ovaries were preserved. One week after the operation, the rats were treated with drugs by oral gavage once daily for 50 days.

The groups were SHAM, OVX, OVX + E2, OVX + TSA, and OVX + SA. The drug concentrations were 1 mg kg⁻¹ of E2 and 50 mg kg⁻¹ of TSA and SA, respectively. The drugs were dissolved in 0.5% carboxymethyl cellulose sodium solution. The solution volume of drugs given to rats was 10 mL·kg⁻¹.

Cell culture

Human liver cell line LO2 was purchased from the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China. The LO2 cells were cultured in Dulbecco’s modified Eagle’s medium (5.56 or 25 mmol·L⁻¹ glucose) (Life Technologies/Gibco, Grand Island, NY, USA). The medium contained 10% fetal bovine serum (Life Technologies/Gibco), 100 mg·mL⁻¹ of streptomycin (Gibco) and 100 U·mL⁻¹ of penicillin (Gibco). The LO2 cells were maintained in the presence of 5% CO₂ at 37 °C.

Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA from the cells and liver were isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA). For the detection of mRNA, total RNA (1 μg) was transcribed into cDNA using AMV Reverse Transcriptase (Promega, Madison, WI, USA). qRT-PCR was performed using the LightCycler Sequence Detection System (Roche Applied Science, Switzerland) with cycling conditions as follows: 95 °C for 30 s, 95 °C 10 s and 60 °C for 30 s for 40 cycles. Fold changes in expression of each gene were calculated by a comparative threshold cycle (Ct) method with the formula 2⁻ΔΔCt.

Western blotting analysis

The proteins were quantified using the BCA Kit (Beiyotime Biotechnology, Haimen, China). 10% SDS-PAGE was used to separate the proteins, which were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA, USA). After blocking with 5% non-fat milk, the membranes were incubated with primary antibodies at 4°C overnight. Antibodies used in the present study were against AKT, p-AKT, STAT-3, and p-STAT-3 (Y705) (Cell Signaling Technology, Boston, MA, USA). The antibodies were diluted 1:1000. β-Actin (Beyotime) served as the internal control to eliminate the differences in protein loading. Followed by incubating them with horseradish peroxidase conjugated secondary antibodies (anti-rabbit IgG and anti-mouse IgG) (Beyotime Co. Ltd, the dilution was 1:1000) for 1 h at the room temperature. The antigen complexes were detected with enhanced chemiluminescence. For densitometric analyse, the bands on the blots were measured by the Image-Pro-Plus 6.0 software.

Cell transfection

siRNA-con and siRNA-STAT-3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA; http://datasheets.scbt.com/sc-37007.pdf and http://datasheets.scbt.com/sc-270027.pdf). The LO2 cells were transiently transfected with the siRNAs in the presence of Lipofectamine 2000 reagent (Invitrogen) for 12 h.

Measurement of hepatic lipids and total cholesterol

The dissected liver tissues (100 mg) were homogenized in 0.9 mL of 0.86% saline on ice. Residual tissue debris was pel-
leted by centrifugation at 2500 r·min$^{-1}$ for 10 min. The supernatant was used to quantify liver triglyceride (TG) and total cholesterol (TC) using the TG and TC Assay Kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The LO2 cells were digested by trypsin-EDTA Solution (Beyotime). The prepared LO2 cell suspension was centrifuged at 1000 r·min$^{-1}$ for 10 min. After washing twice, the cell precipitate was added to 200–300 μL of lysis solution for 30–40 min on ice, then the lysis solution were used for the assay.

**Statistical analysis**

The data were compared using GraphPad version 6.0 (La Jolla, CA, USA) and were presented as means ± SD. $P < 0.05$ was considered statistically significant.

**Results**

**Effects of salvianolic acids on body weight, body weight gain, and food intake**

Bilateral OVX in rats is a well-recognized and widely used animal model of human menopause [21]. We exposed OVX rats to E$_2$, TSA and SA; the initial body weights were not significantly different among all the groups. After 50 days, the OVX group exhibited significantly more weight gain than SHAM group did. In contrast, the weight gain of OVX + E$_2$ group was markedly lower than that of OVX group. Similarly, OVX + TSA and OVX + SA groups also showed less weight gain than OVX group did (Figs. 1A and 1B). There was no difference in OVX-induced body weight gain between TSA and SA groups. The food intake in OVX group was significantly higher than that in SHAM group. Compared with the OVX group, the OVX + E$_2$ group had significantly decreased food intake; however, no apparent difference was found for food intake among OVX, OVX + TSA, and OVX + SA groups (Fig. 1C). These results indicated that salvianolic acids decreased the body weight induced by OVX, not solely dependent on food intake. So, we further determined if the hepatic metabolism was involved in subsequent experiments.

![Fig. 1](image-url)  
Effects of salvianolic acids on body weight (A), body weight gain (B), and food intake (C). The data are expressed as means ± SEM, $n = 7–10$ rats/group. SHAM, sham operated; Vehicle, ovariectomized; E$_2$, OVX + estradiol; TSA, OVX + total salvianolic acids; SA, OVX + salvianolic acid. $^{**}P < 0.01$ vs SHAM, $^#P < 0.05$, $^{##}P < 0.01$ vs OVX

**Effects of Salvianolic acids on lipid levels of OVX rats**

The liver is the first tissue in the body to come in contact with metabolites from ingested diet and an important component in energy balance [15]. The levels of hepatic TG and TC in the OVX group were higher than that in OVX + E$_2$, OVX + TSA, and OVX + SA groups (Figs. 2A and 2B). Lipogenic genes such as hepatic sterol regulatory element binding protein (SREBP)1, fatty acid synthase (FAS), Acetyl-CoA Carboxylase (ACC)1, and stearoyl-CoA desaturase (SCD)1 are related to fat deposition and energy metabolism in the liver after OVX [22-24]. We further determined if OVX had effects on the expressions of lipogenic genes. As shown in Fig. 2C, OVX increased the expression of hepatic SREBP1, but there were marked decreases in hepatic SREBP1 in OVX + E$_2$, OVX + TSA, and OVX + SA groups. Similar results were obtained for the expressions of hepatic FAS and SCD1 (Figs. 2D and 2E). These results indicated that OVX increased the expression of lipogenic genes, which could be inhibited by salvianolic acids, decreasing OVX-induced fat accumulation in liver.

**Relationship between STAT-3 and lipogenesis in OVX rats**

STAT-3 plays an important role in adipogenesis [25]. Previous research has shown that STAT-3 is involved in lipid synthesis by regulating the expression of SREBP1 in high-fat diet [16]. In the OVX model, it was unclear whether STAT-3
was involved in OVX-induced obesity. We found that salvianolic acids inhibited the OVX-induced phosphorylation of STAT-3 in rats. Protein kinase B (PKB/AKT) signaling is involved in lipid metabolism. In the present study, treatment of the OVX rats with salvianolic acids upregulated the phosphorylation of AKT (Figs. 3A and 3B), indicating a classical activation of AKT signaling. Collectively, the in vivo results revealed that the effects of salvianolic acids on lipid metabolism might be associated with STAT-3/SREBP1 signaling.

Fig. 2 Effects of salvianolic acids on lipid levels of OVX rats. (A) Hepatic TG contents. (B) Hepatic TC contents. (C–E) mRNA of SREBP1, FAS and SCD1 were investigated by qRT-PCR. SHAM, sham operated; Vehicle, ovariectomized; E2, OVX + estradiol; TSA, OVX + total salvianolic acids; SA, OVX + salvianolic acid. *P < 0.05, **P < 0.01 vs SHAM, #P < 0.05, ##P < 0.01 vs OVX. The liver tissue of five rats from each group was mixed as a sample, three independent samples each group.

Fig. 3 Effects of salvianolic acids on STAT-3 in liver of OVX rats. (A, B) Western blotting and densitometry analysis of expression of p-STAT-3, STAT-3, p-AKT, and AKT. SHAM, sham operated; Vehicle, ovariectomized; E2, OVX + estradiol; TSA, OVX + total salvianolic acids; SA, OVX + salvianolic acid. ***P < 0.01 vs SHAM, ****P < 0.01 vs OVX. The liver tissue of five rats from each group was mixed as a sample, three independent samples each group.

Effects of Salvianolic acids on lipid levels in LO2 cells

Although STAT-3 signaling is involved in lipid metabolism, the regulatory mechanism of the whole animal model is complex. We determined if salvianolic acids had effects on phosphorylation of STAT-3, contents of TG, and expression of SREBP1 in high-glucose-treated LO2 cells. The used concentration of TSA and SA in the present study had no toxic effects on LO2 cells (data not shown). In accordance with OVX model,
salvianolic acids decreased high-glucose-induced phosphorylation of STAT-3 in LO2 cells (Figs. 4A and 4B). Similar results were found for the expression of SREBP1 and the contents of TG (Figs. 5A and 5B). Furthermore, we verified the relationship between STAT-3 and lipogenic genes, and found that knock down of STAT-3 (Figs. 6A and 6B) significantly attenuated the increased contents of TG and expression of SREBP1 induced by high glucose (Figs. 6C and 6D), but had no effect on FAS (Fig. 6E). These results indicated that salvianolic acids inhibited lipogenesis via STAT-3/SREBP1 signaling.

Discussion

The salvianolic acids, a group of plant-derived chemicals, have been identified having numerous biological functions. Several studies have reported the beneficial effects of salvianolic acids in the management of cerebrovascular diseases, hepatitis, diabetes, and diabetic complications [3, 27]. Our current study revealed that salvianolic acids inhibited the OVX-induced body weight gain and lipid accumulation in vivo, and the TG contents in LO2 cells in vitro. For the
molecular mechanisms, the STAT-3/SREBP1 signaling pathway was involved. Based on these findings, we suggested that the salvianolic acids played an essential role in lipogenesis in OVX rats and in LO2 cells.

Previous studies have indicated that OVX can induce body weight gain and liver TG accumulation due to estrogen deficiency [28]. Increasing food intake via hypothalamus is responsible for OVX-induced body weight gain. E2 has an anorexigenic functions in OVX rats [29]. In our study, E2 could inhibit the OVX-induced body weight gain and food intake. We also found that salvianolic decreased the body weight gain induced by OVX, but had no effect on food intake. Meanwhile, salvianolic acids inhibited the liver TG contents. These results indicated that salvianolic acids inhibited the OVX-induced body weight gain possibly by improving the hepatic lipid metabolism disorders. There were no differences in the inhibitory effects on OVX-induced body weight gain between TSA and SA, due to that Salvianolic acid B is the major constituent of the TSA. The results indicated that salvianolic acid B in TSA and SA was responsible for inhibiting OVX-induced body weight gain.

The synthesis of triglycerides requires the involvement of adipogenic genes [30]. SREBP1, an important nuclear transcription factor, is responsible for the biosynthesis of cholesterol, fatty acids, and TG, favoring the development of hypertriglyceridemia and fatty liver by means of activating the key enzymes of lipogenesis, such as FAS and ACC1 [31]. We found that salvianolic acids suppressed the upregulation of SREBP1, FAS, and SCD1 in OVX rats, suggesting that inhibition of adipogenic genes contributed to suppression of lipid accumulation and thus the improvement of metabolic function.

STAT-3 is an important transcription factor and contributes to various physiological processes [15]. Former studies have confirmed that overexpression of STAT-3 in the liver can increase the levels of atherogenic lipoproteins, likely through alteration of the expression of FAS and ACC involved in lipid metabolism [32]. It has been reported that STAT-3 plays an important role in regulating hepatic insulin resistance induced by palmitate [33]. We found that salvianolic acids inhibited the phosphorylation of STAT-3 induced by OVX. Different with our results, previous studies showed high-fat diet induced obesity owing to inhibition of phosphorylation of STAT-3 [17]. Therefore, we further identified the relationship between lipid metabolism and STAT-3 and explored whether salvianolic acids affected lipid metabolism through STAT-3.

The OVX, due to estrogen deficiency, could increase food intake and much energy storage in body. The high-glucose-treated LO2 cell model in vitro meaning much energy storage in cells, was in accordance with in vivo OVX model. It is reported that high glucose induces adipogenesis and enhances phosphorylation of STAT-3 [34-35]. Previous research has shown that STAT-3 is involved in lipid synthesis by regulating the expression of SREBP1 in high-fat diet [16]. In our study, we found that salvianolic acids inhibited the upregulation of phosphorylation of STAT-3 and SREBP1 and increased contents of TG by high-glucose-treatment in LO2 cells. Furthermore, knock down of STAT-3 significantly attenuated the increased contents of TG and upregulation of SREBP1 in-
duced by high glucose, but had no effect on FAS. Therefore, it demonstrated that phosphorylation of STAT-3 is involved in lipid synthesis. Our in vitro study also confirmed the improvements in lipid metabolism by salvianolic acids in LO2 cells. STAT-3/SREBP1 signaling pathway was involved in salvianolic acids-inhibited synthesis of TG.

In conclusion, our results indicated that STAT-3 played a pivotal role in the regulation of lipid metabolism by salvianolic acids. Salvianolic acids inhibited O VX-induced body weight gain via inactivating STAT-3/SREBP1 signaling. This study provided a new perspective on biochemical and molecular aspects of metabolic complications associated with postmenopausal women.

References


