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Salvianolic acid B accelerated ABCA1-dependent cholesterol efflux by targeting PPAR-γ and LXRα

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ABSTRACT

Objectives: Cholesterol efflux has been thought to be the main and basic mechanism by which free cholesterol is transferred from extra hepatic cells to the liver or intestine for excretion. Salvianolic acid B (Sal B) has been widely used for the prevention and treatment of atherosclerotic diseases. Here, we sought to investigate the effects of Sal B on the cholesterol efflux in THP-1 macrophages.
Methods: After PMA-stimulated THP-1 cells were exposed to 50 mg/L of oxLDL and [^{3}H] cholesterol (1.0 μCi/mL) for another 24 h, the effect of Sal B on cholesterol efflux was evaluated in the presence of apoA-1, HDL_2 or HDL_3. The expression of ATP binding cassette transporter A1 (ABCA1), peroxisome proliferator-activated receptor-gamma (PPAR-γ), and liver X receptor-alpha (LXRα) was detected both at protein and mRNA levels in THP-1 cells after the stimulation of Sal B. Meanwhile, specific inhibition of PPAR-γ and LXRα were performed to investigate the mechanism.
Results: The results showed that Sal B significantly accelerated apoA-I- and HDL-mediated cholesterol efflux in both dose- and time-dependent manners. Meanwhile, Sal B treatment also enhanced the expression of ABCA1 at both mRNA and protein levels. Then the data demonstrated that Sal B increased the expression of PPAR-γ and LXRα. And the application of specific agonists and inhibitors of further confirmed that Sal exert the function through PPAR-γ and LXRα.
Conclusion: These results demonstrate that Sal B promotes cholesterol efflux in THP-1 macrophages through ABCA1/PPAR-γ/LXRα pathway.

1. Introduction

Reverse cholesterol transport (RCT) is a pathway which is constituted of the efflux of excess cellular cholesterol from peripheral tissues and the return of the cholesterol to the liver for excretion [1,2]. RCT, as well as cholesterol efflux, has been confirmed to be the major mechanism by which HDL protects against atherosclerosis [3]. Cholesterol has been thought difficult to be catabolized by most peripheral cells and tissues [1], and thus its metabolism needs effluxing transporters, such as ABCA1 [4,5] and extra cellular acceptors, such as apoA1 and HDL [1]. Cholesterol-loaded macrophages, which subsequently developed into foam cells, are the most important participant of the atherosclerotic lesion, and cholesterol efflux from macrophages can protect against the progression of atherosclerosis [7]. Evidences have shown the critical effect that cholesterol efflux played in preventing atherosclerosis in humans and animals [2,8]. Therefore, acceleration of cholesterol efflux by increasing HDL, apoA1 or ABCA1 levels may result in amelioration of atherosclerosis.

Salvianolic acid B (Sal B), also known as lithospermic acid B or tanshinoate B, is isolated from the root of the Chinese herb Danshen (Salvia miltiorrhiza Bunge). In the past several years, Sal B, as well as the other substance extracted from Danshen, have been widely used for the prevention and treatment of atherosclerotic vascular diseases including coronary artery disease and stroke in China even the western countries. As a water-soluble compound of three molecules danshennol and one molecule of caffeic acid, Sal B is the main constituent of Salvia phenolic acid substances. The previous studies have confirmed that Sal B can improve the blood hemorrheology, decrease oxidative injury and repair the function of blood vessel endothelium. Sal B was able to scavenge against free

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hydroxyl radicals (HO), superoxide anion radicals (O^{2-}), and inhibit lipid peroxidation, and thus it has been seen as an antioxidant [9]. Sal B also inhibited macrophage uptake of modified low density lipoprotein in a scavenger receptor CD36-dependent manner and was confirmed to be an effective CD36 antagonist. The potential of Sal B in the inhibition LDL oxidation, decrease of plasma cholesterol level, reduction of endothelial damage and the severity of atherosclerosis in diet-induced hypercholesteremic rabbits have been also elucidated [10].

Based on these results, cholesterol efflux has been confirmed to be the major mechanism of lipid metabolism by which HDL protects against atherosclerosis, while Sal B played many kinds of important roles in lipid metabolism and protection against atherosclerosis. However, there is still no investigation focused on the effects of Sal B on cholesterol efflux. The present study was designed to clarify the relationship of Sal B and cholesterol efflux.

2. Material and methods

2.1. Reagents

Salvianolic acid B was purchased from Sigma–Aldrich (St Louis, MO). Rabbit polclonal to ABCA1, PPAR-γ, LXRα and GAPDH antibodies were from Abcam (Cambridge, UK). PPAR-γ antagonist GW9662, LXRα antagonist geranylgeranyl pyrophosphate (GGPP) ammonium salt, PPAR-γ agonist Rosiglitazone and LXRα agonist GW3965 were all purchased from Sigma–Aldrich (St Louis, MO).

2.2. Cell culture

THP-1 cell line was purchased from American Type Culture Collection (Manassas, VA, USA). THP-1 cells were maintained in RPMI 1640 (Hycolone) supplemented with 10% fetal bovine serum (FBS, Hycolone), streptomycin (100 mg/mL), and penicillin (100 U/mL) at 37 °C in a humidified atmosphere 5% CO_2. When the concentration of THP-1 cells in the 6-well culture dishes reached 5×10^5 cells per well, medium containing 160 nmol/ml of phorbol 12-myristate 13-acetate (PMA) was added into the cells for 72 h to stimulate the cells differentiation into macrophages.

2.3. Cholesterol efflux assay

After differentiated into macrophages under PMA-stimulation, THP-1 cells were exposed to 50 mg/L of oxLDL and [^{3}H] cholesterol (1.0 μCi/mL) for 24 h in RPMI 1640 supplemented with 0.2% BSA [11]. The cholesterol-loaded macrophages were then washed with PBS for three times and exposed to different concentrations of Sal B for 24 h or 10 μM Sal B for varying times in the presence of apoA-1 (10 μg/mL), HDL_2(50 μg/mL) or HDL_3(50 μg/mL) [11]. For analysis of cholesterol efflux to apoA-1, HDL_2 and HDL_3, the medium and the THP-1 macrophages were collected respectively, and the radioactive content in and out of the cells was determined by liquid scintillation. The percentage of cholesterol efflux was calculated by dividing the radioactive content in the medium by the sum of the radioactive content in the medium and in the cells.

2.4. Western blot analysis

The protein of THP-1 macrophages after stimulation was extracted with radioimmuno-precipitation assay (RIPA) (Beyotime, China). After measurement of the concentration, protein lysates were subjected in 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) at 90 V for electrophoresis and then transferred to the nitrocellulose membranes (Millipore, USA) with 200 mA for 2 h. Subsequently, the nitrocellulose membranes were incubated with primary antibodies overnight at 4 °C and secondary antibodies for 2 h. Finally, the antigen–antibody complex was detected with electro chemiluminescence (ECL) detection system (Millipore, USA).

2.5. Real-time PCR

Total RNA was extracted with TranZol Up (Trans, China) from the THP-1 macrophages after stimulation. One microgram of mRNA was reversely transcribed by using the First Strand cDNA Synthesis Kit (Fermentas, UAB). After that, total cDNA was amplified using the First Start Universal SYBR Green Master (ROX) (Roche, Swiss Confederation) for 40 cycles at 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 30 s in the Light Cycler (Roche, Basel, Switzerland) real-time PCR detection system. 18s was chosen as the reference gene and the primer sequences for real-time PCR analyses were as follows: 18S, forward primer: 5′-CTTAGTTGGTGGAGCGATTTG-3′, reverse primer: 5′- GCTGAACGCCACTTGTCC-3′ [12]. ABCA1, forward primer: 5′-AACAGTTTGTGGCCCTTTTC-3′; reverse primer: 5′-AGTTCCAGGCTGGGGTACTT-3′.

2.6. Statistical analysis

Predictive analytics software (PASW) Statistics 18.0 (SPSS Inc, Chicago, IL) was used to analyze the data. The normally distributed data were analyzed by one-way ANOVA and the nonparametric variables were analyzed by Mann–Whitney U test. Statistical significance was confirmed as $P < 0.05$.

3. Results

3.1. Salvianolic acid B increases cholesterol efflux to ApoA1, HDL_2 and HDL_3 in THP-1 macrophages

We firstly wanted to determine whether the percentage of cholesterol efflux could be changed by Sal B. The cholesterol-loaded THP-1 macrophages were exposed to different concentrations of Sal B (0, 0.1, 1 and 10 μM) for 24 h in the presence of apoA-1, HDL_2 or HDL_3 in the medium. The results confirmed the ability of Sal B to accelerate cholesterol efflux to the mediums. As shown in Fig. 1A and B, 1 μM of Sal B could significantly accelerate cholesterol efflux to ApoA1 and HDL_2 ($P < 0.05$), and 10 μM of Sal B dramatically accelerate cholesterol efflux to ApoA1 and HDL_2 approximately 2.5 and 3.2 folds respectively compared with control group ($P < 0.01$). As shown in Fig. 1C, Sal B could also increase cholesterol efflux to HDL_3 ($P < 0.05$) when its concentration reached 10 μM.

Subsequently, 10 μM of Sal B was used to stimulate the THP-1 macrophages for varying times (0, 3, 6, 12, 24 h). As shown in Fig. 1D and E, Sal B could not exert its role on cholesterol efflux to the three mediums for 6 h, while cholesterol efflux was significantly enhanced after 12 h incubation ($P < 0.05$), and dramatically enhanced after 24 h to ApoA1 and HDL_2 ($P < 0.01$). The results above indicated that Sal B could concentration- and time-dependently accelerate cholesterol efflux to ApoA1, HDL_2 and HDL_3 in THP-1 macrophages.

3.2. Salvianolic acid B enhances the expression of ABCA1 in THP-1 macrophages at both protein and mRNA levels

ABCA1 has been seen as the major transporter and regulator of cellular cholesterol and phospholipid homeostasis. The binding of apoA-I to ABCA1 leads to the formation of phospholipid-apoA-1 complexes, which subsequently promote cholesterol efflux [13]. Thus, we examined the effect of Sal B on the expression of ABCA1 in THP-1 macrophages. In order to investigate the effect of Sal B on

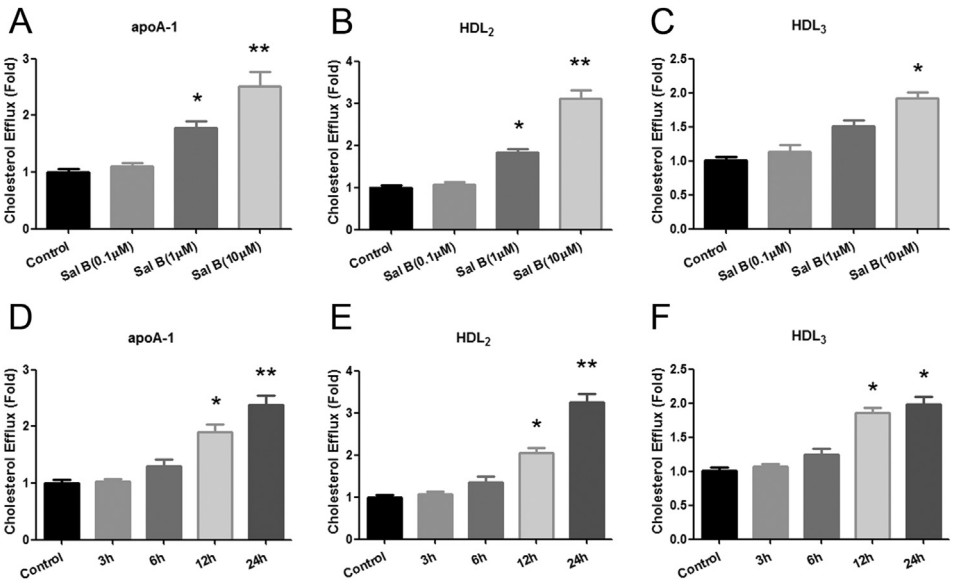

Fig. 1. Effects of Sal B on cholesterol efflux in THP-1 macrophages. After PMA-stimulated THP-1 cells were exposed to ox-LDL and [^{3}H] cholesterol for 24 h, the effect of Sal B on cholesterol efflux was evaluated in the presence of apoA-1, HDL_2 or HDL_3. (A–C) The cells treated with different concentrations of Sal B (0, 0.1, 1 and 10 μM) for 24 h, and Sal B enhanced cholesterol efflux in a concentration-dependent manner. (D–F) The cells treated with 10 μM of Sal B for different times (0, 3, 6, 12, 24 h), and Sal B also enhanced cholesterol efflux in a time-dependent manner. *P < 0.05 versus control group; **P < 0.01 versus control group. Data shown are means ± SEM from three independent experiments in duplicate.

ABCA1 expression in THP-1 macrophages, the cells were treated with different concentrations (0, 0.1, 1 and 10 μM) of Sal B for 24 h. ABCA1 was detected by western blot and real-time PCR respectively at protein and mRNA levels. As shown in Fig. 2A and C, compared with the control group, the expression of ABCA1 was significantly increased under the stimulation of 1 ($P < 0.05$) and 10 μM Sal B ($P < 0.01$), while 0.1 μM of Sal B failed to enhance the expression of ABCA1. Simultaneously, real-time PCR was used to examine the

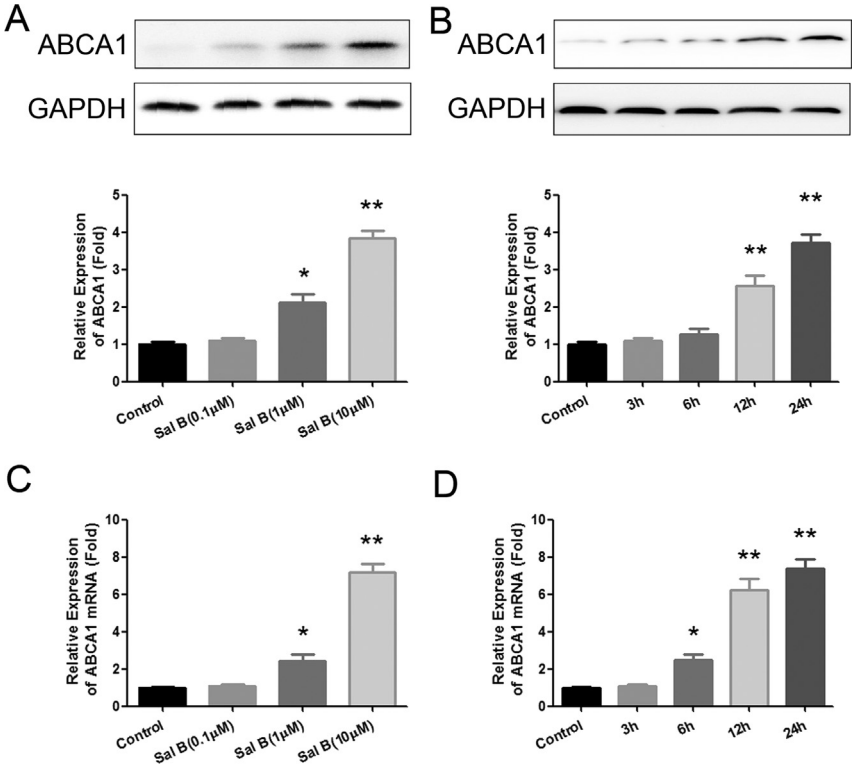

Fig. 2. Sal B up-regulates ABCA1 expression in THP-1 macrophages. Sal B of different concentrations (0, 0.1, 1 and 10 μM) were used to stimulate THP-1 macrophages for 24 h. Western blot (A) and real-time PCR (C) analysis were used to detect the expression of ABCA1 respectively. Then, 10 μM Sal B was used again for different times (0, 3, 6, 12, 24 h), and western blot (B) and real-time PCR (D) analysis were used to detect the expression of ABCA1. *P < 0.05 versus control group; **P < 0.01 versus control group. Data shown are means ± SEM from three independent experiments in duplicate.

mRNA level of ABCA1 after the treatment of Sal B, and the similar results were obtained. Thus ABCA1 production was induced by Sal B in a concentration-dependent manner. Furthermore, Sal B of 10 μM was used to stimulate the cells for different time (0, 3, 6, 12 and 24 h). As shown in Fig. 2B and D, the relative expression of ABCA1 was not increased significantly compared with the control group until the stimulation time was prolonged to more than 12 h ($P < 0.01$). In the other hand, the relative expression of ABCA1 mRNA was significantly increased from 6 h. As a result, we concluded that Sal B induced ABCA1 up-regulation in a time-dependent manner. All the data above showed that Sal B induced ABCA1 production both at protein and mRNA levels.

3.3. The expression of ABCA1 induced by salvianolic acid B is mediated through PPAR-γ and LXRα

PPAR-γ agonists were reported to increase the expression of ABCA1, which has been seen as a downstream target gene of PPAR-γ [14]. And in our previous study, we also confirmed the effect of PPAR-γ on the expression of ABCA1 [15]. LXRα was identified as the target gene of PPAR-γ, which was reported to directly up-regulate the expression of LXRα by binding the PPRE within the promoter region of LXRα [16]. In order to affirm whether PPAR-γ and LXRα were involved in the up-regulation of ABCA1 by Sal B, western blot was performed to examine the expression of PPAR-γ and LXRα in THP-1 macrophages after the stimulation of Sal B. As shown in Fig. 3A and B, the expression of PPAR-γ was significantly increased under the stimulation of 1 and 10 μM Sal B for 24 h ($P < 0.01$), while 0.1 μM of Sal B didn't statistically enhance the expression of PPAR-γ. In the following experiments involved in the expression of LXRα, we also found that Sal B played a similar regulating role just as PPAR-γ.

Then, to further clarify whether Sal B induced ABCA1 production through PPAR-γ/LXRα pathway, western blot and real-time PCR were performed to examine the expression of ABCA1 after the agonists and antagonists of PPAR-γ and LXRα were respectively applied. As shown in Fig. 3B and C, both the PPAR-γ antagonist GW9662 (20 μM) and LXRα antagonist GGPP (5 μM) dramatically inhibited the up-regulating effect of Sal B on the expression of ABCA1 at both protein and mRNA levels ($P < 0.01$), while PPAR-γ agonist rosiglitazone (1 μM) and LXRα agonist GW3965 (5 μM) could drammatically enhance the expression of ABCA1($P < 0.01$).

3.4. Specific inhibition and activation of PPAR-γ and LXRα attenuates the effect of Salvianolic acid B on cholesterol efflux

In order to further ascertain the effect of PPAR-γ/LXRα pathway played in the course of cholesterol efflux, the agonists and antagonists of PPAR-γ and LXRα were respectively applied again to treat the THP-1 macrophages. As shown in Fig. 4A–C, after 24 h incubation, Sal B could exert its role on cholesterol efflux to apoA1 ($P < 0.01$), HDL_2 ($P < 0.01$) and HDL_3 ($P < 0.05$), while cholesterol efflux was significantly inhibited after co-incubation with Sal B and PPAR-γ antagonist GW9662 or LXRα antagonist GGPP.

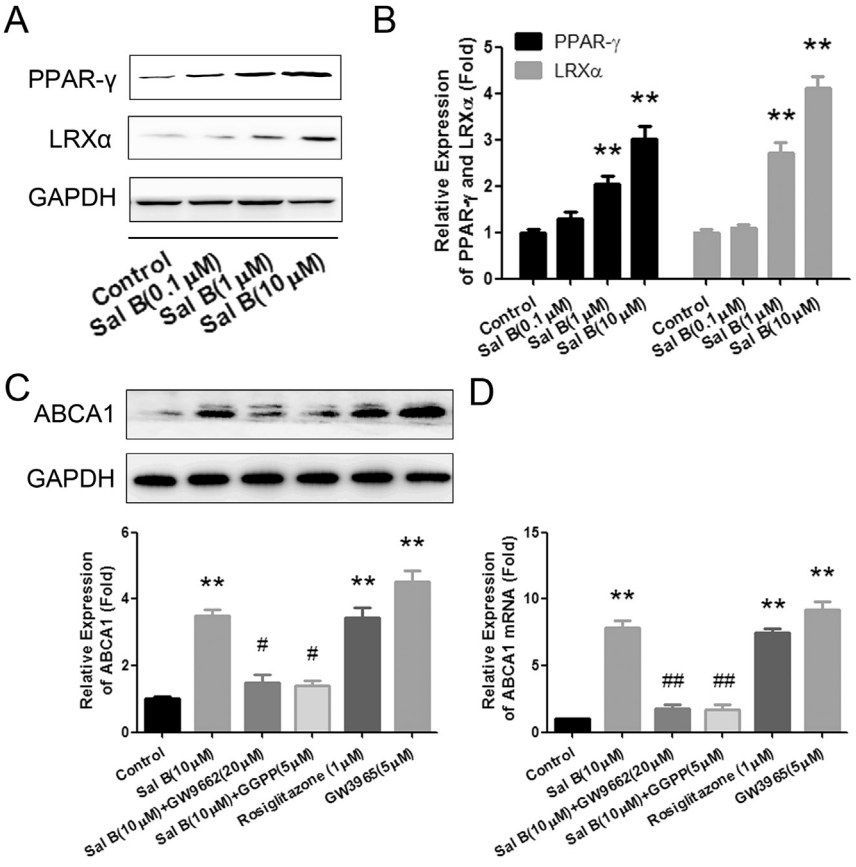

Fig. 3. PPAR-γ and LXRα mediated the regulation of ABCA1 by Sal B. THP-1 macrophages were stimulated with 0.1, 1 and 10 μM of Sal B for 24 h, and the expression of PPAR-γ and LXRα were significantly increased in a concentration-dependent manner (A–B). Pre-incubation with PPAR-γ antagonist GW9662 (20 μM) and LXRα antagonist GGPP (5 μM) dramatically inhibited the up-regulating effect of Sal B (10 μM) on the expression of ABCA1 at both protein and mRNA levels, while PPAR-γ agonist Rosiglitazone (1 μM) and LXRα agonist GW3965 (5 μM) could dramatically enhance the expression of ABCA1 (C–D). *$P < 0.05$ versus control group; **$P < 0.01$ versus control group; #$P < 0.05$ versus the corresponding Sal B group; ##$P < 0.01$ versus the corresponding Sal B group. Data shown are means ± SEM from three independent experiments in duplicate.

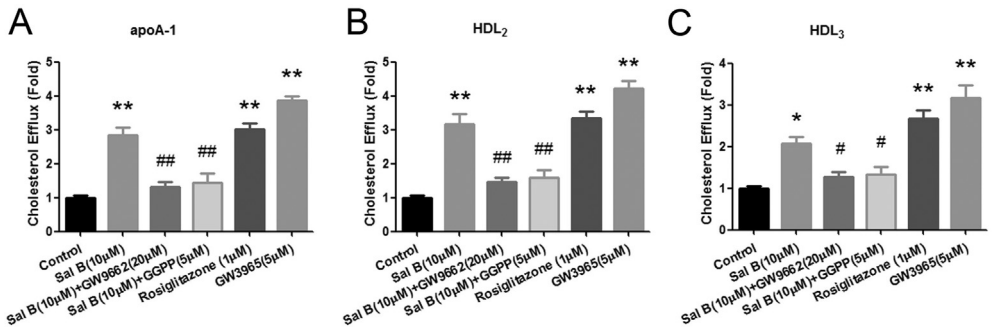

Fig. 4. Sal B-induced cholesterol efflux was mediated by PPAR-γ and LXRα. Pre-incubation with PPAR-γ antagonist GW9662 (20 μM) and LXRα antagonist GGPP (5 μM) dramatically inhibited Sal B-induced cholesterol efflux in THP-1 macrophages, while PPAR-γ agonist Rosiglitazone (1 μM) and LXRα agonist GW3965 (5 μM) dramatically enhance Sal B-induced cholesterol efflux (A–C). *P < 0.05 versus control group; **P < 0.01 versus control group; #P < 0.05 versus the corresponding Sal B group; ##P < 0.01 versus the corresponding Sal B group. Data shown are means ± SEM from three independent experiments in duplicate.

Subsequently, when the THP-1 macrophages were treated with PPAR-γ agonist rosiglitazone and LXRα agonist GW3965, cholesterol efflux to the three mediums were all dramatically increased (P < 0.01). The results above indicated that PPAR-γ/LXRα pathway played a pivotal role in cholesterol efflux, and Sal B-accelerated cholesterol efflux was mediated by PPAR-γ/LXRα pathway in THP-1 macrophages.

4. Discussion

Reverse cholesterol transport has been thought to be the main and basic mechanism by which free cholesterol from extra hepatic cells, such as macrophages, is transferred to the liver or intestine for excretion in the bile and feces. As the most important part and process of RCT, cholesterol efflux mediated the transport of cholesterol out of the cholesterol-loaded macrophages within the vessel wall to the acceptors such as HDL and apoA-1, under the existence and assistance of ABCA1, by which the accumulation of cholesterol in the artery is decreased and the development of atherosclerosis is prevented or decelerated at least.

Salvianolic acid B, a condensate of three molecules danshennol and one molecule of caffeic acid, is extracted from the Salvia miltiorrhiza's dried roots and rhizomes with large consumption worldwide. There is an accumulation of evidence that Sal B may exert cardiovascular protecting effects through inhibition of oxidative stress [17] and lipid peroxidation [18], reducing the generation of ROS, increasing the release of NO, inhibition of platelet aggregation and adhesion [19], anti-inflammatory activity [20] and suppression of apoptosis induced by other factors. Thus, although there is still no direct evidence of Sal B involved in modulating lipid metabolism and improving dyslipidemia, we want to know whether Sal B could improve the cholesterol transport. In the present study, apoA-1, HDL2 and HDL3 were used as the acceptors of the cholesterol in THP-1 macrophages. After stimulation, we found that Sal B could concentration- and time-dependently enhanced apoA-1 and HDL-mediated cholesterol efflux in oxLDL-stimulated THP-1 macrophages.

Afterwards, in order to clarify the mechanism of Sal B-induced cholesterol efflux in THP-1 macrophages, the expression of ABCA1 at both protein and mRNA levels were detected. Previous work has implicated that ABCA1, as a 250 kDa transmembrane protein and one of the most important member of the ABC family of transporter proteins, has been proved to be expressed ubiquitously in human tissues [21], especially in the lipid-loaded macrophages [22]. Research on humans with deficient ABCA1 suggested that compromised ABCA1 activity leads to accelerated cholesterol deposition in macrophages within the arterial wall and cholesterol efflux in humans [23]. Study on atherosclerosis-susceptible mice demonstrated that the selective absence of ABCA1 has a major impact on plasma lipoprotein homeostasis and markedly increased lesion size [24]. These findings further confirmed that ABCA1-mediated cholesterol efflux is an important anti-atherosclerotic process which prevents excess cholesterol accumulation in macrophages and ABCA1 dysfunction promote atherogenesis. In the present study, data showed that Sal B induced ABCA1 production both at protein and mRNA levels in concentration- and time-dependent manners. After the stimulation of Sal B of 10 μM for 24 h, the expression of ABCA1 was elevated approximately 4 folds compared with the control group.

PPAR-γ, one of the most important members of the nuclear receptor super-family, is now considered to be involved in regulating lipid metabolism and cell differentiation in macrophages [25]. PPARγ functioned as a key transcriptional regulator controlling the expression of multiple genes that mediated the homeostasis cholesterol transports [26]. Using PPARγ conditional gene knockout mice, the expressions of ABCA1, ABCG1 and apo E were lowered and cholesterol efflux from cholesterol-loaded macrophages to HDL was significantly reduced [26]. Recent evidence indicated that PPAR-γ could be expressed in all major cell types in the atherosclerotic lesions [14], especially at high levels in macrophages and foam cells [27]. Consistent with these results, PPAR-γ agonists were confirmed to suppress the differentiation from macrophage to foam cell in the peritoneal cavity through distinct ABCA1-independent pathways [14]. In addition, PPAR-γ activators diminished triglyceride accumulation in human monocyte-macrophages via the repression of apoB-48R pathway [28]. As a nuclear receptor, LXRα also functioned as a key regulator to maintain cellular cholesterol homeostasis in macrophages. The activation of LXRα led to induction of genes involved in the cholesterol efflux, such as ABCA1 and ABCG1 [29], to reduce the intracellular cholesterol overload. Synthetic LXR activators could significantly induce cholesterol mobilization to the plasma membrane and increase the amount of cholesterol in the plasma membrane through NPC1 and NPC2 pathway, which was thought to be a determinant step for cholesterol efflux [30]. In the previous study involved in cholesterol efflux, all of pioglitazone [31], niacin [32], and miR-613 [6] were potent activators of cholesterol efflux and played roles through activating PPAR-γ/LXRα pathway.

In the present study, we have provided evidence that PPAR-γ/LXRα pathway participates in the regulation of Sal B on macrophage cholesterol efflux. The expression of PPAR-γ and LXRα were both significantly increased under the stimulation of Sal B in a concentration-dependent manner. Subsequently, the agonists and antagonists of PPAR-γ and LXRα were respectively applied to treat

the THP-1 macrophages to further ascertain the effect of PPAR-γ/LXRα pathway played in the course of cholesterol efflux. The results demonstrated that both PPAR-γ antagonist GW9662 and LXRα antagonist GGPP could suppress the effect of Sal B, while PPAR-γ agonist rosiglitazone and LXRα agonist GW3965 could significantly promote cholesterol efflux to the mediums. Accordingly, the similar tendency was also found in the expression of ABCA1 under the same stimulation above.

Taken together, our data indicate that Sal B promotes the expression of ABCA1 and subsequently cholesterol efflux in macrophages through PPAR-γ/LXRα signaling. These results provide new insight for the role of Sal B on cholesterol efflux, which might be a new approach to protect against the formation of foam cells and atherosclerotic plaques.

Conflict of interest

None declared.

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Transparency document

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