

Rosuvastatin protects against oxidized low-density lipoprotein-induced endothelial cell injury of atherosclerosis *in vitro*

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Received March 9, 2018; Accepted October 18, 2018

DOI: 10.3892/mmr.2018.9666

Abstract. Atherosclerosis-induced cardiovascular diseases (CVDs) are accompanied by substantial morbidity and mortality. The loss and injury of endothelial cells is the primary cause of atherosclerosis. Rosuvastatin is an alternative agent used to reduce the risk of cardiovascular disease. Subsequently, the present study aimed to investigate the protective effects of rosuvastatin on oxidized-low-density lipoprotein (ox-LDL)-induced human umbilical vein endothelial cell (HUVEC) injury. The viability of ox-LDL-cultured HUVECs with or without rosuvastatin (0.01, 0.1 and 1 μ mol/l) pretreatment, and pretreatment at different time points (3, 6, 12 and 24 h) was determined using an MTT assay. Morphological changes and the extent of apoptosis were detected; the anti-oxidase activity, including superoxide dismutase (SOD) and catalase (CAT), was examined, and the contents of malondialdehyde (MDA) and nitric oxide (NO) were measured. The phosphorylation levels of endothelial nitric oxide synthase (eNOS), protein kinase B (Akt) and phosphoinositide 3 kinase (PI3K) were detected using western blot analysis. The results demonstrated that pretreatment with 0.01-1 μ mol/l rosuvastatin decreased cell apoptosis caused by ox-LDL. Notably, pretreatment with 1 μ mol/l rosuvastatin for >12 h increased cell viability.

Additionally, DAPI staining revealed that rosuvastatin inhibited HUVEC apoptosis. Rosuvastatin treatment also resulted in increased SOD and CAT activities and decreased MDA content in ox-LDL-stimulated HUVECs. Furthermore, pretreatment with 0.01-1 μ mol/l rosuvastatin significantly increased the NO content compared with HUVECs treated with ox-LDL alone. Western blot analyses demonstrated that rosuvastatin upregulated the phosphorylation of eNOS, Akt and PI3K. These findings indicated that rosuvastatin could protect HUVECs against ox-LDL-induced injury through its anti-oxidant effect and its ability to upregulate the expression of vascular endotheliocyte-protecting factors.

Introduction

At present, cardiovascular diseases (CVDs) remain the predominant causes of morbidity and mortality in a number of countries. Notably, atherosclerosis (AS) is a leading cause of CVD (1). According to a previous study, the morbidity of coronary heart diseases caused by AS has increased in the past decade and almost 400 out of every 100,000 people succumb to the disease in Asia per year (2). Furthermore, coronary heart disease caused by AS is considered the primary cause of non-infectious disease-associated mortality worldwide (2). Endothelial dysfunction is a typical early manifestation of atherogenesis, as well as the basic pathogeny of multiple CVDs, including hypertension, coronary disease, angina pectoris and cardiac failure (3-5). Notably, lipid metabolism disorders are among the most important causes of endothelial cell function impairment and result in a series of oxidative stress reactions (6).

Nitric oxide (NO) is the vital vasoactive mediator for protecting vascular endothelial cells and its production is catalyzed by endothelial nitric oxide synthase (eNOS). Lipid metabolism disorders can immediately promote the uncoupling of eNOS and catabolism of NO, thereby generating superoxide anions, increasing oxidative stress and reducing NO bioavailability (7,8). Under conditions of oxidative stress, low-density lipoprotein (LDL) is oxidized to form ox-LDL, which penetrates and is deposited under the intima, leading to increased endothelial permeability and impaired endothelial

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Key words: rosuvastatin, atherosclerosis, endothelial dysfunction, nitric oxide synthase

cell function; endothelial cells ingest increased amounts of lipids and develop into foam cells which serve a role in proinflammatory and immune stimulatory effects (9), thereby promoting the occurrence and development of AS (10). Additionally, ox-LDL can act as a carrier of oxygen free radicals and continue to induce reactive oxygen species generation, aggravating atherosclerotic lesions (11).

Protein kinase B (Akt), which is a type of serine/threonine kinase involved in the phosphoinositide-3-kinase (PI3K)-Akt signaling pathway (12), is considered a key mediator of cell proliferation, migration, apoptosis, angiogenesis and metabolism (13). Importantly, Akt can directly phosphorylate eNOS at a serine phosphorylation site, resulting in the enhancement of eNOS enzymatic activity and altered sensitivity of the enzyme to Ca^{2+} (14). Under the stimulation of sustained lipid metabolism, oxidative stress or other factors lead to the inactivation of PI3K and inhibit Akt phosphorylation, thereby affecting the synthesis of eNOS and aggravating endothelial cell dysfunction (15).

Rosuvastatin, an inhibitor of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, is the current paradigm for lipid management that is used for ameliorating abnormal lipid levels to improve lipid metabolism (16). A study by Qian *et al* (17) demonstrated that rosuvastatin was more effective in lowering LDL-C compared with atorvastatin and that it decreased plaque volume and vascular volume in vulnerable coronary artery plaques of AS and stabilized angina pectoris, which are closely associated with endothelial dysfunction (18). To the best of our knowledge, the effects of rosuvastatin on AS have only been macroscopically investigated in previous studies (16,19) and the effects of rosuvastatin on HUVEC dysfunction in atherogenesis remain unclear. In this study, the effects of rosuvastatin on ox-LDL-induced HUVEC injury and hyposecretion of NO were investigated. Furthermore, the possible protective mechanism of rosuvastatin was investigated.

Materials and methods

Chemical reagents. Human umbilical vein endothelial cells (HUVECs) and endothelial cell culture medium (Ham's F-12K) were purchased from Procell Life Science and Technology Co., Ltd., (Wuhan, China), rosuvastatin (purity: >98%) was purchased from Lunan Better Pharmaceutical Co., Ltd., (Linyi, China) and ox-LDL was purchased from Yiyuan Biotechnologies Co., Ltd., (Guangzhou, China). MTT reagent was obtained from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany), DAPI and the Hypersensitive ECL chemiluminescence Kit and bicinchoninic (BCA) protein assay kit were supplied by Beyotime Institute of Biotechnology (Shanghai, China); Nitric Oxide (NO) assay kit (A013-2), Superoxide Dismutase (SOD) assay kit (WST-1, A001-3), Catalase (CAT) assay kit (Ultraviolet, A007-2) and Malondialdehyde (MDA) assay kit (TBA method, A003-1) were purchased from Nanjing Jiancheng Bioengineering Institute (Nanjing, China). Anti-eNOS polyclonal antibody (BS3571), anti-phospho-PI3K p85 α (BS4605) and anti-B-cell lymphoma (Bcl)-2 polyclonal antibody (BS1511) were obtained from Bioworld (Minneapolis, MN, USA). Anti-phospho-eNOS (S1177) polyclonal antibody (ab195944) was purchased from Abcam (Cambridge, UK),

anti-PI3K p85 monoclonal antibody (cat. no. 4257), anti-Akt polyclonal antibody (cat. no. 9272), anti-p-Akt (Ser473) polyclonal antibody (cat. no. 4060) and anti-Bax polyclonal antibody (cat. no. 2772) were from Cell Signaling Technology, Inc., (Danvers, MA, USA) and horseradish peroxidase (HRP)-conjugated anti-rabbit immunoglobulin (Ig)G (cat. no. IH-0011) or anti-mouse IgG (cat. no. IH-0031) were obtained from Dingguo Changsheng Biotechnology Co., Ltd., (Beijing, China).

Cell culture. HUVECs were cultured with endothelial cell culture medium (Ham's F-12K), which contained 10% fetal bovine serum (FBS; Procell Life Science and Technology Co., Ltd.), 0.05 mg/ml endothelial cell growth supplement, 0.1 mg/ml heparin and 1% penicillin/streptomycin at 37°C in an atmosphere containing 5% CO_2 .

Cell viability assay. HUVECs in the logarithmic growth phase were dispersed by trypsinization and seeded into 96-well plates at a density of 4×10^4 cells/ml and 200 μl /well overnight. HUVECs were treated with 0, 0.01, 0.1, 1 or 10 $\mu\text{mol/l}$ rosuvastatin for 48 h in order to estimate whether this agent induced HUVEC injury. In addition, HUVECs were pretreated with the indicated concentrations of rosuvastatin for 24 h and then treated with or without ox-LDL and incubated for a further 24 h to estimate the effect of rosuvastatin on ox-LDL induced HUVECs injury. Subsequently, 20 μl MTT (5 mg/ml in PBS) solution was added into each well and the samples were incubated for 4 h. A total of 150 μl dimethyl sulfoxide was added to each well and the plates were placed on a shaker for 10 min. The absorbance at 570 nm was measured with a microplate reader (SpectraMax Plus384; Molecular Devices, LLC, Sunnyvale, CA, USA). The percentage of surviving cells was calculated as a fraction of the negative control which were treated with an equal volume of cell culture medium alone.

DAPI staining. HUVECs in the logarithmic growth phase were dispersed by trypsinization and seeded at a density of 1×10^5 cells/ml in the coverslips. After treatment, the coverslips were washed three times with PBS, fixed in 4% paraformaldehyde at room temperature for 15 min, permeabilized with 0.1% Triton X-100, stained with 5 $\mu\text{g/ml}$ DAPI and shielded from light at room temperature for 10 min. Finally, the cells were observed under a fluorescence microscope (Nikon TE-2000U; Nikon Corporation, Tokyo, Japan).

Biochemical assays. HUVECs in the logarithmic growth phase were dispersed by trypsinization, seeded into 6-well plates at a density of 1×10^5 cells/ml (2 ml/well) overnight. After treatment, the levels of NO in the supernatant, the activity of SOD and CAT and the content of MDA were estimated using commercial kits according to the manufacturer's protocol. For the measurement of SOD activity, HUVECs in 6-well plates were harvested in pre-cooled PBS using a cell scraper and lysed by ultrasonic decomposition at 300 W for 5 sec, and dissociated for 4 times. Subsequently, 20 μl cell lysis solution, 20 μl enzyme working liquid, 20 μl enzyme diluent and 200 μl substrate working liquid were added to each well of the 96-well plates; the plates were incubated at 37°C for 20 min and the absorbance at 450 nm was measured with a

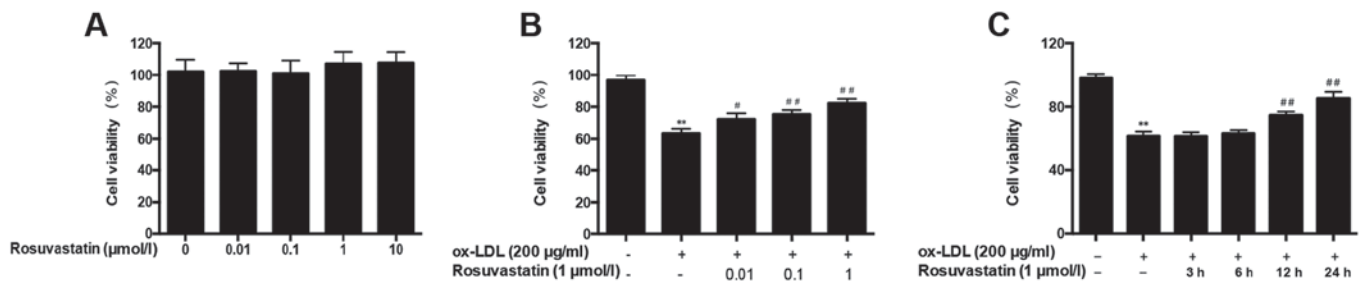


Figure 1. Effects of rosuvastatin on cell viability. (A) HUVECs were treated with different concentrations of rosuvastatin (0.01, 0.1, 1 and 10 $\mu\text{mol/l}$) for 48 h. (B) HUVECs were pretreated with different concentrations of rosuvastatin for 24 h and treated with ox-LDL (200 $\mu\text{g/ml}$) for a further 24 h. (C) HUVECs were pretreated with 1 $\mu\text{mol/l}$ rosuvastatin for various times (3, 6, 12 and 24 h) and then cultured with ox-LDL. Values are expressed as the mean \pm standard deviation from three independent experiments. ** $P<0.01$ vs. the control group; # $P<0.05$ and ## $P<0.01$ vs. the ox-LDL group. HUVECs, human umbilical vein endothelial cells; ox-LDL, oxidized low-density lipoprotein.

microplate reader. The results were calculated and expressed as SOD activity. For the measurement of CAT activity, 20 μl cell lysis solution was added in a 1-cm optical path cuvette and rapidly combined with 3 ml substrate working liquid prior to measuring the absorbance at 240 nm for optical density (OD)₁. The absorbance for OD₂ was measured 1 min later. The substrate (H_2O_2) in HUVECs can be degraded by CAT. Notably, the H_2O_2 concentration was gradually reduced in the reaction liquid and the corresponding absorbance also gradually declined. The results were calculated and expressed as CAT activity. For the measurement of MDA content, 100 μl cell lysis solution, 100 μl NO. 1 working liquid, 1.5 ml NO. 2 working liquid and 1.5 ml NO. 3 working liquid were added in test tubes that were placed in 95°C water baths for 40 min. The tubes were centrifuged at 4,000 \times g for 10 min under room temperature, and the OD values of the supernatants were measured in 1-cm optical path cuvettes at 532 nm. Notably, the MDA in HUVECs can combine with thiobarbituric acid through a condensation reaction, resulting in the development of a red product that has a maximum absorption peak at 532 nm. The results were calculated and expressed as MDA content. Values were expressed as the mean \pm standard deviation from three independent experiments.

Western blot analysis. HUVECs in the logarithmic growth phase were treated with the indicated concentrations of rosuvastatin and incubated with or without ox-LDL. Subsequently, HUVECs were harvested and lysed in radioimmunoprecipitation assay buffer (containing moderate protease inhibitor) for 10 min on ice. The protein concentration was determined using the BCA protein assay kit. Cell extracts were centrifuged at 14,000 \times g at 4°C and equal amounts of protein samples (40 μg) were loaded onto 10-12% polyacrylamide-SDS gel. After electrophoresis, the gel was blotted onto a polyvinylidene difluoride membrane and blocked with 5% (w/v) non-fat milk for 1 h at room temperature. The membranes were incubated with rabbit anti-eNOS polyclonal antibody (1:500), rabbit anti-phospho-eNOS (phospho S1177) polyclonal antibody (1:500), rabbit anti-phospho-PI3K p85 α polyclonal antibody (1:500), rabbit anti-PI3K p85 monoclonal antibody (1:1,000), rabbit anti-Akt polyclonal antibody (1:1,000), rabbit anti-p-Akt (Ser473) polyclonal antibody (1:1,000), rabbit anti-Bcl-2 polyclonal antibody (1:500) and rabbit anti-Bax polyclonal antibody (1:1,000) at 4°C overnight. Primary antibody binding

was detected with using a secondary antibody conjugated to HRP (1:5,000) for 1 h at room temperature. Bands were visualized using ECL chemiluminescence. Finally, densitometric analysis of the bands was conducted using Image J 1.51 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Statistical analysis was performed using the SPSS 19.0 statistical package (IBM Corp., Armonk, NY, USA). The results are expressed as the mean \pm standard deviation. Statistical differences among all groups were evaluated using one-way analysis of variance with Tukey's post hoc test. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effects of rosuvastatin on cell viability. To evaluate the protective effects of rosuvastatin on HUVECs, cell viability was assessed using an MTT assay. First, HUVECs were treated with the different rosuvastatin concentrations for 48 h. However, no concentration of rosuvastatin significantly altered the HUVEC viability compared with the control group (Fig. 1A).

HUVECs were treated with 0.01-1 $\mu\text{mol/l}$ rosuvastatin prior to stimulation with ox-LDL (200 $\mu\text{g/ml}$) and compared with the group only stimulated with ox-LDL. It was demonstrated that 0.01 $\mu\text{mol/l}$ rosuvastatin enhanced HUVEC viability significantly ($P<0.05$) and 0.1-1 $\mu\text{mol/l}$ rosuvastatin demonstrated a more significant effect on enhancing the viability of ox-LDL-induced HUVECs ($P<0.01$; Fig. 1B). Furthermore, HUVECs were pretreated with 1 $\mu\text{mol/l}$ rosuvastatin for different times to assess the time required for rosuvastatin to effect HUVECs with ox-LDL-induced injury. As presented in Fig. 1C, the viability of HUVECs pretreated with rosuvastatin for 12 and 24 h was significantly improved ($P<0.01$); furthermore, rosuvastatin pretreatment for 24 h exerted the most pronounced effect in terms of increasing cell viability ($85.29\pm1.54\%$; $P<0.01$). These results demonstrated that rosuvastatin can enhance the viability of HUVECs treated with ox-LDL.

Effects of rosuvastatin on the morphological alterations of HUVECs. The present study investigated the morphological changes of HUVECs. Cell growth was almost homogeneous in a monolayer and the cells appeared to be organized in a cobblestone-like manner (Fig. 2). After HUVECs were

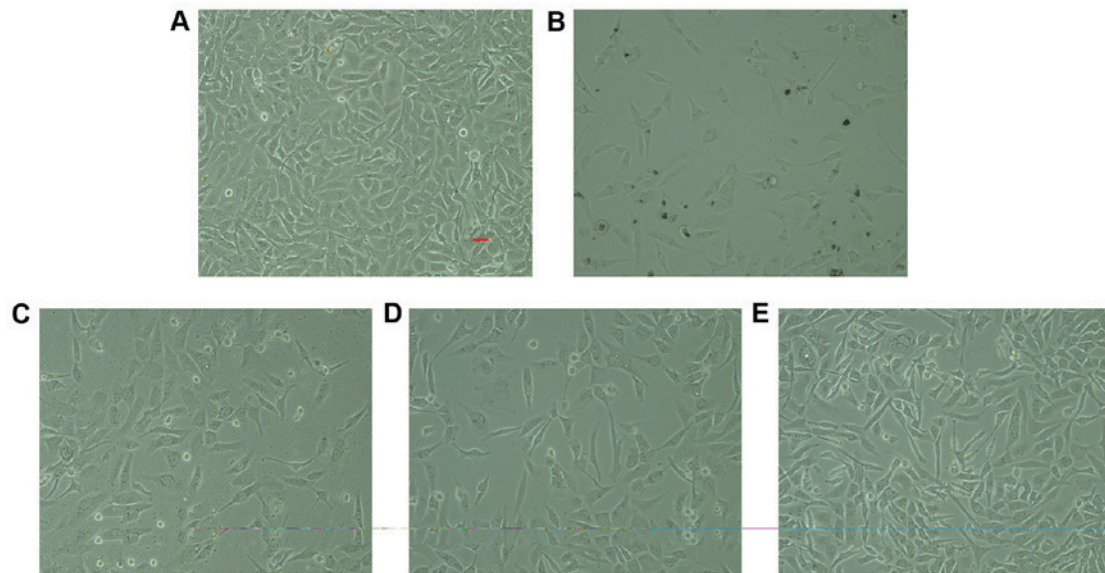


Figure 2. Effects of rosuvastatin on HUVEC morphology. HUVECs were pretreated with different concentrations of rosuvastatin for 24 h and then exposed to ox-LDL (200 $\mu\text{g}/\text{ml}$) for a further 24 h. The morphology of HUVECs was observed under a light microscope (magnification, $\times 100$; scale bar= $100\text{ }\mu\text{m}$). (A) Control group; (B) ox-LDL-induced group; (C) 0.01 $\mu\text{mol}/\text{l}$ rosuvastatin pretreated group; (D) 0.1 $\mu\text{mol}/\text{l}$ rosuvastatin pretreated group; (E) 1 $\mu\text{mol}/\text{l}$ rosuvastatin pretreated group. HUVECs, human umbilical vein endothelial cells; ox-LDL, oxidized low-density lipoprotein.

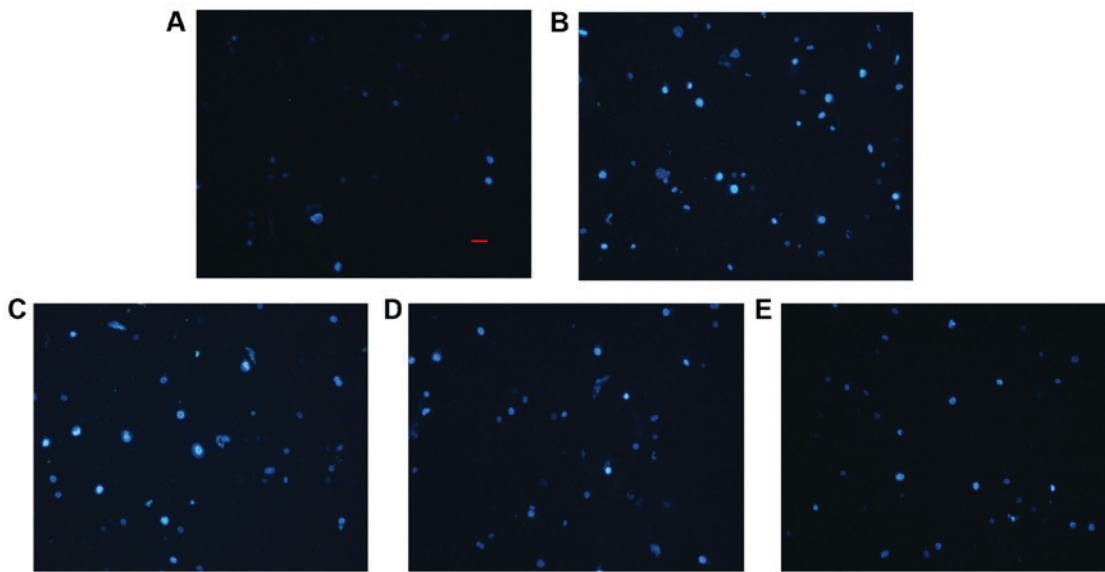


Figure 3. Effect of rosuvastatin on apoptosis in HUVECs treated with ox-LDL. HUVECs were pretreated with different concentrations of rosuvastatin for 24 h and then exposed to ox-LDL (200 $\mu\text{g}/\text{ml}$) for a further 24 h. Cell injury was evaluated by DAPI staining (magnification, $\times 100$; scale bar= $100\text{ }\mu\text{m}$). (A) Control group; (B) ox-LDL-induced group; (C) 0.01 $\mu\text{mol}/\text{l}$ rosuvastatin pretreated group; (D) 0.1 $\mu\text{mol}/\text{l}$ rosuvastatin pretreated group and (E) 1 $\mu\text{mol}/\text{l}$ rosuvastatin pretreated group. HUVECs, human umbilical vein endothelial cells; ox-LDL, oxidized low-density lipoprotein.

cultured with ox-LDL, the cell morphology became irregular, the outline was not clear and nuclear condensation, and fragmentations were observed under an optical microscope. However, HUVECs that were pre-incubated with 0.01-1 $\mu\text{mol}/\text{l}$ rosuvastatin in the presence of ox-LDL had started to display signs of normalization, suggesting that rosuvastatin exerts protective effects against ox-LDL-induced HUVEC injury.

Effect of rosuvastatin on HUVEC apoptosis induced by ox-LDL. The morphological characteristics of HUVECs in which apoptosis was induced by ox-LDL were assessed using DAPI staining. DAPI-positive cells produced a

brighter and steady fluorescence. As presented in Fig. 3, control HUVECs emitted tiny areas of blue fluorescence. Compared with the control, ox-LDL induced severe HUVEC functional impairment and apoptosis, manifesting as intense blue fluorescence that indicated extensive nuclear injury and fragmentation, which was in accordance with the MTT results. In contrast, treatment with rosuvastatin decreased nuclear injury induced by ox-LDL to different degrees. Notably, the highest rosuvastatin concentration was associated with less apoptotic fluorescence. The results indicated that rosuvastatin serves a protective role in ox-LDL-induced apoptosis in HUVECs.

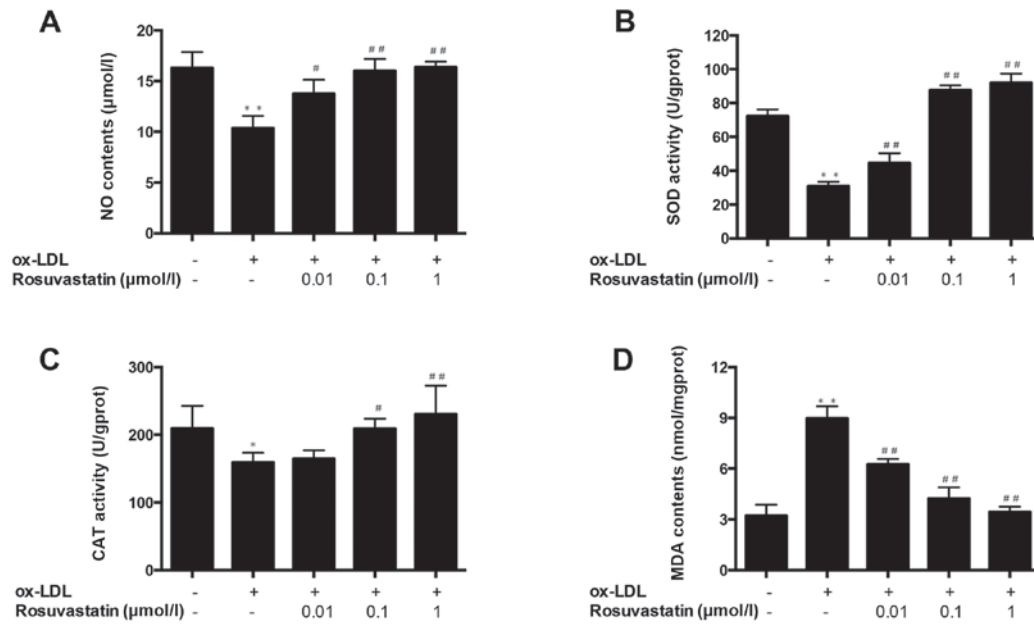


Figure 4. Effect of rosvastatin on the levels of NO and oxidative stress in HUVECs treated with ox-LDL. (A) Culture supernatants of HUVECs were collected to detect the NO content using an NO assay kit (Microwell plate method) and the absorbance was measured at 570 nm. HUVECs were collected for ultrasonic cell disruption in an ice bath to detect the activity of (B) SOD using a SOD assay kit. (C) The activity of CAT was determined with a Catalase assay kit and (D) MDA content was assessed using an MDA assay kit. Values are expressed as the mean \pm standard deviation from three independent experiments. * $P < 0.05$ and ** $P < 0.01$ vs. the control group. # $P < 0.05$ and ## $P < 0.01$ vs. the ox-LDL group. CAT, catalase; SOD, superoxide dismutase; MDA, malondialdehyde; NO, nitric oxide; HUVECs, human umbilical vein endothelial cells; ox-LDL, oxidized low-density lipoprotein.

Effect of rosvastatin on NO levels in HUVEC injury induced by ox-LDL. To elucidate the implication of NO in ox-LDL-induced injury of HUVECs, the contents of NO in the cell culture supernatant were detected. Compared with the control group, the NO level was significantly decreased in HUVECs with ox-LDL-induced injury ($P < 0.01$; Fig. 4A), whereas 0.01 $\mu\text{mol/l}$ rosvastatin pretreatment significantly increased NO secretion compared with the ox-LDL-induced injury group ($P < 0.05$). Notably, the 0.1 and 1 $\mu\text{mol/l}$ rosvastatin pretreatment groups demonstrated superior effects ($P < 0.01$). These results indicated that rosvastatin may increase the levels of the endothelial protective factor NO.

Effect of rosvastatin on oxidative stress in HUVEC injury induced by ox-LDL. To elucidate the implications of oxidative stress in HUVEC injury induced by ox-LDL, the intracellular MDA content and SOD and CAT activities were measured. Compared with the control group, the activity of SOD and CAT were significantly decreased ($P < 0.01$ and $P < 0.05$, respectively; Fig. 4B and C) and MDA content was significantly increased ($P < 0.01$; Fig. 4D) in HUVECs with ox-LDL-induced injury. While HUVECs pretreated with rosvastatin (0.01-1 $\mu\text{mol/l}$) exhibited a significant improvement of SOD activity and a reduction of MDA content ($P < 0.01$), 0.1 and 1 $\mu\text{mol/l}$ of rosvastatin significantly improved the CAT activity compared with HUVECs with ox-LDL-induced injury ($P < 0.05$ and $P < 0.01$, respectively). The results suggested that rosvastatin has antioxidant properties and may relieve the oxidative stress of ox-LDL-induced injury in HUVECs.

Effect of rosvastatin on the phosphorylation of eNOS in HUVEC injury induced by ox-LDL. The phosphorylation of

eNOS, which is the regulatory factor of NO production was measured. As presented in Fig. 5A and B, the phosphorylation of eNOS in ox-LDL-stimulated HUVECs was significantly decreased compared with the control group ($P < 0.01$). Furthermore, HUVECs were pretreated with different concentrations of rosvastatin and the phosphorylation levels of eNOS were significantly enhanced ($P < 0.01$). However, the expression levels of total eNOS were not notably different between these groups. Subsequently, HUVECs were pretreated with rosvastatin 1 $\mu\text{mol/l}$ for different times (3, 6, 12 and 24 h) and then stimulated with ox-LDL to determine the pretreatment time of rosvastatin that altered the phosphorylation of eNOS. The results demonstrated that rosvastatin pretreatment for 3 h could significantly affect the phosphorylation of eNOS ($P < 0.01$) and as the pretreatment time of rosvastatin increased, the phosphorylation of eNOS also significantly increased ($P < 0.01$; Fig. 5C and D). The results suggested that rosvastatin exerts a prominent effect on enhancing eNOS phosphorylation in HUVECs with ox-LDL-induced injury and pretreatment with rosvastatin for only 3 h can significantly promote the phosphorylation of eNOS, which is a shorter time compared with that required for enhancing HUVEC viability following ox-LDL stimulation (Fig. 1D).

Effects of rosvastatin on the phosphorylation of the PI3K/Akt signaling pathway. The PI3K/Akt signaling pathway is considered as a key mediator of eNOS activity that is implicated in the secretion of NO in HUVECs. In the present study, the phosphorylation of PI3K and Akt was determined. As presented in Fig. 6, compared with the control group, the phosphorylation of PI3K (Fig. 6A and B) and Akt (Fig. 6A and C) were significantly decreased in ox-LDL-stimulated HUVECs ($P < 0.01$),

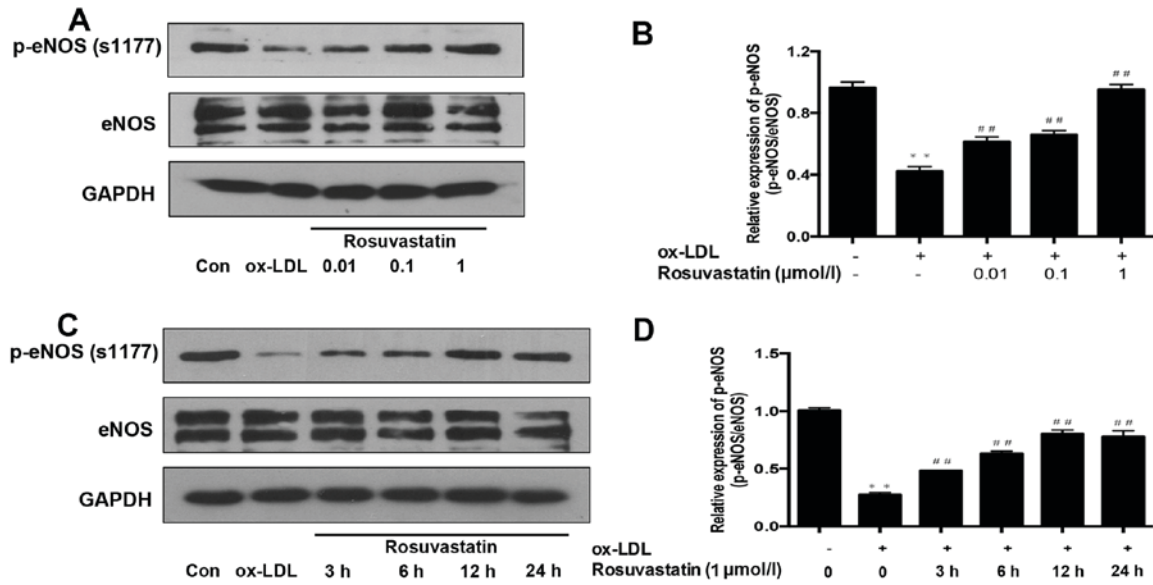


Figure 5. Effect of rosuvastatin on the phosphorylation of eNOS in HUVECs treated with ox-LDL. (A) The effect of 0.01-1 $\mu\text{mol/l}$ rosuvastatin on the phosphorylation of eNOS. (B) The phosphorylation level of eNOS with 0.01-1 $\mu\text{mol/l}$ rosuvastatin pretreatment was assessed using densitometric analysis. (C) The effect of 1 $\mu\text{mol/l}$ rosuvastatin treatment at various time points on the phosphorylation of eNOS. (D) The phosphorylation level of eNOS with 1 $\mu\text{mol/l}$ rosuvastatin pretreatment at various time points was conducted using densitometric analysis. The phosphorylation of eNOS was expressed as the ratio of p-eNOS to total eNOS, the subjacent band is target band of eNOS and were used to calculate the ratio of p-eNOS expression. GAPDH was presented as a loading control. The densitometric analysis values are expressed as the mean \pm standard deviation from three independent experiments. ** $P < 0.01$ vs. the control group; ## $P < 0.01$ vs. the ox-LDL group. HUVECs, human umbilical vein endothelial cells; ox-LDL, oxidized low-density lipoprotein; p-eNOS, phosphorylated endothelial nitric oxide synthase.

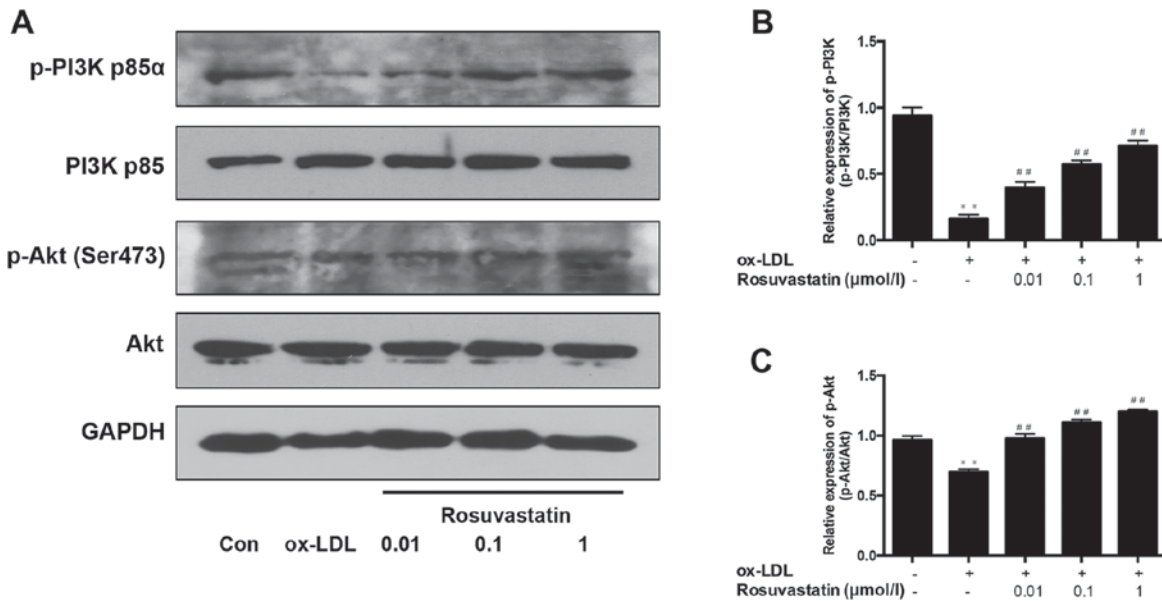


Figure 6. Effect of rosuvastatin on the phosphorylation of the PI3K/Akt signaling pathway mediators. (A) The effect of rosuvastatin on the phosphorylation of PI3K/Akt. The phosphorylation of (B) PI3K/(C) Akt was conducted by densitometric analysis, as well as expressed as the ratio of p-PI3K to total PI3K and p-Akt to total Akt. GAPDH was used as a loading control. The densitometric analysis values are expressed as the mean \pm standard deviation from three independent experiments. ** $P < 0.01$ vs. the control group; ## $P < 0.01$ vs. the ox-LDL group. Akt, protein kinase B; PI3K, phosphoinositide 3 kinase; con, control; ox-LDL, oxidized-low-density lipoprotein; p-, phosphorylated.

and 0.01-1 $\mu\text{mol/l}$ rosuvastatin significantly enhanced the phosphorylation of PI3K and Akt to varying degrees ($P < 0.01$). Furthermore, the effects of rosuvastatin were dose-dependent. The expression of total PI3K and total Akt did not notably differ between the control and HUVECs with ox-LDL-induced injury. These results suggested that rosuvastatin may affect the PI3K/Akt signaling pathway.

Effect of rosuvastatin on the expression of Bcl-2/Bax. Bcl-2 and Bax are important hallmarks of apoptosis that are regulated by the PI3K/Akt signaling pathway. As presented in Fig. 7, it was demonstrated that the expression of apoptotic protein (Bax) increased and the expression of antiapoptotic protein (Bcl-2) was significantly decreased in HUVECs with ox-LDL-induced injury ($P < 0.01$). Notably, rosuvastatin

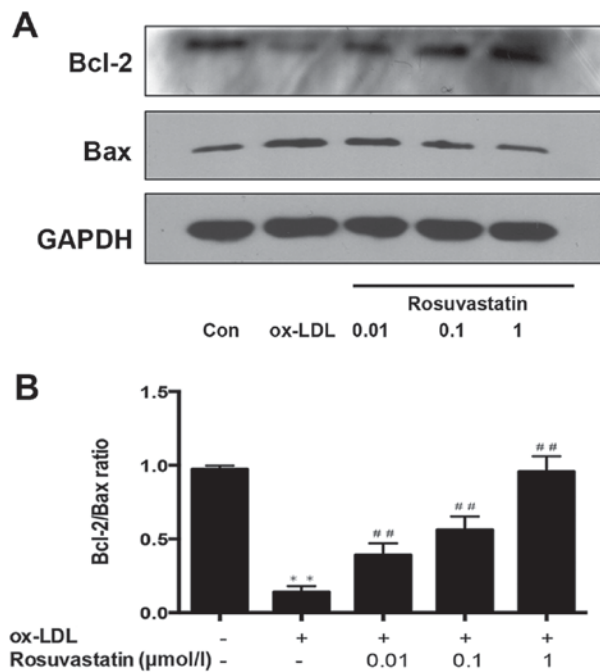


Figure 7. Effect of rosuvastatin on the expression of Bcl-2/Bax. (A) The effect of rosuvastatin on expression of Bcl-2/Bax. (B) The ratio of Bcl-2/Bax was assessed using densitometric analysis. GAPDH was presented as a loading control. Densitometric analysis values are expressed as the mean \pm standard deviation from three independent experiments. ** $P < 0.01$ vs. the control group; ** $P < 0.01$ vs. the ox-LDL group. Bcl-2, B-cell lymphoma-2; con, control; ox-LDL, oxidized-low-density lipoprotein.

enhanced the expression of Bcl-2 and significantly decreased the expression of Bax ($P < 0.01$) in the ox-LDL-stimulated HUVEC group. The results suggested that rosuvastatin may protect HUVECs against ox-LDL-induced apoptosis by regulating the expression of Bcl-2/Bax.

Discussion

The pathogenesis of AS may be initiated with systemic inflammation and acute lipid oxidation (20). Under conditions of high blood lipid levels, the lipids invade and are deposited into subintimal cells, leading to macrophage infiltration under the vascular intima to phagocytose lipids, which promotes the formation of atherosclerotic plaques and thrombi (2). The endothelial cell is an important barrier of blood vessels, which can resist the damage caused by inflammatory cell infiltration, disturbed blood flow and any other external stimulating factors (21). The excessive LDL modified by oxidation or enzymes (ox-LDL) disturbs endothelial function, including disruption of the endothelial barrier, impairment of NO release followed by ox-LDL penetrating into the intima in the earliest stages of AS (21). AS can accelerate the progression of CVDs (6). Notably, chronic lipoprotein abnormalities induce a decline in kidney function (22), with the exception of hyperlipemia induced by AS. AS has become a high-risk complication in a number of other diseases, including diabetes; persistent hyperglycemia suppresses the phosphorylation levels of Akt and eNOS (23) and disrupts L-arginine-NO metabolism, which is a key protective factor associated with the inhibition of apoptosis in HUVECs. It

has been demonstrated that tumor necrosis factor- α -induced apoptosis was inhibited by low concentrations of NO in a cyclic guanosine monophosphate-independent manner and the cellular suicide program was inhibited in HUVECs via S-nitrosylation of members of the caspase family (24). Furthermore, NO protects cells from apoptosis by stimulating the production of vascular endothelial growth factor (25). According to the results above, NO bioavailability disruption may induce a series of endothelial cell dysfunctions (26), including aggravated inflammation (27), cardiac cell death and acute coronary syndrome (28). Ongoing research has focused on investigating the mechanisms underlying endothelial cell injury and improving endothelial cell dysfunction in different diseases.

Oxidative stress is the etiology behind arterial wall alterations. Oxidative stress is due to an imbalance between the enzymatic activity of antioxidants and free radicals, which causes the decreased bioavailability of the endothelial protective factor NO (20). Furthermore, endothelial cell dysfunction increases the production of oxygen free radicals derived from NO catabolism, resulting in a vicious circle of endothelial cell injury (7). Oxidative stress is also implicated in apoptosis-associated protein expression that results in cell apoptosis. It has been demonstrated that ox-LDL-induced apoptosis may be achieved by regulating the expression of Bcl-2 and Bax protein within human fatty streaks (29). Based on the above-mentioned findings, clearing the circulating reactive oxygen species or increasing the antioxidant capacity are considered as key points in the prevention and therapy of AS. The role of eNOS dysfunction in AS is well understood. eNOS dysfunction in endothelial cells can disrupt vascular tone and structure regulation (30). A previous study demonstrated that increasing the expression and activation of eNOS can promote neointimal growth, cell migration and re-endothelialization following arterial balloon catheter injury (31). In endothelial cells, Akt phosphorylates eNOS directly which promotes cell survival by nitrosylating the reactive cysteine residue in caspases (13) and critically regulates apoptosis in endothelial cells including activation of the pro-apoptotic proteins Bax and caspases 3 and 9 (32). In addition, the activation of Akt stimulated with activated phosphoinositide-dependent kinase-1 (PDK1) and mammalian target of rapamycin complex 2 by direct binding and phosphorylation on threonine 308 and serine 473 (13). Notably, PI3K activation is the key regulator of Akt activation by regulating PDK1. The expression and activation of eNOS and Akt can be suppressed by PI3K inhibitors (23,32). The PI3K/Akt signaling pathway exerts a strong regulatory effect on expression and activation of eNOS.

The 2013 American College of Cardiology/American Heart Association cholesterol guidelines recommended using 'high-intensity' statin therapy for reducing the risk of hypercholesterolemia-induced cardiovascular events due to its beneficial effects on LDL-C reduction (33,34). Rosuvastatin has multiple advantages compared with other statins, including stronger regulatory effects on dyslipidemia, a shorter half-life and high bioavailability (19). In addition to lowering LDL-C, it is plausible that there are more pronounced benefits of rosuvastatin therapy in coronary

atherosclerotic plaque regression (17). These results called attention to the effects of rosuvastatin on endothelial cell dysfunction. In the present study, the influence of ox-LDL on endothelial cell protection factor eNOS/NO was measured and the effects of rosuvastatin on ox-LDL induced insufficient expression levels of eNOS/NO, as well as PI3K/Akt signaling pathway-regulatory factor of eNOS, aiming to investigate the multiple effects of rosuvastatin on endothelial cells independent of lipid regulation.

Ox-LDL was used to induce HUVEC injury *in vitro*. The present study demonstrated that ox-LDL promoted extensive HUVEC apoptosis, reduced endothelial cell-derived NO levels, increased oxidative stress and reduced the activity of SOD and CAT. Furthermore, the content of MDA was increased compared with the levels in normal HUVECs. These results were in agreement with the findings of Ahsan *et al* (32). Following treatment with rosuvastatin, cell viability rates were increased, NO secretion in HUVECs was increased and the high-dose rosuvastatin-treated group was comparable to the control group; the activity of SOD and CAT was enhanced, and the MDA content was decreased by rosuvastatin. The results suggested that rosuvastatin exerted beneficial antioxidant effects in HUVECs with ox-LDL-induced injury. Furthermore, ox-LDL-induced eNOS activation was markedly decreased compared with control HUVECs; however, rosuvastatin treatment reversed the effects of ox-LDL in a dose-dependent manner. The present study also evaluated the phosphorylation of PI3K and Akt, as well as the expression of Bcl-2/Bax in HUVECs with ox-LDL-induced injury. Notably, phosphorylation of PI3K and Akt was inhibited and the ratio of Bcl-2/Bax was decreased. However, rosuvastatin treatment increased the phosphorylation of PI3K and Akt compared with ox-LDL-stimulated HUVECs. Furthermore, the upregulation of eNOS by rosuvastatin was regulated via the PI3K/Akt signaling pathway and the results suggested that rosuvastatin may protect HUVECs against ox-LDL-induced apoptosis. Furthermore, the biological functions of vascular eNOS and NO are varied, including anti-inflammatory, anti-platelet and vasodilatory actions, as well as endoplasmic reticulum stress (ERs)-derived eNOS dysfunction is a crucial damage factor of endothelial cell through oxidative stress and ERs-induced apoptosis serves a key role in the occurrence of AS (35). Consequently, the authors intend to investigate the effect of rosuvastatin on ERs and the relevant apoptosis signal pathways, which are closely associated with endothelial cell dysfunction in the next step of research.

In conclusion, ox-LDL can induce severe injury and apoptosis of HUVECs, reduce eNOS phosphorylation and eNOS-derived NO. However, rosuvastatin treatment can reverse the effects of ox-LDL, upregulate the phosphorylation of PI3K/Akt, and the expression of Bcl-2/Bax, indicating that rosuvastatin may attenuate endothelial dysfunction and apoptosis in AS and in other diseases accompanied by vascular lesions.

Acknowledgements

The authors would like to thank Dr Wenwen Fu, Mr. Zeyuan Lu and Mr. Yuchen Wang, of the department of Pharmacology,

College of Pharmacy, Jilin University (Changchun, China), for their technical assistance.

Funding

The present study was supported by the Science and Technology development projects of Jilin, China (grant no. 20150101200JC).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

DS and GL conceived and designed the study. JG, HX and XY performed the experiments. JG and HX wrote the paper. GX and HC analyzed data for the study, reviewed and edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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