Metformin inhibits cholesterol-induced adhesion molecule expression via activating the AMPK signaling pathway in vascular smooth muscle cells

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Abstract. Recruitment of lymphocytes to the vascular wall contributes to the pathogenesis of atherosclerosis (AS). The expression of cellular adhesion molecules, such as vascular cell adhesion molecule-1 and intercellular adhesion molecule-1, serves a critical role in mediating lymphocyte adhesion to the vascular wall. Cholesterol loading induces the expression of adhesion molecules in vascular smooth muscle cells (VSMCs), but the underlying mechanism is not completely understood. The present study aimed to investigate the mechanism underlying the effects of cholesterol on adhesion molecule expression, and whether metformin protected VSMCs against cholesterol-induced functional alterations. Human VSMCs were loaded with cholesterol and different concentrations of metformin. The expression levels of adhesion molecules were assessed via reverse transcription-quantitative PCR and western blotting. Reactive oxygen species (ROS) accumulation and levels were quantified via fluorescence assays and spectrophotometry, respectively. AMP-activated protein kinase (AMPK), p38 MAPK and NF-κB signaling pathway-related protein expression levels were evaluated via western blotting. Compared with the control group, cholesterol loading significantly upregulated adhesion molecule expression levels on VSMCs by increasing intracellular ROS levels and activating the p38 MAPK and NF-κB signaling pathways. Metformin decreased cholesterol-induced VSMC damage by activating the AMPK signaling pathway, and suppressing p38 MAPK and NF-κB signaling. The present study indicated the therapeutic potential of metformin for VSMC protection, reduction of monocyte adhesion, and ultimately, the prevention and treatment of AS.

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Introduction

Atherosclerotic plaque rupture is the most common cause of cardiac mortality worldwide (1). Previous studies have reported that vascular smooth muscle cells (VSMCs) are the primary cell type involved in all stages of human atherosclerotic plaque development (2,3). Leukocyte recruitment to the vessel also directly contributes to the progression of atherosclerosis (AS), as well as the expression of adhesion molecules on VSMCs (4).

Following cholesterol loading, VSMCs undergo significant morphological and functional alterations (5,6); in particular, adhesion molecule expression is significantly upregulated (7). Intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)-mediated leukocyte recruitment to the vessel intima is a crucial event in AS (8,9). Since the accumulation of monocyte-derived cells in atherosclerotic plaques directly contributes to the progression of AS, the inhibition of cholesterol-induced adhesion molecule expression on VSMCs may serve as a potential therapeutic strategy.

Hypercholesterolemia, which is considered to be the most common and significant risk factor of AS, has been linked to increased reactive oxygen species (ROS) production (10,11). Abnormal ROS accumulation is associated with numerous pathophysiological conditions, such as diabetes, obesity and heart failure (11). Oxidative stress mediates endothelial dysfunction (12), modifies the phenotype of VSMCs and affects extracellular matrix synthesis (13). Due to the involvement in the phenotypic switching of VSMCs, ROS may also contribute to cellular morphological and functional alterations. However, the role of ROS in cholesterol-induced VSMC functional damage is not completely understood.

Metformin is used extensively as a first-line medication for type 2 diabetes mellitus (14). In addition to its glucose-lowering effect, metformin displays cardiovascular protective effects, including protection against cardiac ischemia-reperfusion

injury (15), and subsequent suppression of the inflammatory response and development of AS (16). However, the effects of metformin on cholesterol loading-induced morphological and functional alterations in VSMCs are not completely understood. The present study aimed to further the current understanding of the mechanisms underlying the damaging effects of cholesterol on VSMC function, and to clarify the protective effect of metformin on VSMCs.

Materials and methods

Materials. Human aortic VSMCs (cat. no. 6110) and smooth muscle cell medium (SMCM; cat. no. 1101) were obtained from ScienCell Research Laboratories, Inc. Cholesterol-methyl-β-cyclodextrin (cholesterol; cat. no. C4951), metformin (cat. no. D150959), paraformaldehyde and 2',7'-dichlorofluorescin diacetate (DCFH-DA; cat. no. D6883) were obtained from Sigma-Aldrich; Merck KGaA. Compound C (cat. no. HY-13418A), SB203580 (cat. no. HY-10256) and BAY11-7082 (cat. no. HY-13453) were obtained from MCE. TRIzol® and CELLTRACE Violet were obtained from Invitrogen (Thermo Fisher Scientific, Inc.). The Transcriptor First Strand cDNA Synthesis kit and FastStart Universal SYBR-Green Master Mix were obtained from Roche Applied Science. Human monocytic THP-1 cells were obtained from National Collection of Authenticated Cell Center (cat. no. TCHu 57).

Cell culture conditions. Human aortic VSMCs were cultured in SMCM (Science Cell Research Laboratories, cat. no. 1101) with 2% FBS, 1% smooth muscle cell growth supplement (SMCGS; both included in the medium), 100 U/ml penicillin and 100 μg/ml streptomycin at 37˚C with 5% CO_2. At 80% confluence, VSMCs were synchronized by replacing the culture media with FBS- and SMCGS-free basic SMCM. For experiments involving pharmacological reagents, VSMCs were pretreated which metformin (10 and 100 nm, 1 μm), Compound C (10 μm), SB203580 (10 μm) or BAY11-7082 (10 μm) for 2 h and subsequently followed by cholesterol (5 μg/ml) for 72 h at 37˚C with 5% CO_2. In all experiments, passage 3-6 VSMCs were used.

Reverse transcription-quantitative PCR (RT-qPCR). Following treatment with 5 μg/ml cholesterol at 37˚C with 5% CO_2 for 72 h, total RNA was extracted from VSMCs using TRIzol according to the manufacturer's protocol. Total RNA (4 μg) was reverse transcribed into cDNA using the Transcriptor First Strand cDNA Synthesis kit. Subsequently, qPCR was performed using FastStart Universal SYBR-Green Master Mix and a quantitative fluorescence PCR system (Bio-Rad Laboratories; CFX96 Real-Time PCR Detection System). The following conditions were used: 95˚C for 30 sec, 95˚C for 5 sec and 40 cycles at 60˚C for 5 sec. The primers used for qPCR are presented in Table I. mRNA expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method and normalized to the internal reference gene β-actin (17). RT-qPCR was performed in triplicate.

Cell adhesion assay. VSMCs were incubated with 5 μg/ml cholesterol at 37˚C with 5% CO_2 for 72 h. Following washing three times with PBS, CELLTRACE Violet-labeled human monocytic THP-1 cells ($1x10^6$ cells/well) were added to the VSMC cultures ($2x10^6$ cells/well) and incubated for 1 h at 10 rpm at 37˚C. Cells were then washed twice with PBS to eliminate non-attached cells. VSMC layers with attached monocytes were fixed with 4% paraformaldehyde at room temperature for 10 min. Adhered THP-1 cells were visualized using a DMI4000B fluorescence microscope (DMI4000B; Leica Microsystems GmbH).

ROS detection. VSMCs were pretreated with cholesterol (5 μg/ml), metformin (1 μM) or Compound C (10 μM) for 10 h at 37˚C. Subsequently, cells were incubated with diluted fluoroprobe DCFH-DA for 20 min at 37˚C with gentle agitation every 5 min. After washing with serum-free culture medium, cells were harvested and examined using the fluorescent microscope at excitation wavelength 502 nm and emission wavelength 530 nm.

Western blotting. RIPA lysis buffer (Beyotime Institute of Biotechnology; cat. no. P0013B) was used to lyse the cultured cells for 30 min at 4˚C, after which a bicinchoninic acid (BCA) kit (Beyotime Institute of Biotechnology; cat. no. P0012) was used to test the protein concentration. Proteins were denatured at 95˚C for 5 min in SDS-PAGE Sample Loading Buffer (Beyotime Institute of Biotechnology; cat. no. P0015). Equal amounts of protein (20 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to PVDF membranes. Subsequently, the membranes were blocked with 5% non-fat dried milk (R&D Systems) in tris-buffered saline Tween-20 (0.1%; TBST) for 1 h at room temperature, and then incubated overnight at 4˚C with the following primary antibodies (diluted with TBST in a ratio of 1:1,000): Anti-ICAM-1 (cat. no. ab53013; Abcam), anti-VCAM-1 (cat. no. ab134047; Abcam), anti-phosphorylated (p)-P38 (cat. no. 4511; Cell Signaling Technology, Inc.), anti-total-P38 (cat. no. 8690; Cell Signaling Technology, Inc.), anti-p-P65 (cat. no. 3033; Cell Signaling Technology, Inc.), anti-total-P65 (cat. no. 8242; Cell Signaling Technology, Inc.), anti-p-AMPK (cat. no. 2535; Cell Signaling Technology, Inc.), anti-total AMPK (cat. no. 2532; Cell Signaling Technology, Inc.) and anti-β-actin (cat. no. sc-8432 Santa Cruz Biotechnology, Inc.). Following TBST washing, the membranes were incubated with HRP-conjugated goat anti-rabbit IgG (cat. no. ZB-2301; OriGene Technologies, Inc.) or anti-mouse IgG (cat. no. ZB-2305; OriGene Technologies, Inc.) secondary antibodies for 1 h at room temperature. Protein bands were visualized using a Tanon 6600 Luminescent Imaging Workstation (Tanon Science and Technology Co., Ltd.; 6600) and BeyoECL Plus (Beyotime Institute of Biotechnology). Protein levels were semi-quantified using ImageJ 1.8.0 software (National Institutes of Health) with β-actin as the loading control. Western blotting was performed in triplicate.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (version 7.0; GraphPad Software, Inc.). For comparisons between two groups, the unpaired Student's t-test was performed. For comparisons among multiple groups, one-way ANOVA followed by Tukey's post

Table I. Sequences of primers used for reverse transcription-quantitative PCR.

Gene	Sequence (5'→3')
ICAM-1	F: ACCTATGGCAACGACTCCTTC
	R: CCTTCTGAGACCTCTGGCTTC
VCAM-1	F: AATGGGAATCTACAGCACCTTTC
	R: GTCTCCAATCTGAGCAGCAATC
β-actin	F: TTCCTGGGCATGGAGTCCT
	R: AGGAGGAGCAATGATCTTGATC

ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

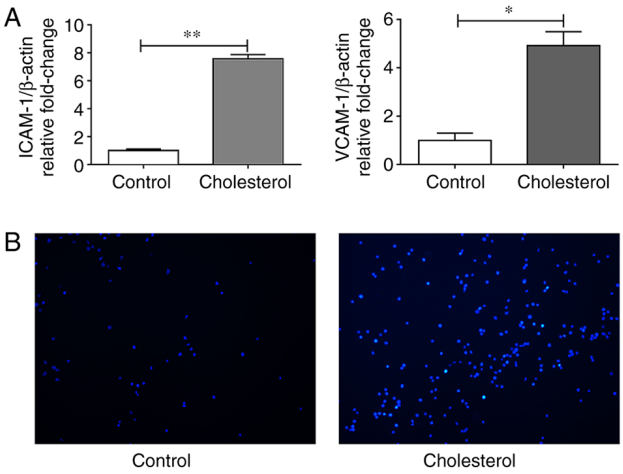

Figure 1. Cholesterol loading in VSMCs activates the expression of adhesion molecules. VSMCs were treated with cholesterol (5 μg/ml) for 72 h. SB230580 and BAY11-7082 were dissolved in DMSO, while compound C was dissolved in DMEM. These groups were negative controls. (A) ICAM-1 and VCAM-1 mRNA expression levels. (B) Representative images of THP-1 cell adhesion to VSMCs (magnification, x50). *P<0.05 and **P<0.01. VSMC, vascular smooth muscle cell; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1.

hoc test was performed. Each experiment was repeated at least three times, and P<0.05 was considered to indicate a statistically significant difference.

Results

Cholesterol loading induces adhesion molecule expression and monocyte adhesion on VSMCs. To investigate the effect of cholesterol loading on the expression of adhesion molecules, VSMCs were incubated with cholesterol for 72 h and the mRNA expression levels of ICAM-1 and VCAM-1 were evaluated via RT-qPCR. The incubation of VSMCs with cholesterol for 72 h significantly upregulated ICAM-1 and VCAM-1 mRNA expression levels compared with control cells (Fig. 1A). Following co-incubation with CELLTRACE-Violet-labeled THP-1 monocytic cells, a marked upregulation of monocyte adhesion on VSMCs after cholesterol treatment for 72 h was observed (Fig. 1B).

Since oxidative stress is an important factor regulating cell function (18,19), the effect of cholesterol on intracellular oxidant levels was assessed. Intracellular ROS were labeled with DCFH-DA probes and measured by conducting fluorescence assays. Compared with the control group, cholesterol significantly increased intracellular ROS accumulation, which may be closely related to cholesterol-induced upregulation of adhesion molecule expression levels (Fig. 2A and B).

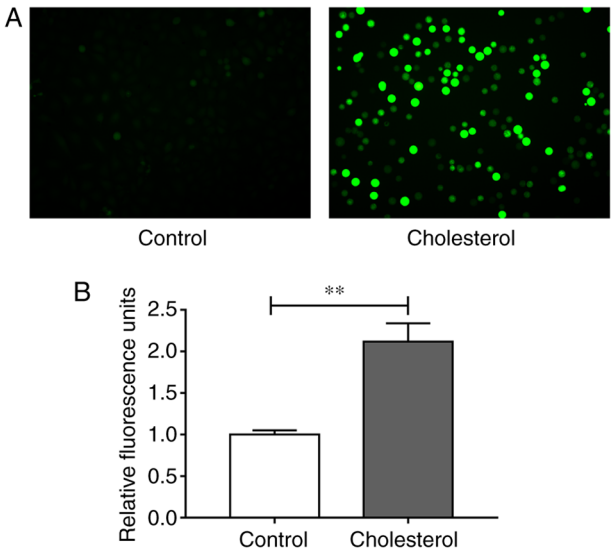

Figure 2. Cholesterol loading in VSMCs induces intracellular ROS accumulation. VSMCs were treated with cholesterol (5 μg/ml) for 10 h. (A) ROS accumulation in VSMCs was examined via immunofluorescence analysis (magnification, x100). (B) ROS levels were measured using a spectrophotometer. **P<0.01. VSMC, vascular smooth muscle cell; ROS, reactive oxygen species.

p38 MAPK and NF-κB signaling pathways are associated with adhesion molecule expression on VSMCs. The redox-sensitive transcription factors p38 MAPK and NF-κB are involved in regulating the expression of ICAM-1 and VCAM-1 (20-24). However, the mechanism underlying the expression of adhesion molecules on VSMCs is not completely understood. To investigate whether the p38 MAPK and NF-κB signaling pathways were involved in ICAM-1 and VCAM-1 expression on VSMCs, the activation of the two signaling pathways was assessed via western blotting. Cholesterol loading significantly increased p38 and p65 phosphorylation levels compared with the control group (Fig. 3A and C). To further evaluate the regulatory effects of p38 MAPK and NF-κB on the expression of adhesion molecules on VSMCs, the p38 MAPK signaling pathway inhibitor SB203580 and the NF-κB signaling pathway inhibitor BAY11-7082 were used to treated VSMCs prior to treatment with cholesterol. BAY11-7082 significantly inhibited cholesterol-induced increases in ICAM-1 and VCAM-1 expression levels, whereas SB203580 only significantly inhibited cholesterol-induced increases in ICAM-1 expression levels (Fig. 3B and D).

Metformin inhibits cholesterol-induced adhesion molecule expression on VSMCs in a dose-dependent manner. To investigate whether metformin inhibited cholesterol-induced adhesion molecule expression, VSMCs were incubated with or without metformin at different concentrations (10 and

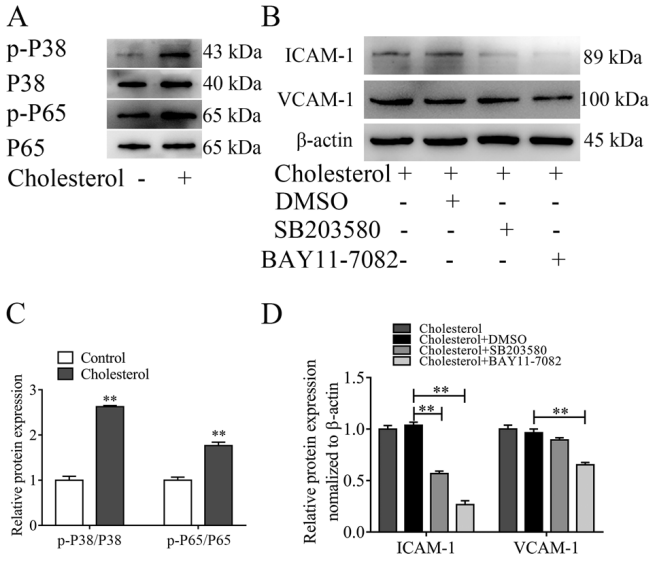

Figure 3. p38 MAPK and NF-κB signaling pathways are associated with cholesterol loading-induced adhesion molecule expression on VSMCs. VSMCs were treated with cholesterol (5 μg/ml) for 72 h. VSMCs were exposed to SB203580 and BAY11-7082 for 30 min prior to treatment with cholesterol. (A) p38 MAPK and NF-κB signaling pathway-related protein expression levels were measured via western blotting. (B) ICAM-1 and VCAM-1 protein expression levels were measured via western blotting. (C) Semi-quantification of p38 MAPK and NF-κB signaling pathway-related protein expression levels. (D) Semi-quantification of ICAM-1 and VCAM-1 protein expression levels. **P<0.01. VSMC, vascular smooth muscle cell; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; p, phosphorylated.

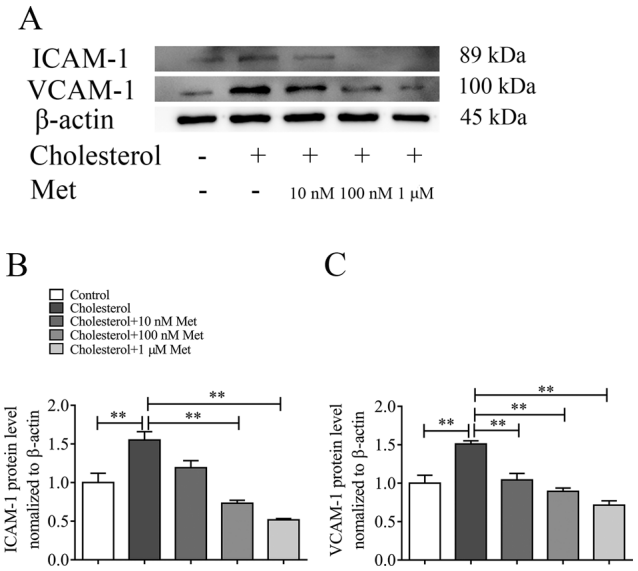

Figure 4. Metformin treatment alleviates cholesterol loading-induced adhesion molecule expression on VSMCs. VSMCs were treated with metformin (10 and 100 nm or 1 μm) in the presence of cholesterol (5 μg/ml). Control cells were untreated. Protein expression levels were (A) determined via western blotting and semi-quantified for (B) ICAM-1 and (C) VCAM-1. **P<0.01. VSMC, vascular smooth muscle cell; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; Met, metformin.

100 nm or 1 μm) for 30 min, and then co-incubated with cholesterol for 72 h. The protein expression levels of ICAM-1 and VCAM-1 were evaluated via western blotting. Metformin treatment inhibited cholesterol-induced upregulation of adhesion molecule expression levels in a dose-dependent manner (Fig. 4A). Compared with the cholesterol group, ICAM-1 and VCAM-1 expression levels were significantly reduced following treatment with 100 nm (Fig. 4B) and 10 nm (Fig. 4C) metformin, respectively.

Metformin inhibits cholesterol-induced ROS accumulation, p38 MAPK and NF-κB activation via the AMPK signaling pathway. To elucidate the mechanism underlying metformin-mediated inhibition of cholesterol-induced expression of adhesion molecules on VSMCs, AMPK signaling pathway-related protein expression levels were measured. Metformin significantly increased the expression levels of p-AMPK in cholesterol-treated VSMCs in a dose-dependent manner, especially at a concentration of 1 μm (Fig. 5A and B). According to the above experimental results, when the concentration of metformin is 1 μm, its effect on VSMCs is the most obvious. Subsequently, whether metformin inhibited cholesterol-induced ROS accumulation in VSMCs was investigated. Metformin notably decreased cholesterol-induced ROS accumulation in a concentration-dependent manner (Fig. 5C), and. To further explore the role of AMPK in the protective effect of metformin, the AMPK signaling pathway inhibitor Compound C was used to suppress AMPK activation. VSMCs were pretreated with Compound C (10 μm) for 30 min, followed by treatment with metformin for 30 min and then cells were exposed to cholesterol for 72 h. First, the expression levels of p-AMPK and AMPK were measured via western blotting. Pre-treatment with Compound C significantly decreased p-AMPK protein expression levels in cholesterol- and metformin-treated VSMCs to a similar level to the control group (Fig. 6A and B), confirming the ability of Compound C to inhibit the phosphorylation of AMPK. Moreover, the effects of Compound C and metformin on the activation of the p38 MAPK and NF-κB signaling pathways were investigated (Fig. 6C and D). Metformin significantly decreased the expression levels of p-P38 and p-P65 in cholesterol-treated VSMCs, whereas Compound C antagonized the effects of metformin, significantly upregulating the expression levels of p-P38 and p-P65 in cholesterol- and metformin-treated VSMCs. In addition, the levels of intracellular ROS were also measured (Fig. 6E). The results demonstrated that treatment with Compound C notably increased ROS levels in cholesterol- and metformin-treated VSMCS to a distinctly higher level compared with the cholesterol + metformin group. Furthermore, the adhesion of THP-1 cells on VSMCs and the expression levels of intracellular adhesion molecules were assessed. In cholesterol- and metformin-treated VSMCs, Compound C treatment significantly increased the expression levels of ICAM-1 and VCAM-1, and elevated the ability of THP-1 cells to adhere to VSMCs, which were both downregulated by metformin (Fig. 6F-H). Collectively, the results indicated that AMPK signaling may serve an important role in the protective effects of metformin.

Discussion

The present study indicated that cholesterol loading led to abnormal ROS accumulation in VSMCs and upregulated

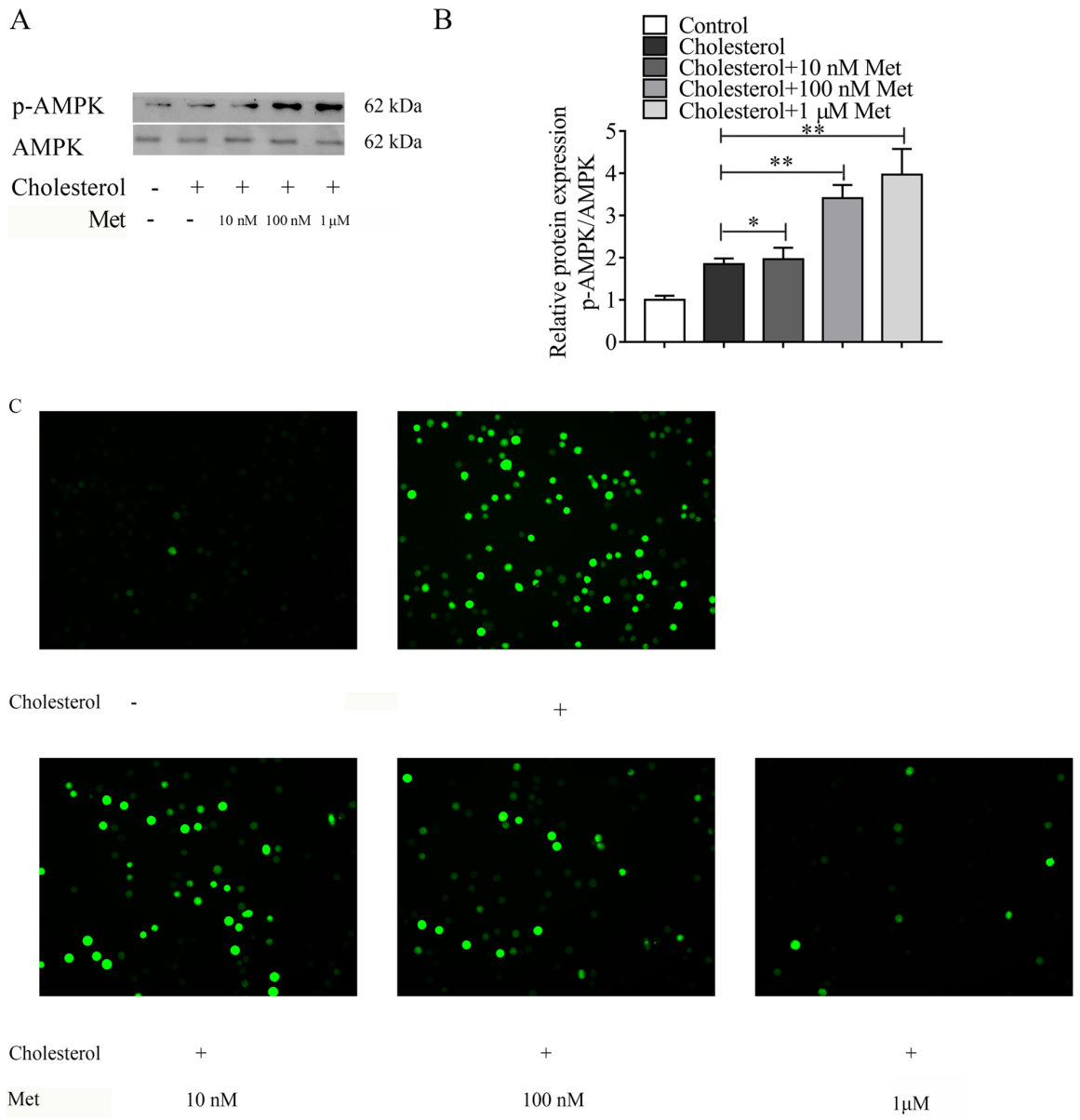

Figure 5. Metformin inhibits cholesterol loading-induced ROS activation via AMPK signaling. VSMCs were treated with metformin (10 and 100 nm or 1 μm) in the presence of cholesterol (5 μg/ml). Control cells were untreated. Protein expression levels were (A) determined via western blotting and (B) the ratio of p-AMPK/AMPK was semi-quantified. (C) Reactive oxygen species accumulation in VSMCs was examined via immunofluorescence analysis (magnification, x100). *P<0.05 and **P<0.01 vs. control or cholesterol. ROS, reactive oxygen species; AMPK, AMP-activated protein kinase; VSMC, vascular smooth muscle cell; p, phosphorylated; Met, metformin.

the expression levels of adhesion molecules via activation of the p38 MAPK and NF-κB signaling pathways. Metformin protected VSMCs against cholesterol loading by decreasing ROS accumulation, downregulating adhesion molecule expression levels, and blocking the activation of the p38 MAPK and NF-κB signaling pathways via AMPK. Collectively, the results indicated that metformin may serve as a novel modulator of vascular inflammation.

Lymphocyte recruitment to the arterial wall accelerates AS progression (25). The adhesion molecules ICAM-1 and VCAM-1 are important functional mediators of the interactions between leukocytes and the vascular wall (26,27). Numerous previous studies reported that ICAM-1 and VCAM-1 are also expressed in VSMCs, especially when cells are exposed to damaging stimuli, such as Salusin-β or cholesterol (7,28). In the present study, the results also demonstrated that, compared with the control group, cholesterol treatment significantly upregulated expression levels of adhesion molecules in VMSCs and promoted the adhesion of monocytes to VSMCs *in vitro*, which may form the basis of the initial development of AS.

NADPH oxidase (Nox) family members contribute substantially to the production of ROS in the cardiovascular system (29). Enhanced Nox expression and subsequent ROS formation are directly associated with the severity of structural-functional alterations at the vascular wall (30). The results of the present study demonstrated that cholesterol loading significantly increased ROS accumulation in VSMCs compared with the control group. ROS levels are a key regulator of the p38 MAPK and NF-κB/p65 signaling pathways (31,32). The present study examined the expression levels of key proteins in the p38

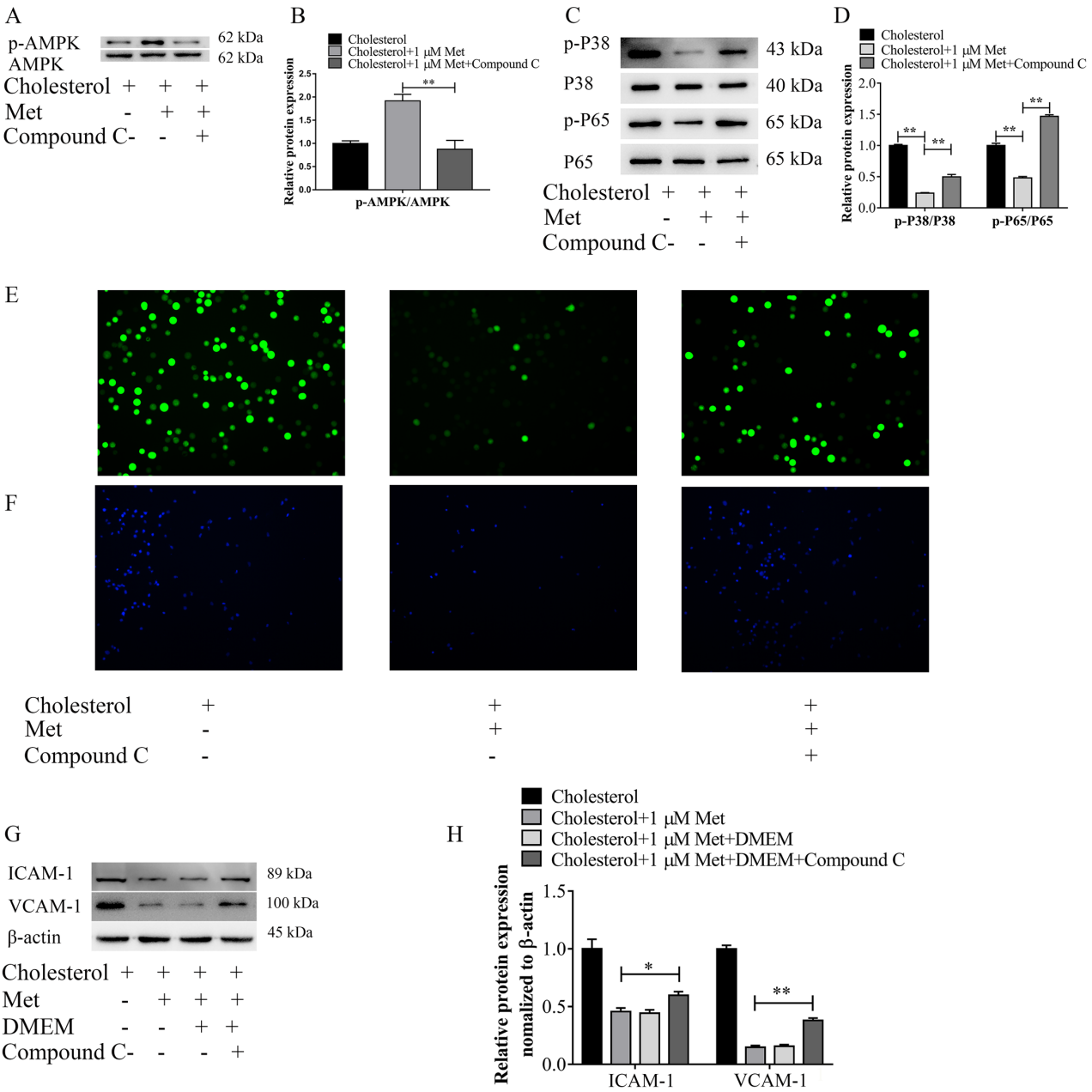

Figure 6. Metformin inhibits cholesterol loading-induced adhesion molecule expression, and p38 MAPK and NF-κB pathway signaling activation via AMPK signaling. VSMCs were exposed to Compound C (10 μm) for 30 min, followed by treatment with metformin (1 μm) and cholesterol (5 μg/ml). Protein expression levels were (A) determined via western blotting and (B) the ratio of p-AMPK/AMPK was semi-quantified. Protein expression levels were (C) determined via western blotting and (D) the ratios of p-P38/P38 and p-P65/P65 were semi-quantified. (E) Reactive oxygen species accumulation in VSMCs was examined via immunofluorescence analysis (magnification, x100). (F) Representative images of THP-1 cell adhesion to VSMCs (magnification, x50). ICAM-1 and VCAM-1 protein expression levels were (G) determined via western blotting and (H) semi-quantified. *P<0.05 and **P<0.01. AMPK, AMP-activated protein kinase; VSMC, vascular smooth muscle cell; p, phosphorylated; ICAM-1, intercellular adhesion molecule-1; VCAM-1, vascular cell adhesion molecule-1; Met, metformin.

MAPK and NF-κB/p65 signaling pathways, and the results suggested that cholesterol loading in VSMCs activated the inflammatory signaling pathways. Hsu *et al* (29) demonstrated that the expression of adhesion molecules is associated with NF-κB/p65 and p38 MAPK phosphorylation. Therefore, it was hypothesized that cholesterol may promote the expression of adhesion molecules in VSMCs by activating the p38 MAPK and NF-κB inflammatory signaling pathways. By using inhibitors of the signaling pathways, the results indicated that ICAM-1 expression in VSMCs was associated with the activation of both inflammatory signaling pathways; however, VCAM-1 expression in VSMCs was exclusively associated with the NF-κB signaling pathway. Further investigation is required to clarify the specific mechanisms underlying the molecular interactions.

In recent years, extensive clinical studies have confirmed the close relationship between diabetes mellitus or impaired glucose tolerance and AS (33,34). Metformin, a hypoglycemic drug, has been reported to reduce cardiovascular events in patients with diabetes (35), which has attracted the attention of scholars in various fields. However, thus far, the mechanisms underlying metformin-mediated inhibition of AS formation and progression have not been previously reported. An oral dose of metformin is

able to induce AMPK activation (36). The results of the present study demonstrated that metformin displayed a dose-dependent effect on the activation of the AMPK signaling pathway in cholesterol-treated VSMCs. AMPK is a highly conserved serine/threonine protein kinase, which is an energy receptor in eukaryotic cells and regulates numerous cell functions, including inhibiting oxidative stress-induced mitochondrial dysfunction (37). In the present study, the results indicated that metformin decreased ROS accumulation and downregulated expression levels of adhesion molecules in cholesterol-treated VSMCs. To investigate whether the aforementioned effects were related to activation of the AMPK signaling pathway, the pathway was inhibited using Compound C. Pre-treatment with Compound C weakened metformin-induced effects on adhesion molecule expression, ROS accumulation and phosphorylation of NF-κB/p65 and p38 MAPK in cholesterol-treated VSMCs, suggesting that metformin exerted anti-inflammatory effects via activation of the AMPK signaling pathway.

The results of the present study demonstrated that cholesterol increased ROS accumulation, and p38 MAPK and NF-κB signaling pathway activation in VSMCs, thereby upregulating the expression levels of ICAM-1 and VCAM-1. By contrast, metformin inhibited cholesterol loading-induced VSMC damage, which may serve as a promising therapeutic strategy for vascular lesions in AS.

Collectively, the present study suggested that cholesterol upregulated adhesion molecule expression levels via ROS accumulation and activation of the p38 MAPK and NF-κB signaling pathways. Moreover, in cholesterol-treated VSMCS, metformin modulated activation of the p38 MAPK and NF-κB signaling pathways by activating AMPK, and reduced abnormal ROS accumulation, thus suppressing adhesion molecule expression.

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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

QL, MY, LZ, RZ and XH performed the experiments. QL and XW performed statistical analyses. WD interpreted the data for the work and reviewed the final version of the manuscript. QL and JH designed the study and drafted the manuscript. QL and JH confirm the authenticity of all the raw data. 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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