

Original Article

Salidroside reduce inflammatory cytokines in atherosclerosis via suppressing MAPK and NF- κ B signaling pathway

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Abstract: Objective: Atherosclerosis is a kind of cardiovascular disease with very high mortality. Recent studies found that inflammatory response in the cardiovascular system accelerated atherosclerosis. The effect and underlying mechanism of salidroside on atherosclerosis remains unclear. The aim of our study was to investigate possible mechanism of salidroside on atherosclerosis. Methods: 60 ApoE^{-/-} mice were divided into four groups: normal diet group (NDG), high fat diet group (HFDG), HFDG with 20 mg/kg/day of salidroside and HFDG with 40 mg/kg/day of salidroside. Weight of the mice was measured through the whole study. ELISA was used to detect the inflammatory factors in the mice serum to evaluate the effect of salidroside on the expression of inflammatory cytokines. Western Blot was used to detect the activation of MAPK and NF- κ B signaling pathway, as well as the expression of eNOS and NRF2, in the aorta of ApoE^{-/-} mice. The phosphorylation of p38 and p65 were also detected by Western Blot. Results: Compared with the HFDG, the level of inflammatory cytokines (TNF- α , IL-1 β , and IL-6) were decreased after the treatment of 20 mg/kg and 40 mg/kg salidroside, in a dose-dependent manner. What's more, phosphorylation of p38 and p65 were increased in HFDG while they were suppressed in the group treated with salidroside. We also found that the expression of eNOS and NRF2 were increased after the treatment of salidroside. Conclusion: Salidroside could inhibit the inflammatory responses induced by HFD in the ApoE^{-/-} mice. Salidroside may reduce the expression of inflammatory cytokines by suppressing MAPK and NF- κ B signaling pathway.

Keywords: Salidroside, atherosclerosis, MAPK, NF- κ B

Introduction

Atherosclerosis is a kind of cardiovascular disease, which has become the leading cause of mortality and disability of cardiovascular related death [1-3]. The cause of atherosclerosis is complex. Recent studies have shown that low-density lipoproteins, extracellular matrix, inflammatory factors, oxidative stress, etc., were among the risk factors of atherosclerosis [4-6]. Among these risk factors, inflammatory related factors were proven to be a key element in the formation of atherosclerosis. According to recent study, inflammatory cytokines could be divided into two groups: pro-atherogenic cytokines (such as TNF- α , IL-1 β , and IL-6) and anti-atherogenic cytokines (such as TGF- β , IL-10, and IL-35) [6]. Atherosclerosis was atten-

uated when these pro-atherogenic cytokines were suppressed in mice model [7, 8]. It has been reported recently that the activated endothelial cells could express adhesion molecules, which attract monocytes and macrophages, thus causing the inflammatory responses [9, 10]. Therefore, suppressing the pro-atherogenic cytokines or increasing the anti-atherogenic cytokines is a promising method of treating atherosclerosis.

At present, studies have shown that anti-inflammation drugs can be applied in treating or preventing atherosclerosis [6]. These drugs can be divided into two major types, i.e. broad anti-inflammatory drugs (BAD) and specific anti-inflammatory drugs (SAD) [6]. BAD (Statins, aspirin, methotrexate) can inhibit various types

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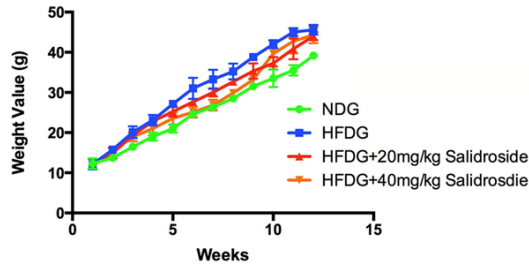


Figure 1. Measure the weight change of mice in different groups. 60 mice were divided into four groups, and treated with different diet. No significant difference was found.

of inflammatory factors. These drugs have been applied in clinical trials or practice, indicating that anti-inflammatory drug is a promising approach of treating or preventing atherosclerosis.

Salidroside is the effective component of *Rhodiola rosea*. It has been reported that salidroside is an effective drug, which could be applied in treatment of many different diseases, including cardiovascular disease. Recent study showed that salidroside had protective effect on coronary artery in myocardial ischemia-reperfusion injury [11]. Salidroside could also protect the pulmonary arterial hypertension via AMPK α 1 signal pathway and A2a receptor pathway [12, 13]. These studies indicated that the salidroside could protect cardiovascular cells in different adverse situations, via inhibiting inflammatory signaling pathways, such as MAPK signaling pathway. However, the effect and underlying mechanism of salidroside in atherosclerosis remains unclear. In this study, we mainly focused on the effect of salidroside on atherosclerosis in ApoE $^{-/-}$ mice model, and discussed the possible mechanism of salidroside in treating atherosclerosis.

Methods

Salidroside

Salidroside used in this study was obtained from National Institute for Food and Drug Control (Purity > 98%).

Animals

This study was performed in accordance with the National Institutes of Health (NIH) Animal Use Guidelines and a protocol that was approved by the Southern Illinois University (SIU) Animal Care Committee. All experimental pro-

cedures were approved by the Institutional Review Board of the Third Military Medical University. Sixty ApoE $^{-/-}$ mice purchased from HFK (Beijing HFK Bioscience Co., Ltd., Beijing) were divided into four groups according to random number table): 1. Normal group (n=15). The mice received a normal diet in this group (10% calories derived from fat in diet) (D12-450B, Research Diets, NJ); 2. High fat diet group (HFDG) (n=15). The mice in this group received a high fat diet (60% calories derived from fat in diet) (D12492, Research Diets, NJ); 3. High fat diet group (n=15), treated with 20 mg/kg/day of salidroside; 4. High fat diet group (n=15), treated with 40 mg/kg/day of salidroside. Water was abundantly supplied. All diets were kept for 12 weeks. All mice were kept in standard SPF environment with a 12 h: 12 h light and dark cycle at a temperature of 21~25°C.

Western blot analysis

The method used to extract protein from the mice cardiovascular was described previously (Chang XY, Inter J Card, 2016). Briefly, tissue from mice was frozen by liquid nitrogen and was pestle into powder, then lysed in RIPA buffer containing PMSF (Protease and Phosphatase inhibitors) and quantified via BCA protein assay. Different kinds of samples were loaded on 10% SDS-PAGE for separation and were electro transferred to the PVDF membranes (Millipore, USA). The target membranes were used to block 5% milk for 2 hours and incubated overnight at 4°C with the specific primary antibodies. After that, the membranes were washed for 5 minutes in TBST (Boster, China) and incubated with secondary antibody. At last, the membranes were visualized by a gel imaging system. The protein bands were quantified using densitometry analysis in Quantity One software (Bio-Rad, USA), and were analyzed using GraphPad Prism software (GraphPad, USA). Primary antibodies includes: p38, phosphorylated p38 (p-p38), p65, phosphorylated p65 (p-p65), antibodies were obtained from Cell Signaling Technology (MA, USA), eNOS and NRF2 antibodies were obtained from Abcam (Cambridge, UK).

Measurement of pro-inflammatory cytokines in serum of ApoE $^{-/-}$ mice

The blood of mice was collected and the serum was extracted and kept in -80°C. TNF- α , IL-1 β ,

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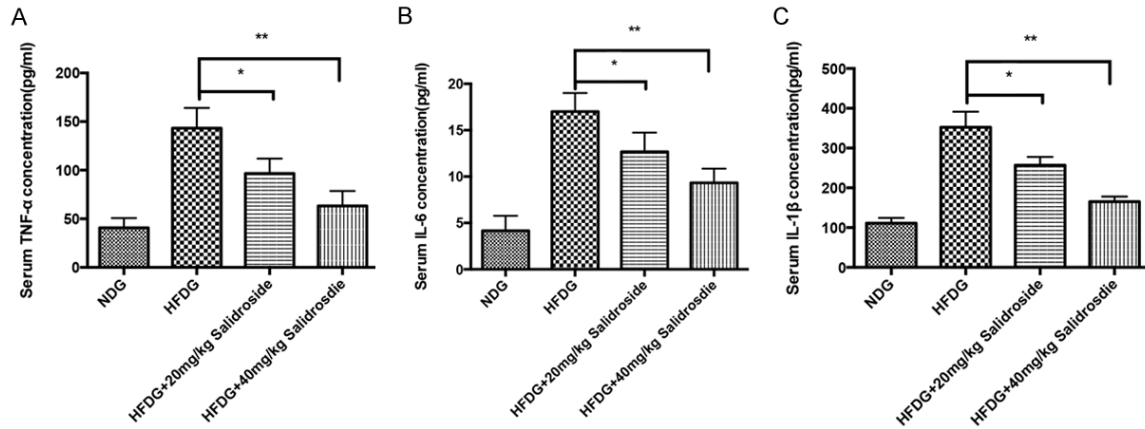


Figure 2. Effect of salidroside on cytokines in serum was measured by ELISA. The expression of TNF- α (A * $p=0.0352$, ** $p=0.0058$), IL-1 β (B * $p=0.0425$, ** $p=0.0062$), and IL-6 (C * $p=0.0233$, ** $p=0.0014$) was measured. Each data point represents the mean standard deviation ($n=3$). An unpaired t-test was used to analyze the data, three times of biological replications were applied.

and IL-6 in the serum of ApoE $^{-/-}$ mice were detected using ELISA kits, according to the instructions.

Statistical analysis

All data were presented as mean \pm SD, which was concluded from repeated measurements for three times. The differences between two groups were analyzed by unpaired t-test, while one-way ANOVA followed by Tukey multiple comparison tests was used for comparisons among more than two groups. All of the cartograms were made using GraphPad Prism (GraphPad, USA). $P<0.05$ was considered with statistical significance.

Results

Effect of salidroside on the weight of ApoE $^{-/-}$ mice

All 60 ApoE $^{-/-}$ mice were randomly selected and divided into four groups of normal diet group (NDG), high fat diet group (HFDG), HFDG treated with 20 mg/kg of salidroside, and HFDG treated with 40 mg/kg of salidroside. Each group consisted of 15 mice, and the weight of each mouse in each group was measured every week. After 12 weeks, the mice were sacrificed; the blood and arteries were collected for further study. No significant difference in weight among three HFDG groups was found (Figure 1). This result indicated that salidroside had no effect on the weight gaining of mice.

Effect of salidroside on the expression of inflammatory cytokines in serum

In this study, we would like to detect whether salidroside could affect the expression of inflammatory cytokines in HFDG. ELISA was applied to detect the expression of TNF- α , IL-1 β , and IL-6 in the serum of mice from different groups. The expression of TNF- α , IL-1 β , and IL-6 was found to be increased in HFDG groups. Interestingly, it was also found that, after being treated with salidroside, the expressions of these three pro-inflammatory cytokines were decreased, (TNF- α , Figure 2A * $p=0.0352$, ** $p=0.0058$; IL-1 β , Figure 2B, $p=0.0425$, ** $p=0.0062$; IL-6, Figure 2C * $p=0.0233$, ** $p=0.0014$), compared with the untreated HFDG group.

Salidroside could inhibit the NF- κ B and MAPK pathway

As reported, the expression of TNF- α , IL-1 β , and IL-6 were mediated by NF- κ B and MAPK pathway [14]. Recent studies also showed that these two pathways played important roles in the formation of atherosclerosis. In our study, it was found that salidroside could reduce the amount of TNF- α , IL-1 β , and IL-6, so it was highly possible that salidroside could suppress the expression of TNF- α , IL-1 β , and IL-6 via inhibiting NF- κ B and MAPK pathway. Western blot was used to detect the expression of p38, phosphorylated p38 (p-p38), p65, phosphorylated p65 (p-p65) in mice coronary artery.

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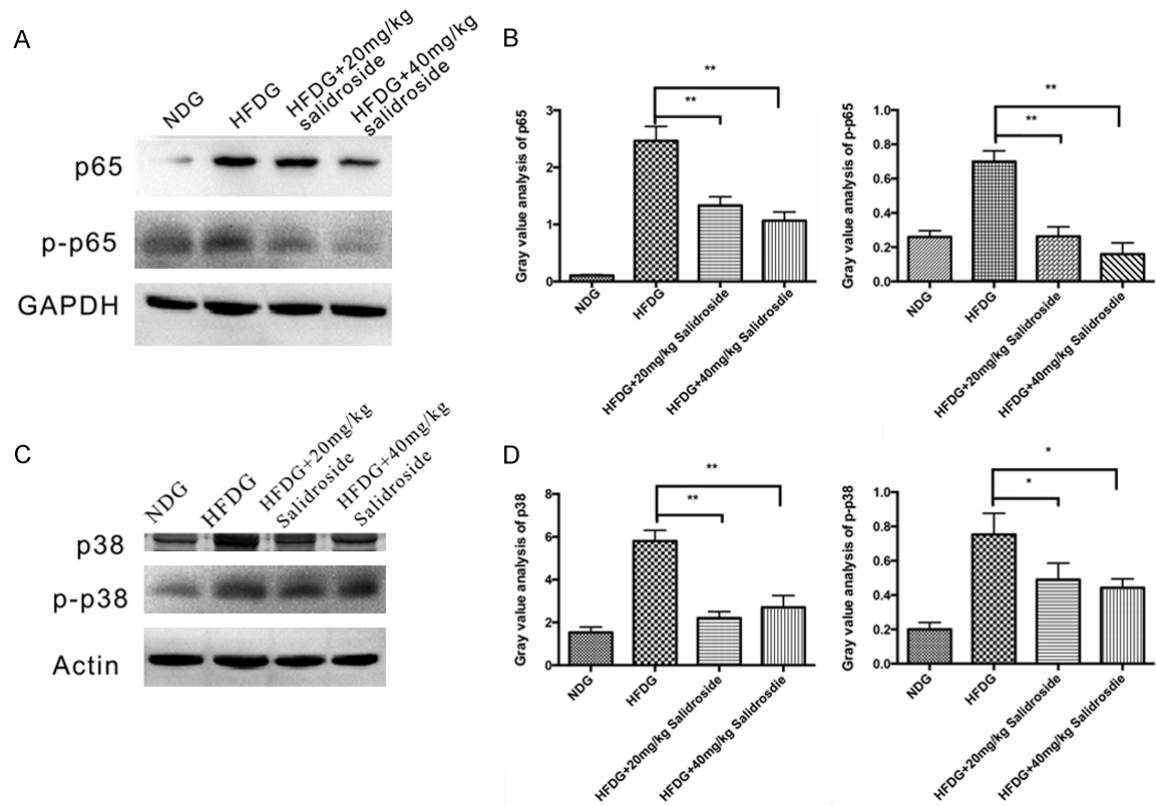


Figure 3. Effect of salidroside on MAPK and NF- κ B was detected by Western blot. (A and B). The expression of p65 and p-p65 were measured by Western blot. A gray value analysis shows each value of the blot (B, left panel, $**p=0.0026$, $**p=0.0012$, from left to right; right panel, $**p=0.0033$, $**p=0.0017$, from left to right). (C and D). The expression of p38 and p-p38 were detected by Western blot. A gray value analysis shows each value of the blot (D, left panel, $**p=0.0011$, $**p=0.002$ from left to right; right panel, $*p=0.0376$, $*p=0.0218$, from left to right). An unpaired t-test was used to analyze the data, three times of biological replications were applied.

It was found that compared with HFD group, salidroside treatment could inhibit the expression of p65 and p-p65 (Figure 3A and 3B, $p=0.0026$, $p=0.0012$; $p=0.0033$, $p=0.0017$), p38 and p-p38 (Figure 3C and 3D, $p=0.0011$, $p=0.002$, $p=0.0376$, $p=0.0218$, from left to right), indicating that salidroside could suppress NF- κ B and MAPK pathway. According to the result, it was possible that salidroside reduced the expression of TNF- α , IL-1 β , and IL-6 by inhibiting NF- κ B and MAPK pathways.

Salidroside could activate eNOS and NRF2 expression

Recent studies have also found that the dysfunction of vascular endothelial cells could lead to atherosclerosis [15-17]. Endothelial dysfunction was another cause of atherosclerosis. Restoring the endothelial function could be a promising method to prevent atherosclerosis. Endothelial nitric oxide synthase (eNOS) can improve function of cardiovascular endothelial,

which has drawn a lot of attention. In our study, we would like to check whether salidroside could affect eNOS expression. It was found that expression of eNOS was highly increased in HFD groups with salidroside treatment, as expected (Figure 4A and 4B, $**p=0.0019$, $**p=0.001$, from left to right).

What's more, it was also shown that NRF2 played a very important role in the redox reaction in cellular activity. Reactive oxygen species (ROS) were inducing factors of atherosclerosis. In our study, it was found that the expression of NRF2 was up-regulated in HFD groups treated with salidroside, compared with that in the control group and HFDG without any treatment (Figure 4C and 4D, $**p=0.0047$, $**p=0.0033$, from left to right).

Discussion

Salidroside is the effective component of *Rhodiola rosea*. Recent studies have showed

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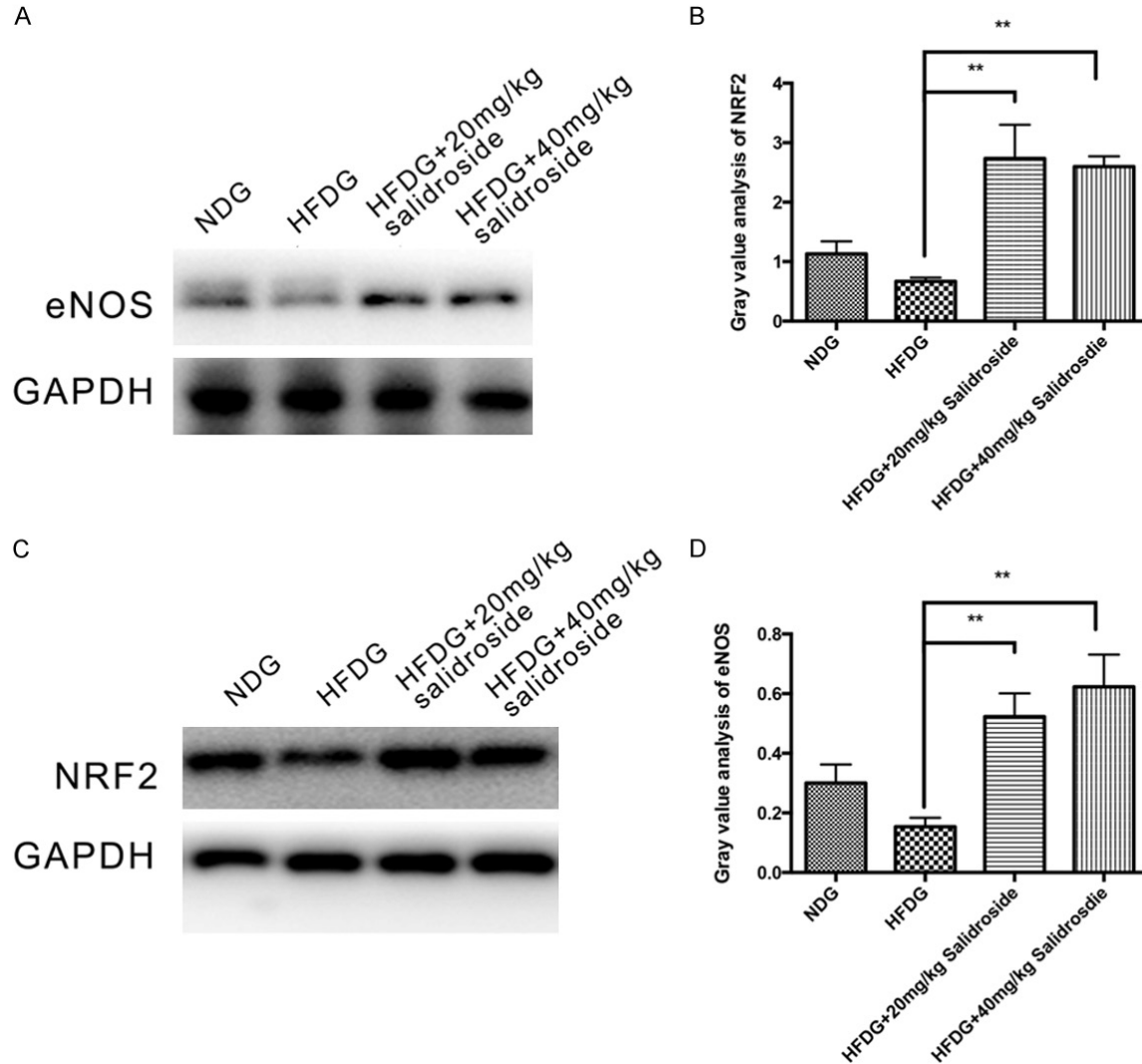


Figure 4. Effect of salidroside on eNOS and NRF2 was measured by Western Blot. (A and B). The expression of eNOS was measured by Western blot. A gray value analysis shows each value of the blot (B, $**p=0.0019$, $**p=0.0015$, from left to right). (C and D). The expression of NRF2 was measured by Western blot. A gray value analysis shows each value of the blot (D, $**p=0.0047$, $**p=0.0033$, from left to right). An unpaired t-test was used to analyze the data, three times of biological replications were applied.

that salidroside could be applied in many different diseases. Yin et al reported that salidroside could protect cortical neurons by inhibiting autophagy [18]. Salidroside was also reported to have neuroprotective ability in Alzheimer's disease via activating PI3K/AKT pathway [19] and suppressing SIRT1/ NF- κ B pathway [20]. It was also found that salidroside could suppress the inflammatory responses in biological activities. Different from its function in cancer, salidroside could inhibit the inflammatory response via suppressing JAK/STAT3 [21]. In conclusion, it is obvious that salidroside is a promising agent, which can be

applied in the clinical practice for different diseases.

The formation of atherosclerosis is complex. Inflammatory response in the epithelial of artery was one of the main causes [6]. Recent studies showed that the abnormal expressed adhesion molecules in endothelial cells, such as intercellular adhesion molecule-1 (ICAM-1) and the vascular cell adhesion molecule-1 (VCAM-1), on the endothelial cells were abnormally expressed at the first stage of atherosclerosis, which in turn could attracted the inflammatory cells, leading further inflammatory

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response [22]. Monocytes and macrophages are the first primary response inflammatory cells in the formation of atherosclerosis. These cells could produce various kinds of inflammatory factors, such as TNF- α , IL-1 β and IL-6, promoting the formation of atherosclerosis [6]. It has been reported that some inflammatory cytokines, such as TNF- α , IL-1 β , and IL-6, could contribute to the formation of atherosclerosis [6]. Therefore, some drugs targeting to inflammatory cytokines have been applied in treating atherosclerosis. As mentioned above, statins, aspirin and methotrexate have a strong anti-inflammatory effect. It was reported that statins and aspirin could reduce the pro-inflammatory cytokines (such as TNF- α , IL-1 and IL-6) and increase the anti-inflammatory cytokines (such as IL-10) [23-26]. In previous studies, methotrexate was reported to be able to reduce the adhesion molecules, such as ICAM-1, E-selectin, VCAM-1 [27]. However, more researches are needed before these drugs applied are applied in preventing or treating atherosclerosis. Therefore, new drugs that target for treating inflammatory reaction in atherosclerosis is needed. In our study, it was found that salidroside could reduce the expression of TNF- α , IL-1 β , and IL-6 in the HFADG (**Figure 2**), indicating that salidroside can contribute to the mitigation of atherosclerosis. This result was consistent with previous studies. Wu et al found that salidroside could inhibit the production of TNF- α , IL-1 β , and IL-6 induced by exposure to solar ultraviolet (SUV) irradiation [28]. Wu et al also found out that salidroside could mitigate myocarditis induced by sepsis in rats, via regulating the expression of inflammatory cytokines [29]. Our results indicated that salidroside can reduce the increased inflammatory factors caused by high fat diet, and salidroside can protect the artery.

As reported, the expression of TNF- α , IL-1 β , and IL-6 are controlled by NF- κ B-p-65 and p38 MAPK pathway [6]. Recent studies also found out that salidroside could affect different signaling pathway in several diseases. Chang et al reported that salidroside could prevent the formation of gastric ulcer induced by ethanol [30]. Yan et al also found out that salidroside had a strong effect on attenuating allergic airway inflammation, via inhibiting MAPK and NF- κ B pathway [31]. In a myocardial study, Zhu et al found that salidroside had protective ef-

fect by decreasing the expression of p65 and p-p65 [32]. In our study, we also found out that after treatment of salidroside, MAPK and NF- κ B pathway were suppressed. This result indicated that salidroside can reduce the expression of inflammatory factors through MAPK and NF- κ B pathway.

It is also reported that redox reaction played a very important role in atherosclerosis. The function of redox reaction genes was closely related with the formation and accumulation of atherosclerosis [33, 34]. Studies have shown that high level of ROS was among the main causes of atherosclerosis. Redox related genes were inactivated in normal conditions, but activated in the presence of pro-inflammatory cytokines [35]. Then, abundant ROS was produced by these genes, such as NADPH oxidase (NOX), which in turn activate various inflammatory cell signaling pathways, such as AKT, ERK, MAPK, Ras, and etc., and positively feedback to the formation of atherosclerosis [36]. It is widely agreed that NRF2 regulate a large numbers of antioxidant response genes, such as heme oxygenase (HO1), γ -glutamyl cysteine ligase-catalytic (GCLC), γ -glutamyl ligase-modulatory (GCLM), glucose-6-phosphate dehydrogenase (G6PD) and etc. [36, 37]. Studies also showed that the increased level of NRF2 in the young mice could multiply redox homeostasis in the heart [38]. In our study, Western blot assay is applied to detect the expression of NRF2 in mice cardiovascular endothelial cell among different groups. We found that salidroside could up-regulate the expression of NRF2, indicating that salidroside could also protect the cardiovascular through regulating redox reaction. eNOS control the generation of NO, which is important in the endothelium-dependent vascular function [39]. We found that treatment of salidroside could increase the expression of eNOS, suggesting that salidroside could also prevent the atherosclerosis by regulating redox reactions.

There were also some limitations in our study. We found that salidroside could reduce the expression of inflammatory cytokines (such as TNF- α , IL-1 β , and IL-6) and inhibit MAPK and NF- κ B pathways, but the evidence was not enough to verify the causal relationship. And the detailed mechanisms of suppressive effect of salidroside on the expression of MAPK and

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NF- κ B pathways remain still unknown. Further studies are needed to solve these problems.

Conclusion

In this study, we found that the treatment of salidroside could reduce the inflammatory factors in the serum possibly by inhibiting MAPK and NF- κ B pathway. Salidroside could also promote the function of vascular via increasing the expression of eNOS and NRF2. These results indicate that salidroside is a promising agent in treating and preventing atherosclerosis.

Disclosure of conflict of interest

None.

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