Schisandrin B Inhibits Endothelial Cell Apoptosis in Mice with Atherosclerosis by Upregulating MiR-26a-5p Expression

Chao Liu¹, Shu-Ping Meng¹, Xiao-Hui Li¹, Fu-Rong Liu¹, Fu-yan Ding¹, Xiao-hang Wang¹, Zhao-Yun Cheng²*

Abstract

Background: Endothelial cell (EC) apoptosis could contribute to plaque erosion and pathological changes of atherosclerosis. The present study primarily aimed to investigate the effect of Schisandrin B on EC apoptosis and the related molecular mechanisms.

Methods: ApoE−/− mice were fed a high fat diet to induce atherosclerosis and treated with 20, 40 and 80 mg/kg of Schisandrin B. Oil red O staining was used to detect lesions in the aortas and aortic sinuses of the mice. A terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay was performed to detect apoptotic EC. The expression of miR-26a-5p, TRPC6 and PTEN were examined by quantitative real-time PCR and western blot, respectively. Human amniotic epithelial cells (HAECs) and epithelial cells ECV304 were treated with ox-LDL or Schisandrin B.

Results: Schisandrin B significantly attenuated the atherosclerotic lesion and atherosclerotic plaque in ApoE−/− mice fed with a high fat diet. Schisandrin B pretreatment reduced EC apoptosis in vivo and in vitro. Furthermore, Schisandrin B upregulated the level of miR-26a-5p and downregulated the expression of TRPC6 and PTEN. MiR-26a-5p knockdown abrogated the effect of Schisandrin B on HAECs and ECV304 cell apoptosis induced by ox-LDL. TRPC6 and PTEN knockdown reduced the HAECs and ECV304 cell apoptosis induced by the miR-26a-5p inhibitor.

Conclusion: Taken together, our results demonstrate that Schisandrin B has an anti-apoptotic effect in endothelial cells in mice with atherosclerosis, as implicated by the regulation of miR-26a-5p and its targets TRPC6 and PTEN.

Keywords: Schisandrin B, endothelial cells, apoptosis, atherosclerosis, miR-26a-5p, Ox-LDL

Introduction

One of the most harmful human diseases, atherosclerosis is a major cause of several cardiovascular diseases, including coronary artery disease, cerebral infarction and peripheral vascular disease (1). Endothelial cell (EC) apoptosis is an early event in atherosclerosis development, contributing to plaque erosion and pathological changes in the arteries (2). Initially, ECs are responsive to vascular function and homeostasis. EC dysfunction might result in the dysregulation of the homeostatic mechanisms (3). EC apoptosis could increase vascular permeability, promote SMC proliferation and enhance blood coagulation, leading to arterial thrombosis or sudden death (4). Therefore, elucidating on the molecular mechanisms underlying EC apoptosis in the process of atherosclerosis might facilitate our understanding of atherosclerosis pathology and its treatment.

Several intrinsic and environmental factors contribute to EC apoptosis during atherosclerosis, including oxidative stress, inflammatory cytokines, reactive oxygen species (ROS) and immunologic factors (5). Previous studies have found that Schisandra chinensis (Turcz.) Bail., a medicinal herb, exerts a positive effect in the treatment of central nervous and cardiovascular disorders, inflammatory and immune diseases, and respiratory disorders through its dibenzocyclooctadiene lignans (6). Furthermore, research has identified Schisandrin B (Sch B) as the most abundant active dibenzocyclooctadiene lignan in Schisandra chinensis. Such studies also demonstrate that Schisandrin B has beneficial effects on cardiac function through its antioxidant and anti-inflammatory properties in vivo and in vitro (7, 8). However, the function of Schisandrin B on EC apoptosis during atherosclerosis remains poorly understood.

MicroRNAs (miRNAs) are a class of small non-coding RNAs with a length of 18-22 nucleotides. MiRNAs are capable of negatively regulating gene expres-
sion by binding to the 3'-untranslated region (3'-UTR) of target mRNAs (9). A large body of research has demonstrated that miRNAs play crucial roles in the pathophysiological processes of atherosclerosis by targeting different factors or signaling pathways (10, 11). In particular, miR-26a plays a central role in cardiovascular disease by regulating critical signaling pathways and targets implicated with angiogenesis, endothelial cell growth and lipid metabolism (12, 13). Recently, researchers demonstrated that miR-26a has an anti-apoptotic effect on EC in atherosclerosis by directly targeting transient receptor potential canonical 6 (TRPC6) (14). In this study, we focused on the effect of Schisandrin B on EC apoptosis in mice with atherosclerosis and explored the molecular mechanisms that might be involved with the regulation of miR-26a in this process.

**Materials and Methods**

**Animal models**

All experimental procedures were approved by the Animal Care and Use Committee of XX. Male ApoE deficient (ApoE−/−) mice (eight weeks old) derived from C57BL/6 were housed under standard animal room conditions with unlimited access to food and water. ApoE−/− mice were divided randomly into four groups (n=10 for each group): the no treatment group, a group treated with 20 mg/kg of Schisandrin B, a group treated with 40 mg/kg of Schisandrin B, and a group treated with 80 mg/kg of Schisandrin B. The mice in all groups were fed with a western diet composed of 21% (w/w) fat and 1.25% (w/w) cholesterol to induce atherosclerosis. C57BL/6J mice fed a western diet served as the control group (n=10). For the ApoE−/− mice in the experimental groups, Schisandrin B was administered intragastrically one time per day for 16 weeks. Obtained from Sigma-Aldrich, the Schisandrin B was dissolved in ethanol. On the last day of administration, the weight of the mice was measured and recorded.

**Evaluation of aortic lesions**

Hearts and proximal aortas were harvested and cleaned immediately following sacrifice of the mice. Aortic atherosclerotic lesion was detected by en face staining of aortas with oil red O. The aortic sinus was cut lengthwise, and the sections were stained with oil-red O for 30 min at 37°C. NIH ImageJ software (National Institute of Health, United States) was used to quantify the lesion areas, and the lesions were expressed as the percentage of lesion areas compared to the total aortic area. The lesion in the aortic sinus was expressed as the cross-sectional aortic sinus.

**Separation of the amniotic endothelial cell from the mice**

Based on a previous report, mouse aortic endothelial cells were isolated from the thoracic aortas (15). After the mice were sacrificed and cleaned, the thoracic cavities were opened and the thoracic aortas isolated. Containing 0.5% Triton X-100, Hank’s buffer salt solution was perfused into the aortas. Then, the aorta was placed into Dulbecco’s Modified Eagle Medium (DMEM) with collagenase type 2 (300 U/mL). The aorta was cut lengthwise and into pieces. Thereafter, the sections were cultured in DMEM containing 20% fetal bovine serum (FBS) at 37°C with 5% CO₂. The cultures were changed after 60 h, and the sections were removed. When cells grew into a single layer, subculture was performed.

**Cell culture and treatment**

Human amniotic epithelial cells (HAECs) and epithelial cells ECV304 were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured in Endothelial Cell Medium supplemented with endothelial cell growth factors, 5% FBS and 1% penicillin/streptomycin. The cells were maintained at 37°C with 5% CO₂. HAECs and ECV304 cells were pretreated with Schisandrin B (1.0 mg/L) for 2 h and then exposed to ox-LDL (25 μg/mL) for 48 h. Ox-LDL was obtained from Sigma-Aldrich.

**Cell transfection**

MiR-26a-5p inhibitor was used to inhibit the expression of miR-26a-5p in HAECs and ECV304 cells. si-TRPC6 and si-PTEN were synthetized by the RiboBio corporation (Guangzhou, Guangdong, China). According to the manufacturer’s instructions, the MiR-26a-5p inhibitor or si-TRPC6 and si-PTEN were transfected into HAECs and ECV304 cells with Lipofectamine 2000. HAECs and ECV304 cells were seeded for 24 h before transfection. DNA was dissolved in Opti-MEM serum-free medium and added to the cells. After 24 h of transfection, the medium was replaced by fresh medium.

**Quantitative real-time PCR**

Whole RNAs were isolated from the cultured cells using Trizol reagent (Invitrogen). The extracted RNA was reversely transcribed into complementary DNA (cDNA) using M-MLV reverse transcriptase. Quantitative real-time PCR was performed using the SYBR® green master mix kit to detect the levels of miR-19b-3p, miR-221-3p, miR-222-3p, miR-129-5p and miR-26a-5p. Expression was normalized to U6. The comparative threshold cycle (Ct) method was used to calculate the data.

**Western blot**

Western blot analysis was carried out to detect the protein level of TRPC6 and PTEN (phosphatase and tensin homolog), which were the targets of miR-26a. The cells were lysed with RIPA buffer, and the total proteins were extracted. After quantifying the protein concentration, the proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene fluoride membrane (Millipore). The
membranes then were blocked with 5% non-fat milk and probed with anti-TRPC6 (Abcam) and anti-PTEN (Abcam) before incubation with a secondary antibody. Signals were observed with ECL detection reagent (Pierce). The data were normalized to that of β-actin. The Gel-Pro Analyzer 4.0 program was used to quantify the protein bands of Western blot.

### TUNEL staining

Apoptotic cells were determined using the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL, Roche) assay kit following the manufacturer's instructions. Cells were fixed with 4% paraformaldehyde for 1 h and washed with PBS 3 times. Then, cells were treated with 0.1% Triton X-100 for permeabilization. The TUNEL reaction mixture was incubated with cells at 37°C for 1 h. Converter-POD was used for signal conversion and 3, 3'-diaminobenzidine (DAB) served as the selenium organic reagent. The stained cells were examined under a confocal laser scanning microscope (FV300, Olympus, Japan).

### Statistical analyses

The data in this study are presented as means ± standard deviations. Data were analyzed using SPSS software version 17 (SPSS, Chicago, IL, USA). One-way analysis of variance (ANOVA) was used to analyze the differences among groups. Comparisons between two groups were performed using the Student’s t test. A P value < 0.05 was accepted as statistically significant.

### Results

**Schisandrin B attenuated the endothelial cell apoptosis in mice with atherosclerosis**

To investigate the effect of Schisandrin B on arterial injuries in mice with atherosclerosis, ApoE⁻/⁻ mice were fed a high fat diet and treated with different doses of Schisandrin B (0, 20, 40 and 80 mg/kg, n=10/group). Doses were given through intragastric administration one time per day for 16 weeks. Then, the lesions in the aortas and aortic sinuses of these mice were detected using en face oil red O staining.
The results showed that the aortic lesion percentage and the atherosclerotic plaque area in the aortic sinus in ApoE−/− mice were significantly larger than that in C57BL/6J mice fed a high fat diet. This finding indicates that ApoE−/− mice fed a high fat diet suffered from atherosclerotic lesions.

In addition, we found that pretreatment with Schisandrin B greatly attenuated the atherosclerotic lesion and atherosclerotic plaque in ApoE−/− mice in a concentration-dependent manner (Figures 1A and 1B). To further explore the effect of Schisandrin B on endothelial cell apoptosis,
endothelial cells were separated from these mice and TUNEL staining assay was performed. The percentage of apoptotic endothelial cells in ApoE\(^{-/-}\) mice was much higher than that in C57BL/6J mice. Schisandrin B abrogated the endothelial cell apoptosis in a concentration-dependent manner (Figure 1C). However, the weight of ApoE\(^{-/-}\) mice with varying treatments showed no significant difference on the last day of Schisandrin B administration (Figure 1D).

**Schisandrin B upregulated the expression of miR-26a-5p in endothelial cells**

We determined the expression of miR-19b-3p, miR-221-3p, miR-222-3p, miR-129-5p and miR-26a-5p in the separated endothelial cells. As shown in Figures 2A-2D, higher expressions of miR-19b-3p, miR-221-3p, miR-222-3p and miR-129-5p were observed in endothelial cells separated from ApoE\(^{-/-}\) mice fed a high fat diet: 3.05 times, 2.41 times, 2.89 times and 3.63 times more, respectively. Meanwhile, the expression of miR-26a-5p was reduced in ApoE\(^{-/-}\) mice (Figure 2E). Schisandrin B pretreatment had no influence on the expression of miR-19b-3p, miR-221-3p, miR-222-3p and miR-129-5p in endothelial cells separated from ApoE\(^{-/-}\) mice. However, the expression of miR-26a-5p was upregulated with the increasing concentration of Schisandrin B (Figure 2E).

**Schisandrin B decreased the expression of TRPC6 and PTEN in endothelial cells**

As TRPC6 and PTEN were identified as the target of miR-26a-5p, we explored the effect of Schisandrin B on the expression of TRPC6 and PTEN in separated endothelial cells. Studies have shown that the protein level of TRPC6 and PTEN in ApoE\(^{-/-}\) mice was significantly higher than that in C57BL/6J mice. Schisandrin B pretreatment decreased the expression of TRPC6 and PTEN in ApoE\(^{-/-}\) mice in a concentration-dependent manner (Figure 3).

**Schisandrin B upregulated the expression of miR-26a-5p and decreased the expression of TRPC6 and PTEN in HAECs and ECV304 treated with ox-LDL**

The current study also sought to explore the effect of Schisandrin B on the expression of miR-26a-5p, TRPC6 and PTEN in HAECs and ECV304 cells. Therefore, cells were pretreated with Schisandrin B for 2 h and then treated with ox-LDL for 48 h. The expression of miR-26a-5p was reduced greatly in HAECs and ECV304 cells treated with ox-LDL compared to that of the control group. Schisandrin B pretreatment reversed this reduced expression of miR-26a-5p induced by ox-LDL in HAECs and ECV304 cells (Figure 4A). In terms of the expression of TRPC6 and PTEN, ox-LDL increased the protein level of TRPC6 and PTEN in HAECs and ECV304 cells, which was abrogated by Schisandrin B pretreatment (Figure 4B).

**MiR-26a-5p knockdown abrogated the effect of Schisandrin B on HAECs and ECV304 cell apoptosis induced by ox-LDL**

To detect the effect of Schisandrin B on HAECs and ECV304 cell apoptosis, HAECs and ECV304 cells were treated with ox-LDL, ox-LDL + ethanol, or ox-LDL + Schisandrin B. The percentage of TUNEL-positive HAECs and ECV304 cells, which represented the apoptotic cells, increased greatly by ox-LDL. Meanwhile, the percentage of apoptotic cells treated with ox-LDL + Schisandrin B significantly reduced compared to the percentage of TUNEL-positive cells treated with ox-LDL + ethanol (Figure 5). We
further examined the effect of miR-26a-5p knockdown on the function of Schisandrin B on HAECs and ECV304 cell apoptosis. HAECs and ECV304 cells were treated with ox-LDL + Schisandrin B + NC or ox-LDL + Schisandrin B + miR-26a-5p inhibitor. As shown in Figure 5, miR-26a-5p knockdown abrogated the effect of Schisandrin B on HAECs and ECV304 cell apoptosis induced by ox-LDL.

TRPC6 and PTEN knockdown reduced the HAECs and ECV304 cell apoptosis induced by the miR-26a-5p inhibitor

We found that miR-26a-5p knockdown could reverse the effect of Schisandrin B on HAECs and ECV304 cell apoptosis induced by ox-LDL. To further detect the effect of TRPC6 and PTEN knockdown on the function of miR-26a-
5p knockdown, HAECs and ECV304 cells were treated with ox-LDL + Schisandrin B + miR-26a-5p inhibitor + si-NC or ox-LDL + Schisandrin B + miR-26a-5p inhibitor + si-TRPC6 + si-PTEN. As shown in figures, TRPC6 and PTEN knockdown reduced the HAECs and ECV304 cell apoptosis induced by the miR-26a-5p inhibitor.

Discussion

In this study, we observed that Schisandrin B significantly attenuated the atherosclerotic lesion and atherosclerotic plaque in ApoE−/− mice fed a high fat diet. In addition, Schisandrin B pretreatment reduced the endothelial cell apoptosis in vivo and in vitro. Furthermore, we found that Schisandrin B upregulated the expression of miR-26a-5p and downregulated the expression of its targets TRPC6 and PTEN. MiR-26a-5p knockdown abrogated the effect of Schisandrin B on HAECs and ECV304 cell apoptosis induced by ox-LDL. TRPC6 and PTEN knockdown reduced the HAECs and ECV304 cell apoptosis induced by the miR-26a-5p inhibitor. Taken together, these findings indicate that Schisandrin B exerts an anti-apoptotic effect on endothelial cells, as implicated by the regulation of miR-26a-5p and TRPC6 and PTEN.

Endothelial dysfunction is an early step in the process of atherosclerosis associated with several traditional cardiovascular risk factors, such as diabetes, dyslipidemia and hyperglycemia (16). EC apoptosis could be induced by many pro-atherogenic factors, including oxidative stress, angiotensin II and the tumor necrosis factor receptor superfamily (17). Recently, research has shown Schisandrin B to hold high antioxidant potential in ischemic diseases, such as cerebral ischemia and ischemia/reperfusion injury (18, 19). Thandavarayan et al. reported that Schisandrin B could prevent doxorubicin-induced cardiac dysfunction by modulating oxidative stress through the inhibition of MAPK/p53 signaling (20). Recent studies also have shown that Schisandrin B increases the expression of the heat shock protein (Hsp) 25 and Hsp 70 to attenuate myocardial ischemia/reperfusion I/R injury in rats (21).

In addition to regulating key factors to exert function, Schisandrin B also has been shown to modulate the expression of miRNAs. In 6-OHDA-induced Parkinson's disease, Schisandrin B demonstrated a neuroprotective effect, negatively regulating the expression of miR-34a (22). Zhang et al. found that Schisandrin B inhibited the proliferation of airway smooth muscle cells via miR-135a suppressing TRPC6 expression (23). According to several recent reports, abnormally expressed miRNAs were related to the regulation of EC apoptosis. For example, miRNA let-7g inhibited the EC apoptosis induced by ox-LDL by targeting caspase-3 (24). ox-LDL-induced endothelial apoptosis was regulated by miR-221/222 via Ets-1/p21 inhibition (25). In this study, we found that Schisandrin B suppressed endothelial cell apoptosis by upregulating the expression of miR-26a-5p both in vivo and in vitro.

MiR-26a initially was identified as an oncogenic or tumor suppressor gene in different cancers (26, 27). In addition to miR-26a's involvement in the progression of various cancers, ectopic expression of miR-26a has been implicated in numerous cardiovascular repair mechanisms (13). In a mouse model of acute myocardial infarction, miR-26a knockdown was found to promote angiogenesis while diminishing the size of acute myocardial infarction.
(28). Zhang et al. reported that miR-26a mediated cardiac hypertrophy suppression by targeting GSK3β (12). MiR-26a also played a protective role in cardiomyocyte ischemia/reperfusion injury, which was involved in the inhibited HMGB1 expression (29). In this study, we found that miR-26a-5p was downregulated in ECs separated from ApoE−/− mice fed a high fat diet and in HAECS and ECV304 treated with ox-LDL. In addition, Schisandrin B reversed this aberrant expression of miR-26a-5p in vivo and in vitro.

We further investigated the expression of two miR-26a-5p targets in ECs, TRPC6 and PTEN. The results showed that TRPC6 and PTEN were significantly upregulated in ApoE−/− mice fed a high fat diet and in HAECS and ECV304 treated with ox-LDL, which could be abrogated by Schisandrin B pretreatment. Zhang et al. reported that miR-26a prevented endothelial cell apoptosis, which was implicated by the regulation of TRPC6 (14). Apart from TRPC6, PTEN also was validated as a target of miR-26a. PTEN overexpression could attenuate angiogenic processes of endothelial cells by blocking endothelin-1/endothelin B receptor signaling (30). The apoptosis of human vascular endothelial cells induced by high glucose could be suppressed by PTEN inhibition (31). Therefore, TRPC6 and PTEN might also be involved in the anti-apoptotic role of Schisandrin B in endothelial cells.

In summary, our study has provided evidence that Schisandrin B plays a protective role in atherosclerotic lesions and inhibited endothelial cell apoptosis. This effect was implicated by the upregulation of miR-26a-5p. These data provide important evidence related to the function of Schisandrin B in the treatment of atherosclerosis. This study further sheds light on how Schisandrin B inhibits endothelial cell apoptosis in atherosclerosis.

**Conflict of interest**

All authors declare that there is no conflict of interest.

**References**

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