European Review for Medical and Pharmacological Sciences 2023; 27: 4369-4377

2,3,4′,5-Tetrahydroxystilbene-2-O-β-d-glucoside attenuates atherosclerotic progression by inhibiting inflammation *via* downregulation of TNF receptor-associated factor 6 expression

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Abstract. – OBJECTIVE: Atherosclerosis (As) is an inflammatory disease, and 2,3,4′,5-tetrahydroxystilbene-2-O-β-d-glucoside (TSG) has been shown to suppress inflammation. However, it is still unclear if TSG alleviates As by inhibiting inflammation.

MATERIALS AND METHODS: Quantitative real-time polymerase chain reaction (qRT-PCR) was performed to assess the mRNA levels of tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), TNF-α and interleukin-6 (IL-6) in lipoprotein E knockout (ApoE -/-) mice with As. Hematoxylin-eosin (H&E) staining was performed to examine the atherosclerotic plaques in the aortic sinus. QRT-PCR and western blotting were used to measure the expression levels of TRAF6, TNF-α, and IL-6 in human umbilical vein endothelial cells (HUVECs), and enzyme-linked immunosorbent assays (ELISAs) were performed to monitor the levels of TNF-α and IL-6 in serum and cell culture medium.

RESULTS: TSG inhibited subendothelial plaques formation in the aortic sinus and inhibited the levels of total cholesterol (TCHO), low-density lipoprotein (LDL), TRAF6, TNF-α and IL-6 in AS mice in a dose-dependent manner. Moreover, TSG attenuated the oxidatively modified LDL (ox-LDL)-induced increases in TRAF6, TNF-α and IL-6 expression, whereas TRAF6 overexpression reversed the TSG-induced decreases in TRAF6, TNF-α, and IL-6 expression in HUVECs.

CONCLUSIONS: TSG attenuates atherosclerotic progression by inhibiting inflammation *via* the downregulation of TRAF6 in ApoE-/- mice and HUVECs.

Key Words:
Atherosclerosis, TRAF6, TSG, ApoE-/- mice, HUVE.

Introduction

Atherosclerosis (As) is a chronic inflammatory disease that commonly occurs in the subendothelial intima of large- and medium-sized arteries, especially in the thoracic aorta, abdominal aorta, and coronary arteries[1]. The balance between proinflammatory cytokines and anti-inflammatory mediators determines the final clinical outcome[2]. A previous study[3] showed that the accumulation of low-density lipoprotein (LDL) initiated vascular inflammation. Reactive oxygen species oxidize LDL to promote the uptake of oxidatively modified low-density lipoprotein (ox-LDL) into macrophages[4]. Then, ox-LDL can trigger inflammation in the subendothelial intima of the arterial wall[5] by combining with Toll-like receptors, which activate proinflammatory signaling[6]. Proinflammatory cytokines can destroy the tight junctions of endothelial cells[7], resulting in a large amount of LDL further accumulating in the subendothelial intima. In summary, inflammation plays a crucial role in initiating and driving As.

Based on the role of inflammation in As, anti-inflammatory drugs or substances have been proposed as a new strategy to fight against As. Genetic knockout or inhibition of inflammatory cytokines can result in a significantly lower burden of As in As-prone mice[8-10]. Statins therapy has been proven to be effective at reducing inflammatory cytokine levels thereby inhibiting the progression of As[11]. Raloxifene protects against As in ApoE-/- mice by inhibiting the interleukin-6/signal transducer and activator of transcription 3 (IL-6/STAT3) pathway[12]. Curcumin inhibited inducible factor-1α-induced inflammation by an extracellular regulated kinase (ERK)-dependent pathway in macrophages[13]. Berberine inhibits the production of proinflammatory cytokines and stimulates the production of anti-inflammatory cytokines by modulating the gut microbiota, thereby treating As[14]. These investigations suggest the potential of anti-inflammatory therapies in the treatment of As.

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2,3,4′,5-tetrahydroxystilbene-2-O-β-d-glucoside (TSG) is an active compound extracted from traditional Chinese medicines, such as *Polygonum multiflorum*, and has been proven to have neuroprotective, anti-inflammatory and anti-atherosclerotic effects[15,16]. A previous study[16] showed that TSG inhibited inflammation by downregulating the expression of proinflammatory cytokines, such as IL-6, tumor necrosis factor (TNF)-α, vascular cellular adhesion molecule-1 (VCAM-1), and monocyte chemoattractant protein-1 (MCP-1), to combat As in ApoE-/- mice. TNF receptor-associated factor 6 (TRAF6) can promote inflammation, and the expression of TRAF6 was positively associated with the progression of As. Many studies[17,18] have shown that downregulation of TRAF6 expression can inhibit inflammation, thereby preventing the progression of As. It was reported that major vault protein suppressed As by inhibiting TRAF6-mediated inflammation. MiR-345-3p attenuated inflammation by targeting the TRAF6/transforming growth factor-beta-activated kinase-1 (TAK1)/p38/nuclear factor NF-kappaB (NF-κB) signaling pathway[18]. However, whether TSG prevents As progression by inhibiting inflammation through regulation of TRAF6 expression levels in ApoE-/- mice is unclear.

Here, an As model was established and treated with different concentrations of TSG. Human umbilical vein endothelial cells (HUVECs) were preincubated in ox-LDL to induce inflammation and were then treated with TSG. We verified that TSG reduced the expression levels of IL-6 and TNF-α by regulating TRAF6 to alleviate the process of As, which provided a new theoretical basis for TSG treatment of As.

Materials and Methods

Animals and Diets

Eighty male 6-week-old weighted (160-200 g) ApoE-/- mice were purchased from Changzhou Cavens Laboratory Animal Co., Ltd. (Changzhou, Jiangshu, China). The animals were housed in clean cages (32×22×16 cm, four mice per cage) in a 25°C room with a constant 12 h light-dark cycle and 50±5% humidity at the Laboratory Animal Research Centre of Fujian Medical University. Animals were acclimated at least 1 week prior to the experiment. This research was approved by the Animal Experimental Committee of Fujian Medical University (number: FJMU IACUC 2020-015). The procedures abided by the Ethical Principles of Animal Research in compliance with the ARRIVE guidelines. The mice fed standard diets were defined as the control group (n=20). The mice fed a high-fat diet (HFD, 21% fat, and 0.21% cholesterol) for 12 weeks were defined as the As group (n=60). The As mice were intragastrically administrated 125 and 500 mg/ml TSG (ChemicalBook, Beijing, China) at a dose of 1 ml/100 g per day for 7 days (As model, n=20; 125 mg/ml TSG, n=20; 500 mg/ml TSG, n=20). Then, all mice were sacrificed under 1% sodium pentobarbital (Sigma, St. Louis, MO, USA), and the blood, hearts, and thoracic aortas were collected for further analysis.

Cell Culture and Transfection

HUVECs were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco, Carlsbad, CA, USA). All mediums were supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin (Sigma) at 37°C in a 5% CO_2 incubator. The pcDNA3.1-TRAF6 (#66930) and pcDNA3.1 plasmids were purchased from Addgene (Addgene, Cambridge, MA, USA). The plasmid DNAs were transfected into HUVECs preincubated with 150 μg/ml ox-LDL for 24 h[18], and then the transfected cells were subjected to 40 μg/ml TSG[19].

Quantitative Real-time Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from tissues with TRIzolTM reagent (15596026; Invitrogen, Camarillo, CA, USA). The concentration of total RNA was measured using a NanoDrop ND-2000 spectrophotometer (Thermo, Wilmington, DE, USA). The cDNAs were synthesized with All-In-One MasterMix (G492; Abm, Vancouver, Canada) in accordance with the manufacturer's protocol. The qRT-PCR was performed using Bsetar SYBR Green qPCR Mastermix (DBI-2044; DBI Bioscience, Ludwigshafen, Germany) to analyze TRAF6, TNF-α, and IL-6 expression in accordance with the manufacturer's protocol. The reaction parameters of qRT-PCR were as follows: pre-denaturation at 95°C for 2 min and amplification for 40 cycles at 95°C, 15 s, 60°C, 20 s and 72°C for 20 s. The 18S was used as an internal reference. The raw data were analyzed by $2^{-\Delta\Delta Ct}$ method[20]. All primers are listed in Table I.

Table I. Primers used for qRT-PCR in this study.

Primer names	Nucleotide sequence (5′-3′)
TRAF6 Forward	GATCCAGAGTTTGCCGTCCA
TRAF6 Reverse	GCTGGGTCCCTTCAGAAGTT
TNF-α Forward	CATCTTCTCAAAATTCGAGTGACAA
TNF-α Reverse	TGGGAGTAGACAAGGTACAACCC
IL-6 Forward	AGGATACCACTCCCAACAGACCT
IL-6 Reverse	CATCATTGTTGCTACTACGTGAAC
18S Forward	AGAAACGGCTACCACATCCA
18S Reverse	CACCAGACTTGCCCTCCA-3

Western Blotting

Samples were collected and lysed in RIPA buffer (P0013B; Beyotime, Shanghai, China) supplemented with complete™ Protease Inhibitor Cocktail (11697498001; Roche, Mannheim, Germany) on ice for 30 min. The lysates were centrifuged at 13,300 × g for 30 min at 4°C. Supernatant of lysates was collected. The concentration of total protein was measured with a BCA kit (PA101-01; Beyotime) by a microplate reader according to the manual's protocol. The proteins were separated by 10% SDS-PAGE and transferred to a polyvinylidene fluoride membrane (IPV00010; Millipore, Billerica, MA, USA). The membranes were incubated in 5% defatted milk at 37°C for 1 h to block heterogenetic antigens. Based on the molecular weight of the proteins, the membranes were cropped prior to hybridization. Then, the membranes were incubated with mouse anti-TRAF6 antibody (66498-1-Ig, 1:5,000; Proteintech, Wuhan, Hubei, China) or mouse anti-β-actin antibody (66009-1-Ig, 1:5,000; Proteintech) at 4°C overnight. Subsequently, the membranes were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (A0216, 1:5,000; Beyotime) at room temperature for 1 h. Finally, the protein bands were visualized by chemiluminescence. Protein quantification was analyzed by ImageJ software (Bethesda, MD, USA).

Hematoxylin and Eosin (H&E) Staining

Heart tissues were collected from ApoE-/- mice with or without As, fixed with 4% paraformaldehyde, and embedded in paraffin. The sections were cut at 5 μm thickness. The sections with tissues were dewaxed in dimethylbenzene for 5-10 min, and sequentially soaked in gradient ethanol (100% ethanol for 2 min, 95% ethanol for 1 min, 80% ethanol for 1 min, and 75% ethanol for 1 min), subsequently washed in ddH_2O for 2 min. The sections were stained with Hematoxylin (C0105S-1, Beyotime) for 2-5 min, and then stained with Eosin (C0105S-2, Beyotime) for 2 min. The images were obtained by microscope (Olympus, Tokyo, Japan).

ELISA Analysis

The plasma of mice and medium of cells were collected to detect total cholesterol (TCHO), LDL, TNF-α and IL-6 levels using ELISA analysis kits (E1005, E1018, H052, H007, respectively; Applygen, Beijing, China) according to the manufacturer's protocol.

Statistical Analysis

All data from three independent cell experiments and five independent animal experiments were presented as the mean ± standard deviation. The difference between the two groups was analyzed by *t*-test with SPSS 23.0 (IBM, Armonk, NY, USA) and GraphPad Prism 8.0 software (Boston, MA, USA). When the *p*-values were lower than 0.05, the difference was considered statistically significant.

Results

The TRAF6 Expression and Proinflammatory Cytokines Were Increased in ApoE-/- Mice with As

The As model mice were generated by HFD. To verify whether the As model mice were established, H&E staining was performed. Figure 1a showed that atherosclerotic plaque sizes were significantly increased in the aortic sinus subendothelium of ApoE-/- mice with As. TCHO and LDL were measured by ELISA analysis in blood serum. The levels of TCHO and LDL were elevated in ApoE-/- mice with As, compared with those in the ApoE-/- mice (Figure 1b). These results indicated that the As model mice were established successfully.

Then, the expression of TRAF6 was detected by qRT-PCR and western blotting. Compared with

that in ApoE-/- mice, the expression of TRAF6 was increased in As mice (Figure 1c and 1d). The mRNA expression levels of TNF-α and IL-6 were measured by qRT-PCR. As shown in Figure 1e, the expressions of TNF-α and IL-6 in the As mice were higher than those in the ApoE-/- mice. TNF-α and IL-6 in the serum were detected by ELISA. Compared with ApoE-/- mice, the levels of TNF-α and IL-6 were increased in As mice (Figure 1f). These results demonstrated that the expression levels of TRAF6 and proinflammatory cytokines were increased in ApoE-/- mice with As.

TSG Alleviated the Formation of Atherosclerosis in ApoE-/- Mice

ApoE-/- mice with As were treated with 125 mg/ml and 500 mg/ml TSG for 7 days. Samples were collected to analyze the effect of TSG on As mice. As shown in Figure 2 a-b, the sizes of aortic sinus subendothelial plaques in As mice treated with TSG were lessened compared with those in the As model without TSG treatment. Meanwhile, with increasing concentrations of TSG, the plaque areas decreased. Then, the concentrations of TCHO and LDL in ApoE-/- mice were analyzed. Compared with the As model, the TCHO level was decreased

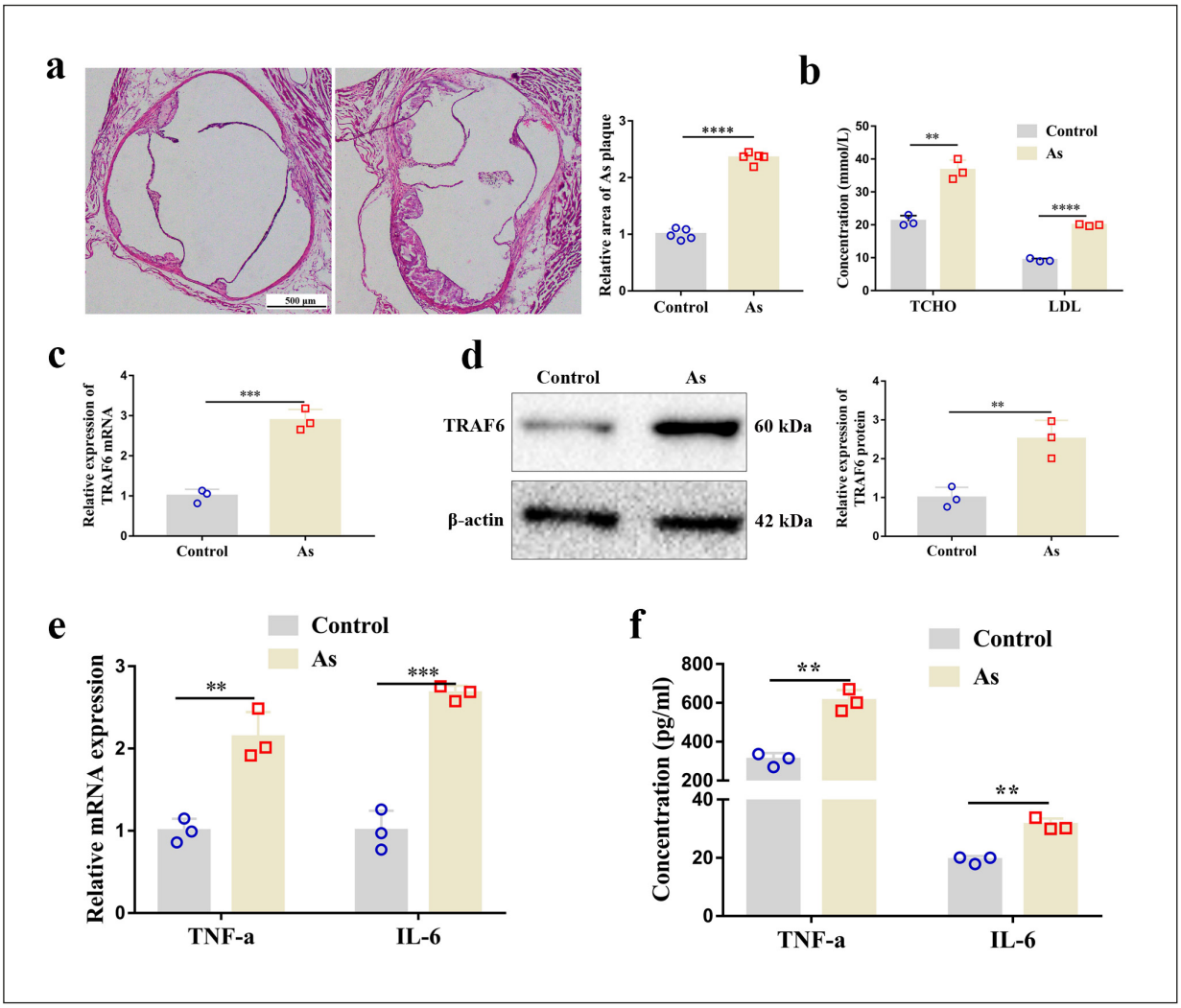

Figure 1. The expression of TRAF6, TNF-α and IL-6 in ApoE-/- mice with As. The ApoE-/- mice fed HFD for 12 weeks to generate As model mice. **a**, The aortic sinus was stained with H&E in the ApoE-/- mice (500 μm). Left: Representative image of the aortic sinus was showed by H&E staining. Right: The plaques of As were analyzed using ImageJ. **b**, The concentration of TCHO and LDL were measured by ELISA analysis. **c**, The expression of TRAF6 mRNA was measured by qRT-PCR. **d**, The expression of TRAF6 protein was analyzed by western blot. Left: Representative image of TRAF6 was shown. Right: The expression of TRAF6 was analyzed by ImageJ. **e**, The expression of TNF-α and IL-6 mRNA was analyzed by qRT-PCR. **f**, The TNF-α and IL-6 levels were detected by ELISA in the serum of ApoE-/- mice. All data were obtained from three or five independent experiments (Student's *t*-test, $^{**}p<0.01$, $^{***}p<0.001$, $^{****}p<0.0001$).

in the As mice treated with 125 mg/ml and 500 mg/ml TSG, and TCHO level was decreased in TSG dose-dependent. The effect of TSG on LDL was the same as that on TCHO in ApoE-/- mice (Figure 2c). These results indicated that TSG alleviated the formation of atherosclerosis in ApoE-/- mice.

TSG Inhibited the Expression of TRAF6 and Proinflammatory Cytokines in ApoE-/- Mice

To explore the mechanism by which TSG alleviates the As, the expression levels of TRAF6, TNF-α and IL-6 were measured in As mice treated with or without 125 mg/ml and 500 mg/ml TSG. The TRAF6 mRNA and protein levels were significantly decreased in the As mice treated with 125 mg/ml and 500 mg/ml TSG, compared with those in the As mice without TSG treatment (Figure 3a and 3b), which suggested that TSG inhibited the expression of TRAF6. Then, the expression of proinflammatory cytokines was analyzed by qRT-PCR. Compared with the As model, the expression levels of TNF-α and IL-6 mRNA were decreased in the TSG groups, and the expression levels of TNF-α and IL-6 were decreased with increasing TSG concentration (Figure 3c). The serum levels of TNF-α and IL-6 proteins were consistent with their mRNA levels (Figure 3d), which showed that TSG downregulated the expression of TNF-α and IL-6 in As mice.

TSG Attenuated Inflammation Induced by ox-LDL via Decreasing TRAF6 in HUVECs

To explore the underlying mechanism by which TSG attenuated inflammation, HUVECs were preincubated in ox-LDL to induce inflammation. The cells were treated with TSG and transfected with or without TRAF6. As shown in Figure 4a, TSG inhibited the upregulation of TRAF6 expression induced by ox-LDL, and the inhibition of TSG on TRAF6 was partially abolished by overexpression of TRAF6 in HUVECs. Then, the concentrations of TNF-α and IL-6 in medium of HUVECs were analyzed by ELISA. The results showed that TSG attenuated the ox-LDL-induced increase in TNF-α and IL-6, and decrease of TNF-α and IL-6 which were abrogated by overexpression of TRAF6 in HUVECs (Figure 4b and 4c).

Taken together, the results indicated that TSG inhibited inflammation by downregulating TRAF6 expression, thereby attenuating As.

Discussion

As is the most common cardiovascular disease; it is characterized by chronic inflammation of the arterial wall and is triggered by the subendothelial

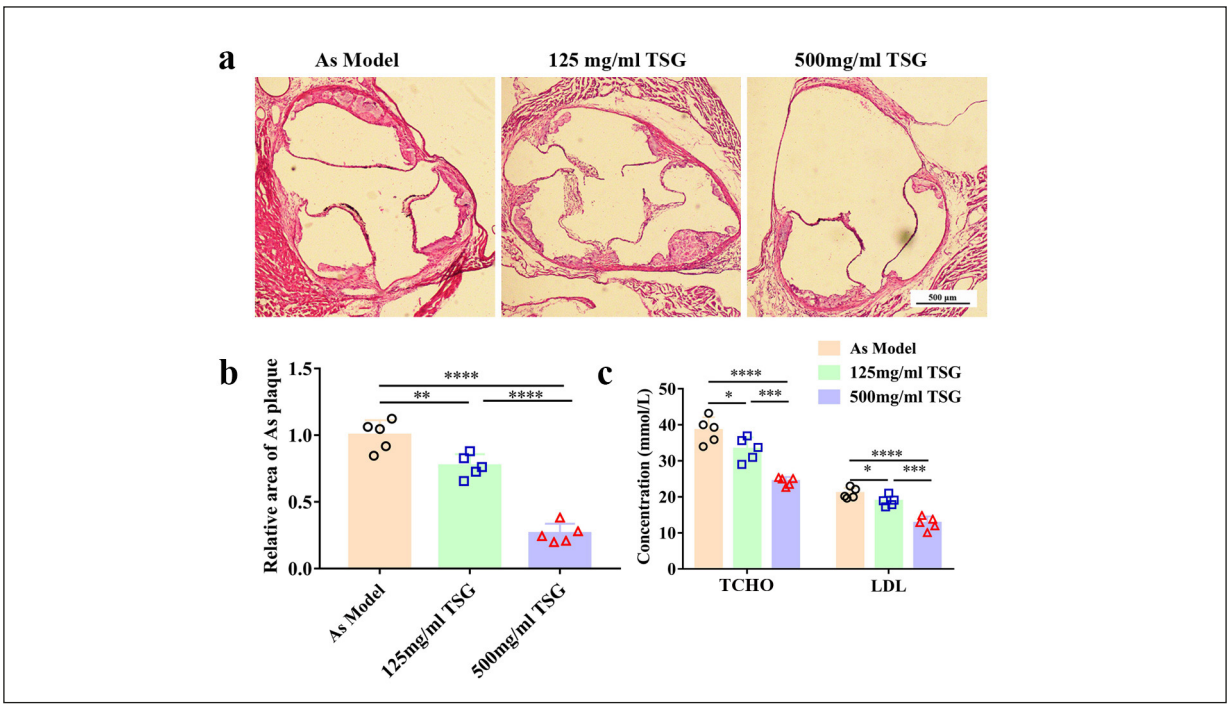

Figure 2. TSG relieved atherosclerosis in the ApoE-/- mice. The ApoE-/- mice with As were treated with or without 125 mg/ml and 500 mg/ml TSG. **a-b**, The aortic sinus was stained with H&E in the ApoE-/- mice (500 μm). **c**, The levels of TCHO and LDL were detected by ELISA in the serum. All data were obtained from five independent experiments (Student's *t*-test, **p*<0.05, ***p*<0.01, *****p*<0.0001).

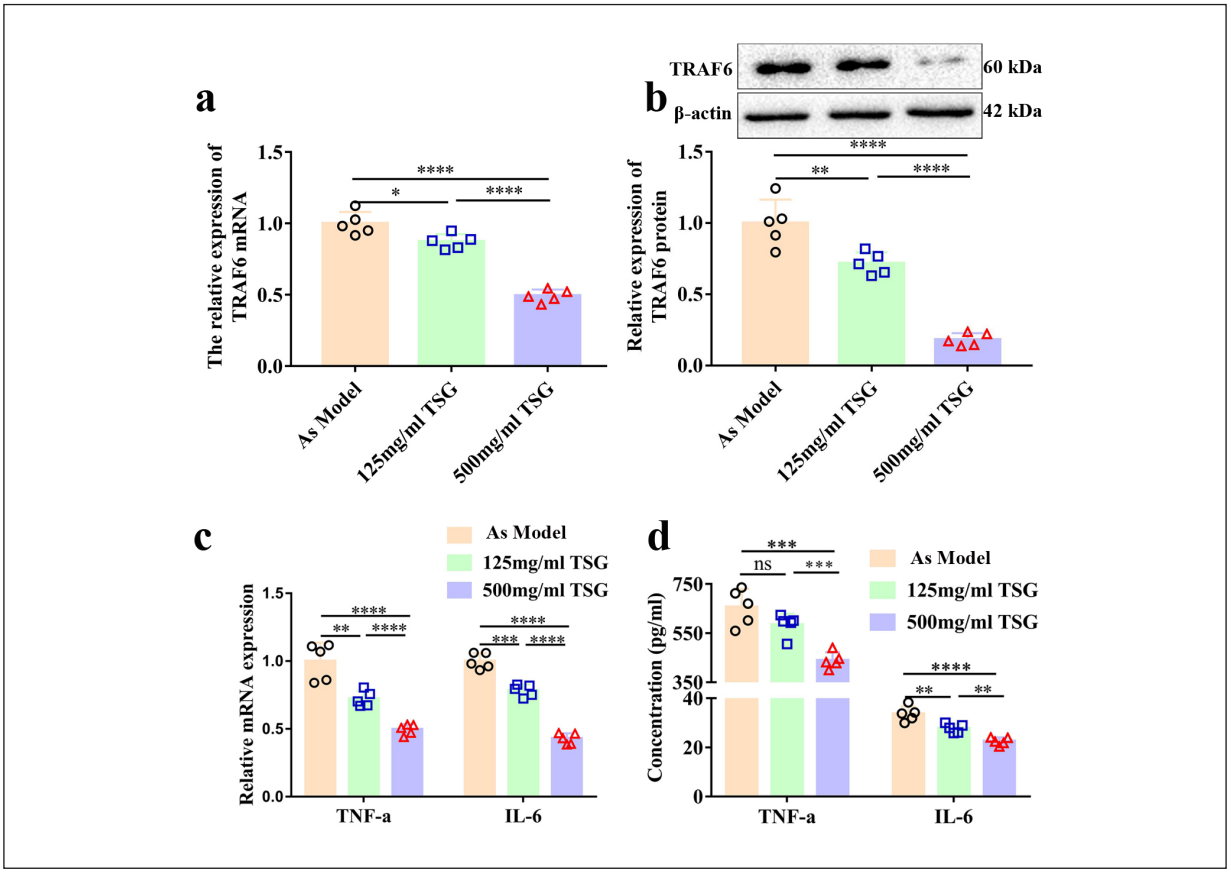

Figure 3. TSG reduced the expression of TRAF6, TNF-α and IL-6, inhibited the release of TNF-α and IL-6 in the ApoE-/- mice. The expression of TRAF6 mRNA (**a**) and protein (**b**) were analyzed by qRT-PCR and western blot, respectively. **c**, The expression of TNF-α and IL-6 mRNA were measured by qRT-PCR. **d**, The concentration of TNF-α and IL-6 were detected by ELISA. All data were obtained from five independent experiments (Student's *t*-test, **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

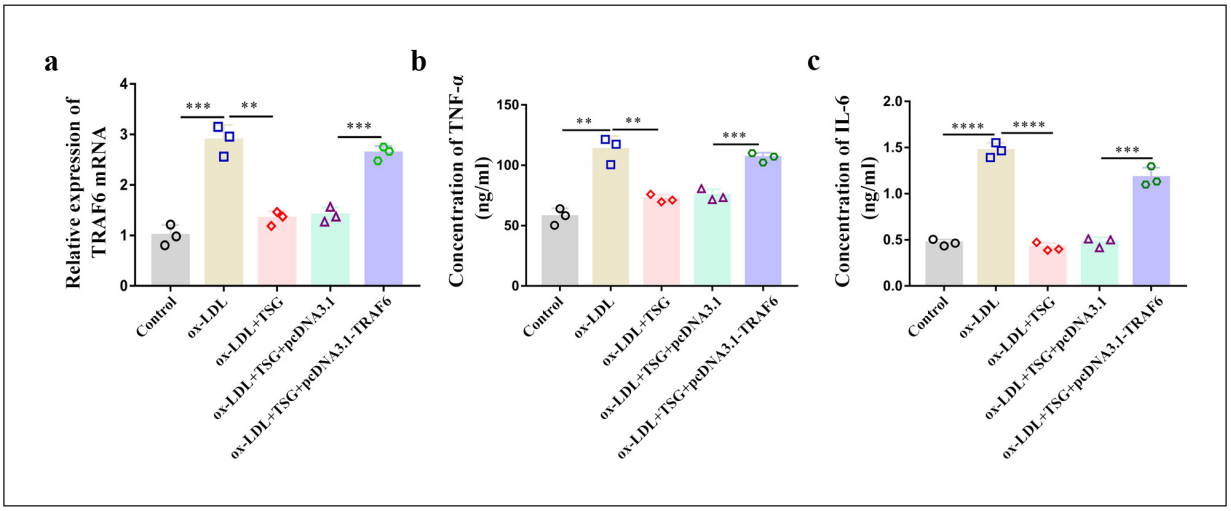

Figure 4. TSG attenuated inflammation by downregulating TRAF6 expression in HUVECs. HUVECs were induced by 150 μg/ml ox-LDL, and then subjected to 40 μg/ml TSG, while, transfected with pcDNA3.1-TRAF6. **a**, The expression of TRAF6 mRNA was detected by qRT-PCR, which showed TSG reduced the ox-LDL-induced increase of TRAF6. The levels of TNF-α (**b**) and IL-6 (**c**) were measured by ELISA in the medium of HUVECs, which indicated that TSG attenuated ox-LDL-induced the increase of TNF-α and IL-6, and TRAF6 overexpression abrogated inhibitory effect of TSG on TNF-α and IL-6. All data were obtained from three independent experiments (Student's *t*-test, **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001).

accumulation of plasma lipoproteins in the affected intima[2,21]. It has been confirmed that inflammation is a major driving force in the initiation and progression of As. The inflammation triggered by lipid accumulation and inflammatory cell infiltration expedited the initiation and progression of As[22-24]. The As model was constructed by HFD induction in our study, and then, the expression levels of proinflammatory cytokines were detected[25]. The results showed that the expression levels of IL-6 and TNF-α were increased in ApoE-/- mice with As, confirming that As is closely related to inflammation.

Based on the promotion of inflammation in atherosclerosis, many studies[26-29] have explored the specific molecular mechanisms of inflammation in the formation and development process of As, which is beneficial for developing new strategies to combat As by inhibiting inflammation. TRAF6, a member of the TNF receptor-associated factor family, is reported to be a signal transducer in the NF-κB pathway and can activate IKK in response to proinflammatory cytokines. TRAF6 can also regulate inflammation in As. Ben et al[17] found that Major Vault proteins can suppress inflammation by inhibiting the activity of TRAF6, thereby preventing As. Notoginsenoside R1 alleviated the ox-LDL-induced inflammatory response in HUVECs by modulating the XIST/miR-221-3p/TRAF6 axis through the NF-κB pathway, thereby treating As[30]. Apigenin significantly decreased the expression levels of TRAF6, NF-κB, and TNF-α, which are responsible for generating inflammation in ox-LDL-induced monocytes[31]. *Abelmoschus esculentus* and metformin ameliorated endothelial inflammation by increasing the expression of miR-146a targeted TRAF632. Literature has indicated that TRAF6 may become a new target to inhibit inflammation for the treatment of As.

Many studies[33-35] have concluded that As is alleviated by reducing lipid accumulation and accelerating cholesterol efflux in the cells and tissues of patients with As or As models. A previous study[33] confirmed that TSG could prevent As by promoting cholesterol efflux, stimulating cholesterol uptake in the liver, increasing the secretion of cholesterol into bile, and improving cholesterol metabolism. Moreover, several studies[34,35] have shown that TSG can treat many diseases through anti-inflammatory effects. It was reported that TSG suppressed the expression of matrix metalloprotease-2 (MMP-2) and MMP-9 and inhibited inflammation in diet-induced atherosclerotic rats. TSG attenuated lipopolysaccharide (LPS)-induced RAW264.7 cell secretion of proinflammatory cytokines by inducing the expression of HO-1[35]. TSG not only promoted reverse cholesterol transport but also inhibited inflammation from attenuating As. In this study, the plaque area, total cholesterol (TCHO), and LDL levels decreased in a TSG dose-dependent manner in ApoE-/- mice with AS, and the expression levels of proinflammatory cytokines, such as IL-6 and TNF-α, were also inhibited by TSG. These results were consistent with the previous conclusion that TSG could attenuate AS by inhibiting inflammation.

The expression of TRAF6 was increased in the As model. Moreover, in this study, TSG alleviated As by inhibiting inflammation and decreasing TRAF6 expression. Furthermore, TRAF6 can promote inflammation. Whether TSG can inhibit inflammation by regulating TRAF6 has not been reported yet. In our study, HUVECs preincubated in ox-LDL were treated with TSG and overexpressed TRAF6. Our results showed that TSG attenuated the ox-LDL- induced increase of TRAF6, TNF-α, and IL-6 levels, whereas the inhibitory effect of TSG was abolished by overexpression of TRAF6 in HUVECs.

Conclusions

Based on our research, we speculated that TSG might attenuate As by inhibiting inflammation *via* the downregulation of TRAF6 expression, and the findings of the present study may provide a novel therapeutic strategy for the treatment of atherosclerosis.

Conflict of Interests

The Authors declare that they have no conflict of interest.

Funding

This research did not receive any specific grants from any funding agency in the public, commercial, or non-profit sectors.

Authors' Contributions

ZY, YL, YC, and ZZ designed the study. ZY, YL, YC, NH, and DK carried out the experiments. ZY, YL, YC, NH, ML, and ZZ analyzed the data. ZY, YC, and ZZ prepared figures and wrote the manuscript. All authors read and approved the final manuscript.

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Ethics Approval

The procedures were performed in accordance with the Ethical Principles in Animal Research, compliance with the ARRIVE guidelines, and approved by the Animal Experimental Committee of Fujian Medical University (number: FJMU IACUC 2020-015).

Informed Consent

Not applicable.

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