G0S2 ameliorates oxidized low-density lipoprotein-induced vascular endothelial cell injury by regulating mitochondrial apoptosis

Zenghui Liang[1], Wenjie Diao[2], Yiyao Jiang[2], Yanrong Zhang[1]^

[1]Department of Vascular Surgery, the Third Hospital of Hebei Medical University, Shijiazhuang, China; [2]Department of Cardiac Surgery, the First Affiliated Hospital of Bengbu Medical College, Bengbu, China

Contributions: (I) Conception and design: Z Liang, Y Zhang; (II) Administrative support: Y Zhang; (III) Provision of study materials or patients: All authors; (IV) Collection and assembly of data: W Diao, Y Jiang; (V) Data analysis and interpretation: Z Liang, Y Zhang; (VI) Manuscript writing: All authors; (VII) Final approval of manuscript: All authors.

Correspondence to: Yanrong Zhang. Department of Vascular Surgery, the Third Hospital of Hebei Medical University, 139 Ziqiang Road, Shijiazhuang 050051, China. Email: Yanrongzhang2022@163.com.

Background: Oxidative low-density lipoprotein (ox-LDL)-induced endothelial cell damage is a major risk factor for atherosclerosis and its related cardiovascular diseases. The G0/G1 switch gene 2 (G0S2) is a multifunctional protein which has been poorly studied in atherosclerosis.

Methods: In this study, ox-LDL was utilized to construct a human aortic endothelial cell (HAEC) injury model.

Results: It was found that ox-LDL impaired cell viability, augmented lactate dehydrogenase (LDH) release, and reduced G0S2 levels in HAECs in a dose-dependent manner. Further, G0S2 overexpression improved the viability and restrained apoptosis of HAECs treated by ox-LDL. Conversely, G0S2 depletion decreased the viability and aggravated apoptosis of HAECs treated by ox-LDL. At the molecular level, G0S2 overexpression significantly increased the secretion of superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPH-Px), promoted intracellular reactive oxygen species (ROS) production and malondialdehyde (MDA) content in HAECs under either normal or ox-LDL conditions. Meanwhile, the ox-LDL-induced mitochondrial dysfunction, as demonstrated by decreased mitochondrial membrane potential, translocation of mitochondrial cytochrome c (Cyt-c) to the cytoplasm, and activation of caspase-3 and caspase-9, was significantly reversed by G0S2 overexpression. In addition, G0S2 overexpression promoted the activation of AMP-activated protein kinase (AMPK) and increased the expression of nuclear factor erythroid-2-related factor-2 (Nrf2), sirtuin 1 (SIRT1) and heme oxygenase 1 (HO-1) under normal and ox-LDL conditions.

Conclusions: This study demonstrated that G0S2 protects against ox-LDL-induced vascular endothelial cell injury by regulating oxidative damage and mitochondrial homeostasis and may be a promising target for the treatment of atherosclerosis.

Keywords: Human aortic endothelial cells (HAECs); G0/G1 switch gene 2 (G0S2); mitochondrial homeostasis; oxidative damage

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^ ORCID: 0000-0001-8960-8917.

Introduction

Atherosclerosis is a multi-factorial, multi-step process characterized by oxidative low-density lipoprotein (ox-LDL) vascular intimal deposition and subsequent endothelial cell damage and plaque formation (1). Ox-LDL-mediated endothelial damage is a prominent event and risk factor for atherosclerosis (2). Ox-LDL promotes the production of a large amount of reactive oxygen species (ROS), damages mitochondrial function, and eventually leads to the occurrence of cell death (3,4). Since ROS is mainly synthesized by mitochondria, when mitochondrial function is impaired, ROS production will be further induced, forming a vicious cycle, and finally leading to the occurrence and progression of atherosclerosis (5). Therefore, exploring the key molecules and mechanism of mitochondrial dysfunction during ox-LDL-induced endothelial damage will contribute to the treatment of atherosclerosis.

G0/G1 switch gene 2 (G0S2) is a multifunctional protein containing 103 amino acids, which is widely expressed and especially abundant in the heart, skeletal muscle, liver, kidney, brain, and adipose tissue (6). Previous studies have shown that G0S2 exists in the cytoplasm, endoplasmic reticulum, mitochondria, or lipid droplets at the subcellular levels, and plays different roles in different cellular contexts (7-9). G0S2 was first identified in activated lymphocytes and was related to the G0/G1 switch of cell cycle (10). Later studies confirmed that G0S2 acted as a lipolytic inhibitor, and contributed to lipid accumulation and fat homeostasis in adipocytes and hepatocytes by inhibiting lipolysis (11-14). Moreover, several studies have revealed that G0S2 modulates oxidative phosphorylation activity (15,16). Kioka *et al.* (17) reported that upregulated G0S2 under hypoxic stimulation increased oxidative phosphorylation activity and mitochondrial adenosine triphosphate (ATP) production in cardiomyocytes. However, Lee *et al.* (18) found that G0S2 maintained homeostatic proliferation of naïve $CD8^+$ T cells through inhibiting oxidative phosphorylation in mitochondria. These findings suggest the important role of G0S2 in mitochondrial function. Mitochondria are important organelles of eukaryotic cells, and their dysfunction is closely related to the initiation and development of atherosclerosis. Many studies have shown that mitochondrial dysfunction could lead to reduced production of ATP and increased production of ROS, and has been considered to be one of the triggers of vascular endothelial injury (19-21). G0S2 has been shown to have a protective function in endothelial cells by shielding them from serum-free starvation stress and hydrogen peroxide-induced apoptosis (22). However, whether G0S2 is involved in ox-LDL-induced endothelial injury and mitochondrial dysfunction has not been reported.

In this study, we discovered that G0S2 was downregulated in human aortic endothelial cells (HAECs) upon ox-LDL condition. G0S2 overexpression reversed ox-LDL-induced viability reduction and apoptosis of HAECs. Notably, restoring G0S2 expression alleviated oxidative damage and mitochondrial dysfunction stimulated by ox-LDL. Together, our findings support the importance of G0S2 in mediating protective effects on ox-LDL-induced endothelial cell injury. We present the following article in accordance with the MDAR reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5618/rc).

Highlight box

Key findings

- G0S2 inhibits oxidative damage and mitochondrial apoptosis via activating the AMPK pathway and ameliorates ox-LDL-induced vascular endothelial cell injury.

What is known and what is new?

- It is known that G0S2 exists in the mitochondria and modulates oxidative phosphorylation activity.
- This study is the first to report that G0S2 can protect vascular endothelial cells from ox-LDL damage. Mechanistically, G0S2 inhibits oxidative damage and mitochondrial apoptosis by activating AMPK pathway.

What is the implication, and what should change now?

- G0S2 may be a promising target for the treatment of atherosclerosis.

Methods

Cell culture

HAECs were purchased from Procell (CP-H080; Procell, Wuhan, China). The cells were cultured in a HAEC complete medium (CM-H080, Procell) at 37 ℃, 5% CO_2, and 97% humidity in an incubator. The medium was changed every 2–3 days. HAECs were treated with ox-LDL (Peking Union-Biology Co., Ltd., Beijing, China) for 24 hours to establish an *in vitro* atherosclerosis model. The final work concentration was 50 μg/mL.

Cell transfection

Small interfering RNAs targeting G2S0 (si-G2S0) and

its negative control (si-NC), pcDNA3.1-G2S0 plasmids (G0S2_OE), and empty vectors (Vector) were obtained from RiboBio (Guangzhou, China). HAECs in the logarithmic growth phase were seeded in 6-well plates at a density of 1×10^5 cells per well. When the cell growth density reached around 60%, the medium was replaced with a fetal bovine serum (FBS)-free medium. These plasmids were transfected into cells using Lipofectamine 2000 kit (Thermo Fisher, Shanghai, China) according to the instructions.

Cell viability assay

The transfected HAECs (1×10^4 cells /mL) were seeded into a 96-well plate, and then cultured with or without ox-LDL (50 μg/mL) for 24 hours. Then, 10 μL Cell Counting Kit-8 (CCK-8) reagent (Beyotime, Shanghai, China) was added to each well and incubated for 2 hours. Finally, the absorbance was detected at 450 nm with a microplate reader.

Lactate dehydrogenase (LDH) assay

The activity of LDH in the culture supernatant was quantified by the colorimetric method with an LDH Assay Kit (Beyotime, Shanghai, China).

Cell apoptosis analysis

Annexin V FITC/PI Apoptosis Detection Kit (Vazyme, Jiangsu, China) was used for cell apoptosis staining. In brief, HAECs were re-suspended in 1× binding buffer, incubated with Annexin V fluorescein isothiocyanate (FITC) for 15 minutes, and 5 μL of propidium iodide (PI) for another 5 minutes in the dark. The stained cells were placed in a flow cytometer and the rate of cell apoptosis was analyzed.

Quantitative reverse transcription polymerase chain reaction (PCR)

Total RNAs were extracted from cells with Trizol reagent, and the concentration and purity of RNAs were determined by a micro-nucleic acid analyzer, and then stored at –20 ℃ for future use. A one-step PCR reverse transcription (RT) kit was used to synthesize complementary DNA (cDNA), and SYBR Greenmaster Mix was used for reverse transcription quantitative polymerase chain reaction (RT-qPCR) amplification. The relative expression level of G0S2 messenger RNA (mRNA) was calculated by the $2^{-\Delta\Delta Ct}$ method with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as an internal reference. The reagents for RNA extraction and PCR amplification were purchased from Tiangen (Beijing, China). The primers were provided by Sangon Biotech (Shanghai, China) and the sequences of primers were as follows: G0S2, forward: 5'-GAGAACGCTGAGGTCGGTC-3', and reverse: 5'-GGGCTCATAGAAAGGCGGAC-3'. GAPDH, forward: 5'-CCTGTTCGACAGTCAGCCG-3', and reverse: 5'-GAGAACAGTGAGCGCCTAGT-3'.

Isolation of mitochondrial and cytosolic fraction

Mitochondrial and cytosolic fractions of HAECs were separated by differential centrifugation using a Cell Mitochondria Isolation Kit (Beyotime, Shanghai, China) according to the kit's protocol. Mitochondrial and cytosolic proteins were obtained for determining the cytochrome c (Cyt-c).

Western blot analysis

Proteins were isolated from cells using radioimmunoprecipitation assay (RIPA) lysis buffer. After protein quantification with bicinchoninic acid (BCA) Protein Assay Kit (Beyotime, Jiangsu, China), an appropriate amount of protein was mixed with an equal volume of loading buffer and boiled for 5 minutes to denature the protein. Then, 30 μg protein was loaded in each lane, and polyacrylamide gel electrophoresis (PAGE) was performed at 100 V for 90 minutes. After the protein was transferred to a nitrocellulose membrane, it was incubated with a blocking solution containing 5% nonfat dry milk at room temperature for 2 hours. The membrane was incubated with anti-G0S2 antibody (#ab236113, Abcam, Cambridge, USA), anti-cytochrome C antibody (#ab133504, Abcam), anti-COX IV antibody (#ab202554, Abcam), anti-cleaved Caspase-3 antibody (#ab2302, Abcam), anti-pro caspase-3 antibody (#ab32499, Abcam), anti-cleaved caspase-9 antibody [#9505, Cell Signaling Technology (CST), Danvers, MA, USA], anti-phosphorylated AMPK (ab133448, Abcam), anti-AMPK (ab207442, Abcam), anti-Nrf2 (#80593-1-RR, Proteintech, Wuhan, China), anti-HO-1 (#66743-1-Ig, Proteintech) anti-SIRT1 (#60303-1-Ig, Proteintech), and anti-beta actin antibody (#ab115777, Abcam) at room temperature for 2 hours, and then incubated with diluted goat anti-rabbit IgG H&L (ab6721,

Abcam) solution at room temperature for 1 hour. The membrane was developed with a chemiluminescence kit. After exposure, ImageJ software (National Institutes of Health, Bethesda, MD, USA) was used to detect the gray value of each band, and the relative expression level of the target protein was represented by the gray value ratio of the target protein and the internal reference protein β-actin.

Detection of ROS

Dichloro-dihydro-fluorescein diacetate (DCFH-DA) staining was used for intracellular ROS detection. DCFH-DA is a general indicator of oxidative stress. The intensity of fluorescence represents the concentration of intracellular ROS. HAECs were incubated with DCFH-DA at 37 ℃ for 20 minutes in dark and then monitored under a fluorescence microscope.

Detection of malondialdehyde (MDA) content, superoxide dismutase (SOD), and glutathione peroxidase (GSH-Px) activities

Lipid Peroxidation MDA Assay Kit, SOD Activity Assay Kit, and Micro GSH-Px Assay Kit were all purchased from Solarbio (Beijing, China) and used for MDA, SOD, and GSH-Px detection. First, HAECs were lysed with the extracting solution and broken by ultrasound (power 200 W, ultrasonic 3 s, interval 10 s, repeat 30 times). Cell lysates were centrifuged at 8,000 g for 10 minutes at 4 ℃. Then, the supernatant was collected and placed on ice for subsequent testing.

For MDA content, the obtained supernatant was mixed with an MDA detection working solution. The mixture was kept at 100 ℃ for 60 minutes, cooled to room temperature, and centrifuged at 10,000 g, for 10 minutes. The absorbance of each sample was determined at 532 and 600 nm.

For SOD activity, the obtained supernatant was fully mixed with the SOD working reagent and kept at 37 ℃ for 30 minutes. Then, the absorbance at 560 nm was measured. According to the formula: inhibition percentage = $(\Delta A^{blank} - \Delta A^{sample})/\Delta A^{blank} \times 100\%$, the inhibition percentage of SOD was calculated.

For GSH-Px activity, the obtained supernatant was fully mixed with the GSH-Px working reagent at room temperature for 15 minutes. Then, the absorbance at 412 nm was measured. According to the formula: inhibition percentage = $(A^{control} - A^{sample})/(A^{control} - A^{blank}) \times 100\%$, the inhibition percentage of GSH-Px activity was calculated.

Evaluation of mitochondrial transmembrane potential (MMP)

A JC-1 probe is used as an indicator for detecting MMP. When apoptosis occurs, the MMP is depolarized, JC-1 is released from the mitochondria, the red-light intensity is weakened, and it exists in the cytoplasm in the form of a monomer to produce green fluorescence. Red fluorescence indicates high MMP, whereas green fluorescence indicates low MMP. Here, HAECs were labeled using JC-1 Mitochondrial Membrane Potential Assay Kit (MedChemExpress, Shanghai, China). Then, cells were monitored under an inverted fluorescence microscope (Leica, Wetzlar, Germany).

Statistical analysis

The software GraphPad Prism version 8.0 (GraphPad Software Inc., San Diego, CA, USA) was used for plotting bar charts and graphs; SPSS 20.0 (IBM Corp., Armonk, NY, USA) was used for statistical analysis. Values were shown as the mean ± standard deviation of 3 independent experiments (conducted in triplicate). Student's *t*-test was used for comparison of 2 groups, and one-way analysis of variance (ANOVA) along with *post-hoc* test was used for comparison of multiple groups. A P value <0.05 was considered statistically significant.

Results

G0S2 expression is reduced in HAECs exposed to ox-LDL

HAECs were incubated with different concentrations of ox-LDL (0, 25, 50, and 100 μg/mL) to mimic endothelial injury in atherosclerosis. The CCK-8 assay showed that ox-LDL led to a significant decline of cell viability of HAECs in a dosed manner (*Figure 1A*). Meanwhile, the ox-LDL treatment also significantly augmented LDH release (*Figure 1B*). The ox-LDL treatment decreased the expression of G0S2 in HAECs in a dose-dependent manner, which was evidenced by the results of RT-qPCR (*Figure 1C*) and western blot (*Figure 1D*). In consideration of cytotoxicity and effect, 50 μg/mL was selected for the subsequent experiments.

Overexpression of G0S2 restored the viability and reduces apoptosis of HAECs under the ox-LDL condition

G0S2-overexpression plasmids (G0S2_OE) were transfected

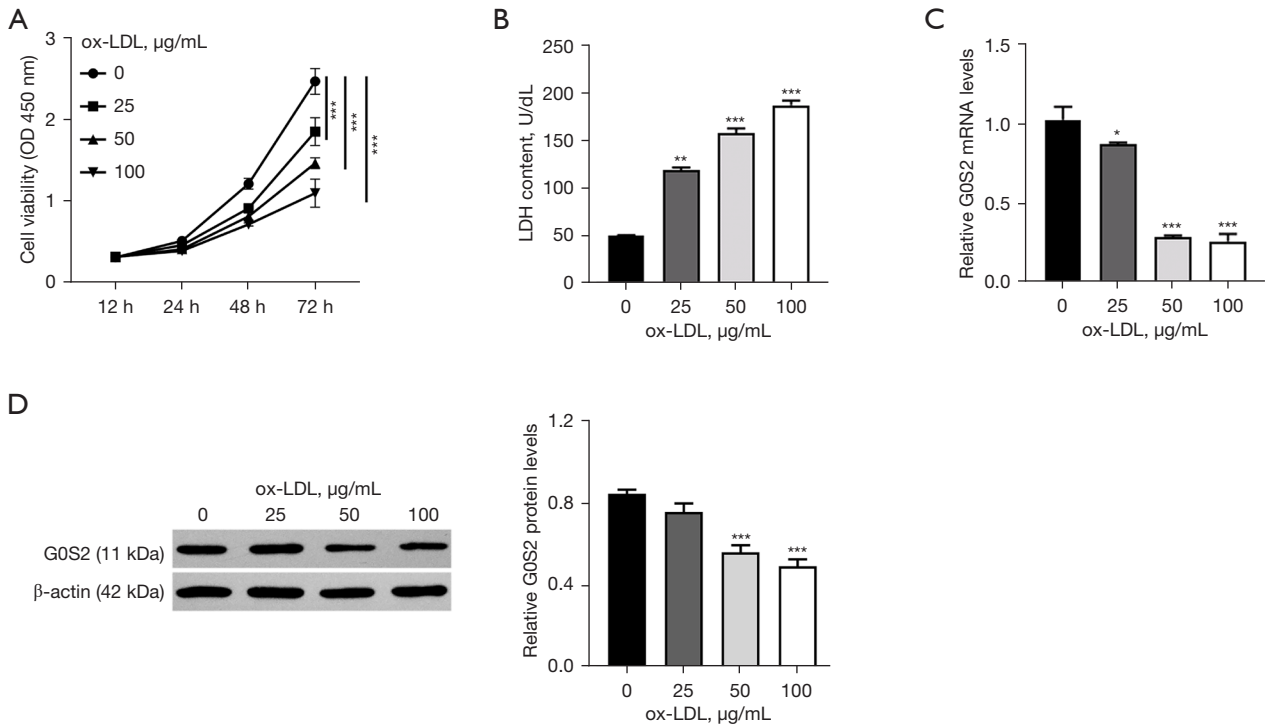

Figure 1 G0S2 expression is reduced in HAECs exposed to ox-LDL. (A) Cell viability was assessed in HAECs exposed to ox-LDL (0, 25, 50 and 100 μg/mL) for 24 hours, using CCK-8 assay. (B) LDH release was detected. (C) G0S2 mRNA expression was measured by RT-qPCR. (D) G0S2 protein were measured by western blot. n=3, *, P<0.05; **, P<0.01; ***, P<0.001 *vs.* the ox-LDL (0 μg/mL) groups. OD, optical density; ox-LDL, oxidative low-density lipoprotein; LDH, lactate dehydrogenase; HAECs, human aortic endothelial cells; CCK-8, Cell Counting Kit-8; mRNA, messenger RNA; RT-qPCR, reverse transcription quantitative polymerase chain reaction.

into HAECs to upregulate the G0S2 expression. RT-qPCR verified that transfection with G0S2-overexpression plasmids significantly enhanced G0S2 mRNA and protein in HAECs (*Figure 2A,2B*). HAECs transfected with G0S2-overexpression plasmid or vector were added with or without ox-LDL, and then cell viability and apoptosis were examined. As shown in *Figure 2C*, G0S2 overexpression slightly promoted cell viability under normal culture conditions, the ox-LDL treatment impaired cell viability, whereas G0S2 overexpression significantly restored cell viability in ox-LDL-induced HAECs. Flow cytometry analysis showed that ox-LDL raised the proportion of HAECs apoptotic cells from 6.18% to 25.1%. However, G0S2 overexpression reduced the proportion of ox-LDL-induced HAECs apoptosis to 18.9% (*Figure 2D*). These results indicate that G0S2 overexpression can alleviate ox-LDL-induced cell damage.

In addition, we also analyzed the effect of G0S2 silencing on HAECs. HAECs were transfected with si-G0S2 or si-NC and then incubated with ox-LDL for 24 hours. As expected, G0S2 expression was decreased in si-G0S2-transfected cells (*Figure 2E,2F*). G0S2 knockdown decreased the viability of HAECs in either normal or ox-LDL-treated conditions (*Figure 2G*). Furthermore, G0S2 deficiency had no obvious effect on cell apoptosis under basal culture conditions. However, G0S2 deficiency increased the cell apoptosis rate from 26.22%±2.07% to 39.62%±4.89% under ox-LDL condition (*Figure 2H*). These results indicate that loss of G0S2 exacerbates the ox-LDL-induced cell damage.

G0S2 overexpression reverses ox-LDL-induced oxidative damage via eliminating ROS accumulation

The intracellular ROS production was assessed by the DCFH-DA probe. As exhibited in *Figure 3A*, G0S2 overexpression decreased ROS levels in comparison to the vector control as can be seen in DCFH-DA green fluorescent. Meanwhile, ox-LDL treatment enhanced the intracellular ROS production as indicated by an obvious

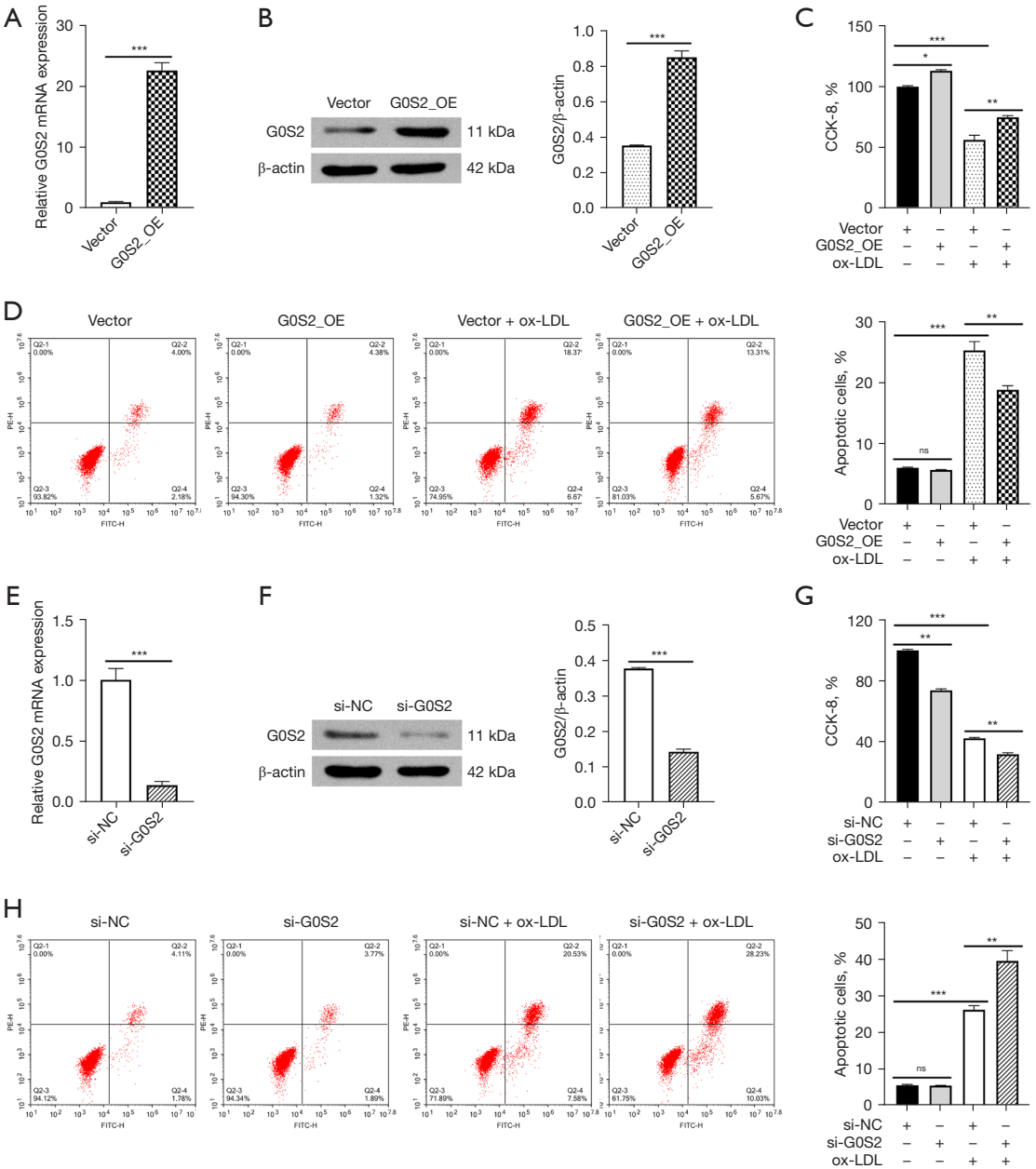

Figure 2 G0S2 improved cell viability and inhibited apoptosis of HAECs under ox-LDL condition. (A) qRT-PCR was used for G0S2 mRNA expression in HAECs transfected with G0S2_OE and then ox-LDL treatment. (B) Western blot was conducted for G0S2 protein expression in HAECs transfected with G0S2_OE and then ox-LDL treatment. (C) CCK-8 assay was performed for cell viability in HAECs transfected with G0S2_OE and then ox-LDL treatment (D) Annexin V-FITC/PI double staining and flow cytometry analysis were used for cell apoptosis in HAECs transfected with G0S2_OE and then ox-LDL treatment. (E) qRT-PCR was used for G0S2 mRNA expression in HAECs transfected with si-G0S2 and then ox-LDL treatment. (F) Western blot was conducted for G0S2 protein expression in HAECs transfected with si-G0S2 and then ox-LDL treatment. (G) CCK-8 assay was performed for cell viability in HAECs transfected with si-G0S2 and then ox-LDL treatment (H) Annexin V-FITC/PI double staining and flow cytometry analysis were used for cell apoptosis in HAECs transfected with si-G0S2 and then ox-LDL treatment. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant. mRNA, messenger RNA; CCK-8, Cell Counting Kit-8; ox-LDL, oxidative low-density lipoprotein; si-G2S0, small interfering RNAs targeting G2S0; si-NC, small interfering RNA-negative control; HAECs, human aortic endothelial cells; qRT-PCR, quantitative reverse transcription polymerase chain reaction; V-FITC/PI, V-fluorescein isothiocyanate/propidium iodide; PE-H, phycoerythrin-height; FITC-H, fluorescein isothiocyanate-height.

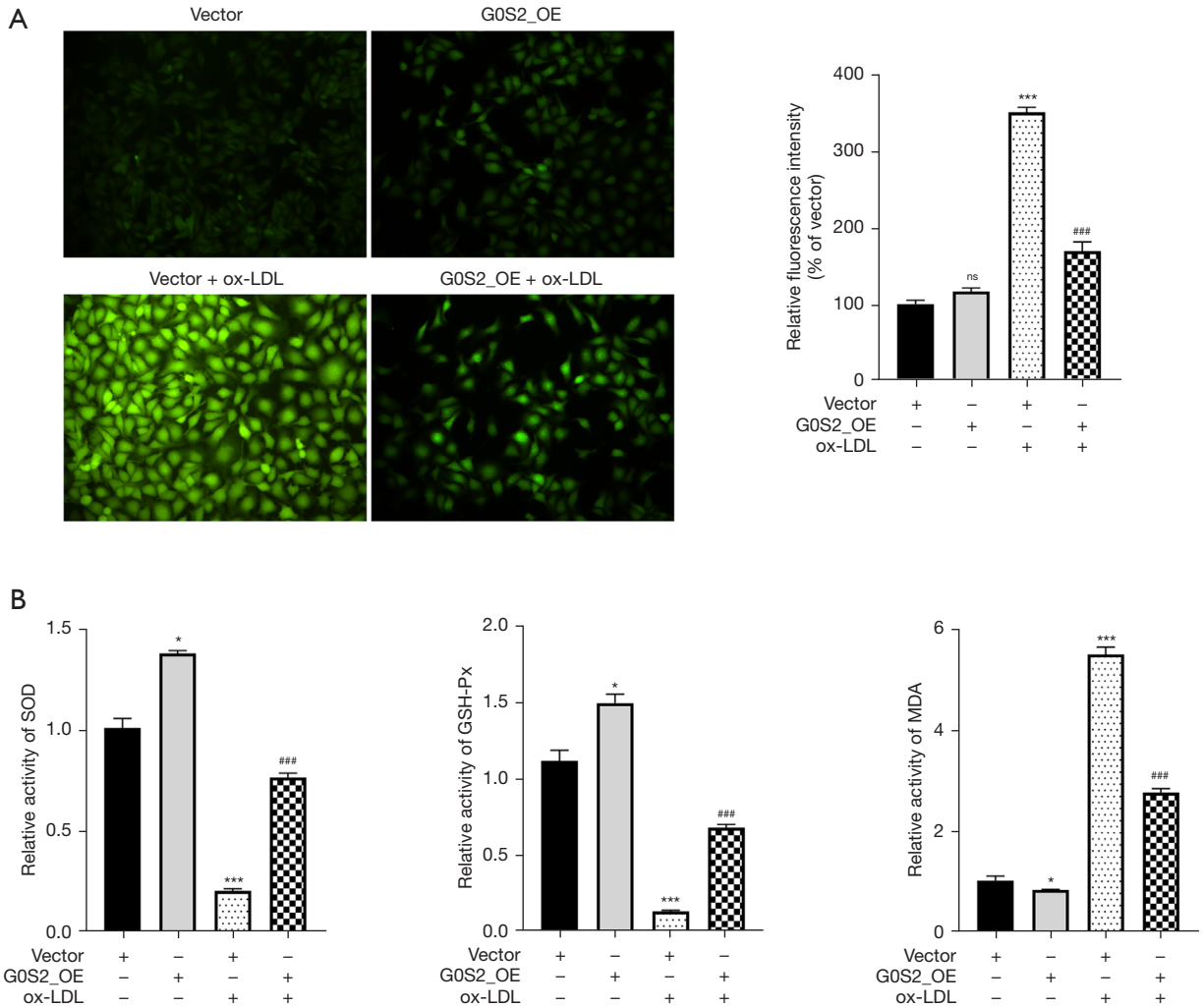

Figure 3 G0S2 mediated ox-LDL-triggered oxidative stress in HAECs. After transfection with G0S2-overexpression plasmids or vectors, HAECs were exposed to ox-LDL (50 μg/mL) or a normal medium for 6 h. (A) Intracellular ROS levels were assessed using the DCFH-DA staining (×200). (B) Cellular SOD and GSH-Px levels, and MDA content were assessed. *, P<0.05; ***, P<0.001 *vs.* the Vector groups; ###, P<0.001 *vs.* the Vector + ox-LDL groups; ns, not significant. ox-LDL, oxidative low-density lipoprotein; HAECs, human aortic endothelial cells; ROS, reactive oxygen species; DCFH-DA, dichloro-dihydro-fluorescein diacetate; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MDA, malondialdehyde.

increase of green fluorescence, which was ameliorated by G0S2 overexpression. Besides, oxidative stress-related factors, including MDA, GSH-Px, and SOD were examined. As shown in *Figure 3B*, ox-LDL treatment reduced cellular SOD and GSH-Px levels and augmented MDA content, and these changes were all reversed by G0S2 overexpression. Overall, these results highlighted that G0S2 overexpression ameliorates ox-LDL-stimulated oxidative stress in HAECs.

Overexpression of G0S2 alleviates ox-LDL-induced mitochondrial dysfunction

Mitochondrial function is closely related to apoptosis and oxidative stress. MMP decrease or loss is one of the typical characteristics of mitochondrial injury. Here, a JC-1 assay was performed to assess MMP change. The results showed that ox-LDL treatment significantly promoted the formation of JC-1 monomer with green fluorescence, indicating MMP loss of HAECs. However, G0S2

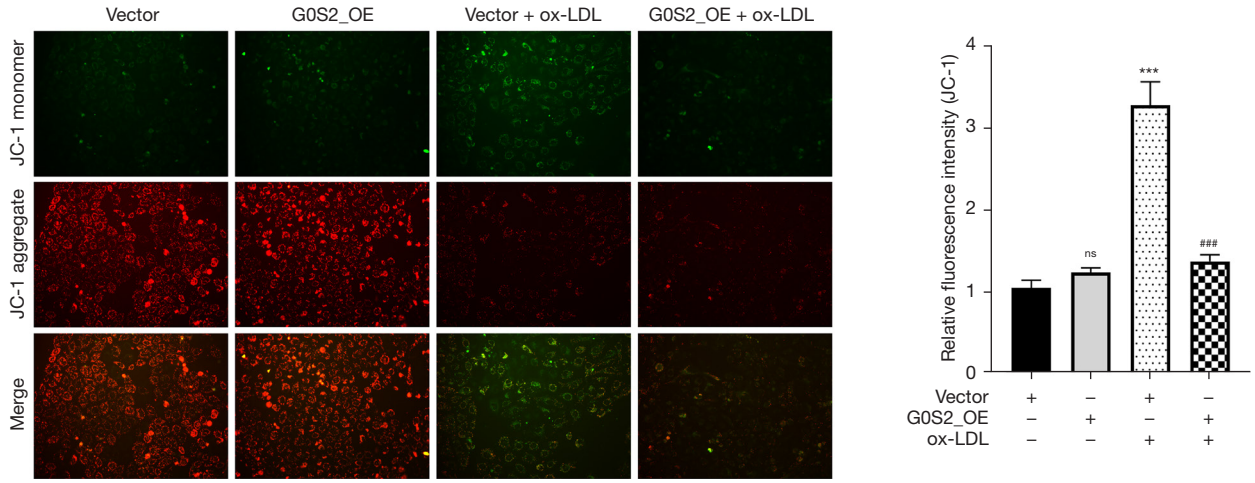

Figure 4 G0S2 attenuates the decrease of MMP induced by ox-LDL. HAECs were transfected with non-targeting control vector (vector), or G0S2-overexpression vector (G0S2_OE) for 24 h and then treated with ox-LDL for 24 h. MMP was measured by JC-1 staining and fluorescent intensity was assayed by fluorescence microscope, scale bar =20 μm. ***, P<0.001 *vs.* the Vector groups; ###, P<0.001 *vs.* the Vector + ox-LDL groups; ns, not significant. ox-LDL, oxidative low-density lipoprotein; MMP, mitochondrial transmembrane potential; HAECs, human aortic endothelial cells.

overexpression resulted in a decreased green fluorescence (*Figure 4*), pointing out that G0S2 is essential to maintain mitochondrial integrity.

Furthermore, western blot results showed that ox-LDL stimulation caused significant translocation of mitochondrial Cyt-c to the cytoplasm of the HAECs, which was remarkably reversed by G0S2 overexpression (*Figure 5A*). In addition, we also found that cleaved caspase-3 and cleaved caspase-9 were up-regulated under ox-LDL conditions, but these changes were balanced by overexpressed G0S2 (*Figure 5B*). Taken together, these results suggested that G0S2 protects against ox-LDL-induced mitochondrial damage and activation of the mitochondrial apoptosis pathway.

G0S2 activates the AMPK signaling pathway

Researchers found that oxidative stress-induced mitochondrial-dependent apoptosis is associated with AMPK signaling pathway activation. The levels of p-AMPK, AMPK, and downstream targets including Nrf2, HO-1 and SIRT1 were detected by Western blot. It was demonstrated that ox-LDL treatment reduced the levels of p-AMPK, Nrf2, HO-1 and SIRT1 proteins. However, G0S2 overexpression remarkably increased the levels of p-AMPK, Nrf2, HO-1 and SIRT1 proteins under normal or ox-LDL conditions (*Figure 6*), indicating that AMPK signaling pathway was activated by G0S2.

Discussion

Healthy vascular endothelial cells are essential for maintaining normal vascular function. Under homeostatic conditions, the endothelium dynamically regulates vascular barrier function, coagulation pathways, leukocyte adhesion, and vasomotor tone to maintain normal vascular function. Vascular endothelial cell dysfunction is a complex process, which is the initiating event for atherosclerosis. Ox-LDL can induce pathological injury of endothelial cells, including oxidative stress injury and cell apoptosis, and then contribute to atherosclerosis progression. Inhibition of oxidative stress and correction of mitochondrial dysfunction have shown effective improvement of endothelial dysfunction. In this study, we found that ox-LDL resulted in a dose-dependent decrease of G0S2 expression in HAECs. Ectopic expression of G0S2 reversed ox-LDL-stimulated HAECs apoptosis and damaged viability, whereas G0S2 depletion showed the opposite effect. In terms of mechanism, overexpression of G0S2 inhibited oxidative damage and maintained mitochondrial homeostasis, thereby protecting HAECs against ox-LDL toxicity. Taken together, our study confirms that G0S2-related mitochondrial homeostasis protects vascular endothelial cells against ox-LDL stimulation.

G0S2 was first identified in blood monocytes after induction of cell cycle progression and is thought to mediate cell transition from the G0 phase to the G1 phase of the cell

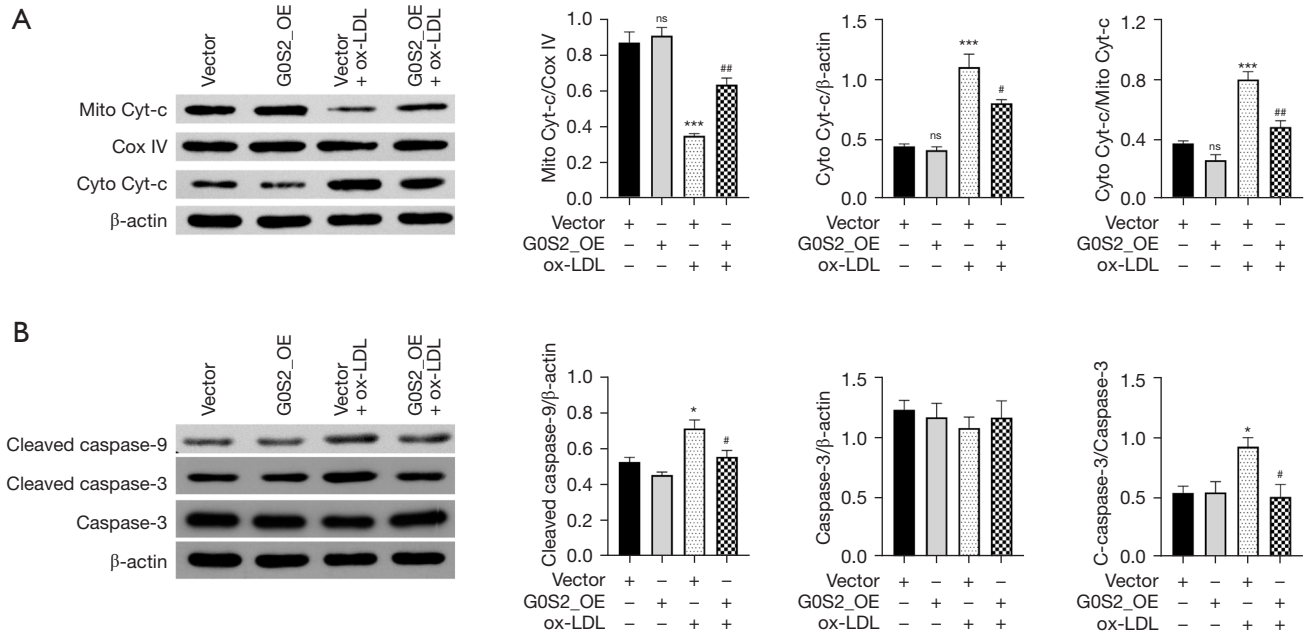

Figure 5 G0S2 attenuates mitochondrial damage in ox-LDL-treated HAECs. (A) The expression of mitochondrial and cytosolic cytochrome c was examined by western blot. (B) The expression of activated forms of caspase-3 and caspase-9 were examined by western blot. *, P<0.05; ***, P<0.001 *vs.* the Vector groups; #, P<0.05; ##, P<0.01 *vs.* the Vector + ox-LDL groups; ns, not significant. ox-LDL, oxidative low-density lipoprotein; HAECs, human aortic endothelial cells.

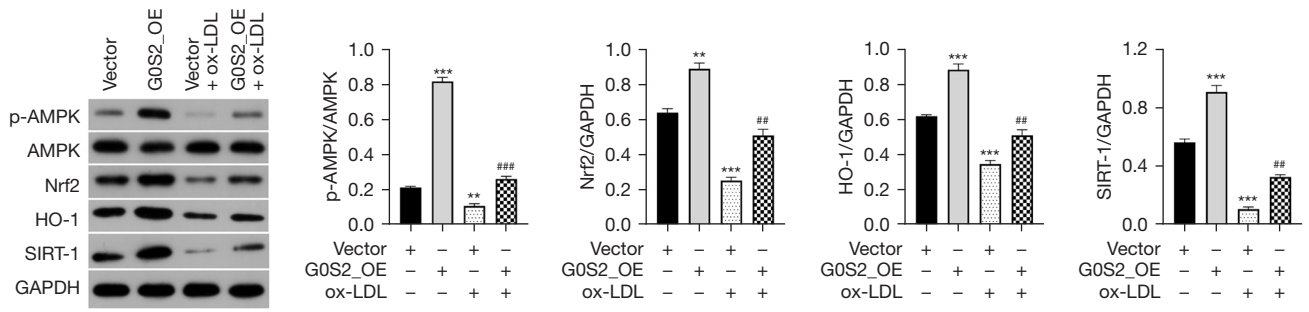

Figure 6 G0S2 activates AMPK signaling pathway in HAECs. The expression of p-AMPK, Nrf2, HO-1 and SIRT-1 proteins was examined by western blot. **, P<0.01; ***, P<0.001 *vs.* the Vector groups; ##, P<0.01; ###, P<0.001 *vs.* the Vector + ox-LDL groups. HAECs, human aortic endothelial cells; ox-LDL, oxidative low-density lipoprotein.

cycle (23). To date, numerous studies have shown that G0S2 and its protein products regulate lipolysis, cell proliferation, apoptosis, oxidative phosphorylation, and other biological processes in different cellular environments (11,17,22). G0S2 levels in the peripheral blood leukocytes of patients with acute myocardial infarction are lower than those of patients with stable coronary heart disease. Kioka *et al.* (17) 2014 showed that G0S2 increased ATP production and protected cardiomyocytes from a critical energy crisis in response to hypoxic stress, and identified G0S2 as a positive regulator of oxidative phosphorylation. A study about zebrafish hearts reveals that G0S2 protected cardiomyocytes against hypoxia *in vitro* and induced ischemic tolerance *in vivo* via maintaining ATP production (24). Wang *et al.* (22) reported the protective function of G0S2 in H_2O_2-induced endothelial cell injury *in vivo*. Consistent with these findings, in our study, ox-LDL stimuli decreased G0S2 expression in HAECs, and overexpression of G0S2 could improve the

viability and suppress apoptosis in HAECs induced by ox-LDL. These results confirmed that G0S2 protected HAECs against ox-LDL-induced cell injury.

Oxidative stress is characterized by the imbalance of intracellular oxygen free radical generation and clearance, which leads to the accumulation of ROS and causes oxidative damage (25,26). Excessive ROS accumulation leads to increased apoptosis of vascular endothelial cells (27). A previous study has revealed that G0S2 regulates the oxidative stress response (22). Similarly, this study demonstrated that G0S2 overexpression significantly alleviated the ox-LDL-stimulated oxidative stress response, as evidenced by decreased ROS production and MDA levels, and increased activity of SOD and GSH-Px in HAECs.

Mitochondrial apoptosis is one of the main pathways of cell apoptosis, and it is a highly conserved death process (28). Stimulated by upstream apoptotic signals, mitochondria membranes are damaged and MMP decreases or loses, leading to the release of mitochondrial Cyt-c into the cytoplasm and activation of caspase-3 and caspase-9, which are key steps to induce apoptosis (29). Mitochondrial damage is a key event in the pathological process of atherosclerosis (30). Previous studies have revealed that ox-LDL exposure results in mitochondrial damage (30-32). G0S2 also has several roles within mitochondria (33). Herein, we found that ox-LDL exposure decreased MMP, resulted in the transfer of Cyt-c from the mitochondria to the cytoplasm, and increased cleaved caspase-3 and caspase-9 levels of HAECs, indicating the activation of the mitochondrial apoptosis pathway. In the G0S2-overexpressing HAECs, these effects were reversed. These data confirm that G0S2 alleviates ox-LDL-triggered mitochondrial damage and mitochondrial apoptosis.

AMPK is a metabolic sensor and regulates cellular energy metabolism, growth, inflammation, cytokine production, and apoptosis. Growing evidence has demonstrated that the AMPK/SIRT1 and AMPK/Nrf2 pathway serve protective roles in the progression of atherosclerosis (34,35). SIRT1, a member of the conserved sirtuin family, delay the progression of atherosclerosis via inhibiting apoptosis and oxidative stress, and SIRT1 deficiency exacerbates endothelial dysfunction (36). Nrf2 is a nuclear transcription factor that binds to antioxidant-response element and promotes the transcription of antioxidant genes including GSH-Px, SOD, and HO-1 in response to oxidative stress (37). Several studies demonstrated that the expression of Nrf2 and its downstream antioxidant genes were downregulated in ox-LDL-induced vascular endothelial cells. Nrf2 overexpression inhibits inflammation and attenuates ox-LDL-induced endothelial dysfunction (38-40). Consistent with these findings, our results showed that the expression of p-AMPK, Nrf2, HO-1 and SIRT1 proteins was inhibited in HAECs exposed to ox-LDL. However, overexpression of G0S2 could significantly improve the expression of these molecules. Therefore, we conclude that G0S2 inhibits ox-LDL-induced inflammation, oxidative stress and ameliorates endothelial dysfunction by activating the AMPK signaling pathway.

In this study, we elucidated the mechanism by which G0S2 protects vascular endothelial cells from ox-LDL-induced oxidative stress and mitochondrial apoptosis through AMPK signaling pathway. However, this study has potential limitations. Firstly, considering the multifunctional characteristics of G0S2, the functional role of G0S2 in other aspects, including vascular endothelial metabolism and angiogenesis remains to be further explored. Secondly, non-coding RNAs including miRNAs, lncRNAs and circRNAs have been found to play a variety of roles in atherosclerosis and are also important regulators of RNA and protein expression. Thus, the regulatory mechanism of non-coding RNA on G0S2 remains to be elucidated. And, we hope, in the future, to employ endothelial-specific G0S2 conditional knockout mice to further determine the role and mechanism of G0S2 *in vivo*.

Conclusions

In conclusion, this study provides evidence that G0S2 overexpression prevents ox-LDL-induced endothelial cell injury. Further studies have confirmed that G0S2 overexpression protects mitochondria against ox-LDL-induced dysfunction in vascular endothelial cells. This study confirms that G0S2 is critical for the maintenance of vascular endothelial cell function and may be a promising target for the treatment of atherosclerosis.

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Footnote

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