Berberine inhibits tumor necrosis factor-α-induced expression of inflammatory molecules and activation of nuclear factor-κB via the activation of AMPK in vascular endothelial cells

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Abstract. Berberine, which is a well-known drug used in traditional medicine, has been demonstrated to exert diverse pharmacological effects, including anti-inflammatory effects. However, whether berberine can affect the production of inflammatory molecules in vascular endothelial cells remains to be elucidated. Therefore, the present study aimed to determine the effects of berberine, and the underlying molecular mechanisms of these effects. The effect of berberine on tumor necrosis factor (TNF)-α-induced inflammatory molecule expression was examined in cultured human aortic endothelial cells (HAECs). The HAECs were stimulated with TNF-α and incubated with or without berberine. The activation of nuclear factor (NF)-κB and adenosine monophosphate-activated protein kinase (AMPK) were analyzed using western blotting, and the protein secretion of intercellular adhesion molecule (ICAM)-1 and monocyte chemoattractant protein (MCP)-1 was measured using ELISA kits. The mRNA expression levels of ICAM-1 and MCP-1 were analyzed using reverse transcription-quantitative polymerase chain reaction. The results of the present study demonstrated that berberine significantly inhibited the TNF-α-induced expression of ICAM-1 and MCP-1, as well as the activation of NF-κB in the HAECs. These effects were attenuated following co-treatment with AMPK inhibitor compound C, or specific small interfering RNAs. In conclusion, the results of the present study indicated that berberine inhibits the TNF-α-induced expression of ICAM-1 and MCP-1, and the activation of NF-κB in HAECs *in vitro*, possibly through the AMPK-dependent pathway.

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Abbreviations: HAECs, human aortic endothelial cells; AMPK, adenosine monophosphate-activated protein kinase; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemotactic protein-1

Key words: berberine, tumor necrosis factor-α, monocyte chemoattractant protein-1, intercellular adhesion molecule-1, vascular endothelial cells

Introduction

Maladaptive remodeling of arteries following injury, including balloon angioplasty and endovascular stent implantation, is characterized by inflammation, neointima formation and media hypertrophy, which may result in restenosis or re-narrowing of the affected artery (1). Vascular injury-induced inflammation involves complex interactions between multiple vascular cell types, among which vascular endothelial cells are essential by releasing various types of growth factors, chemokines and cytokines (2).

Berberine, is one of the main active alkaloids isolated from plants, including *Coptis chinensis* and *Hydrastis canadensis*, and has long been used to treat gastrointestinal disorders, including diarrhea (3). Previous studies have demonstrated that berberine exerts multiple pharmacological effects, including anti-microbial (4,5), anti-inflammatory (6,7) and anti-tumor (8,9) activities. Increasing evidence has indicated that berberine may possess beneficial cardiovascular effects, due to its anti-hyperglycemic (10,11), anti-cardiac hypertrophic (12) and anti-ischemia-reperfusion injury (13) effects. Previous studies have demonstrated that berberine exerts beneficial vascular effects by inhibiting vascular smooth muscle cell proliferation, following various pathogenic conditions, including restenosis (14,15). Furthermore, a previous study indicated that berberine exerts an anti-inflammatory effect in patients with acute coronary syndrome following percutaneous coronary intervention (16), suggesting that the anti-inflammatory effects of berberine may also contribute to its vascular protective activity.

Tumor necrosis factor (TNF)-α is a major proinflammatory factor in the development of vascular inflammation (17). Due to the inhibitory effect of berberine on vascular inflammation, the present study aimed to investigate whether berberine downregulates the expression of inflammatory molecules. The present study also aimed to determine how berberine exerts anti-inflammatory effects in TNF-α-stimulated human aortic endothelial cells (HAECs). The present study investigated the

effects of berberine on the expression of intercellular adhesion molecule (ICAM)-1, monocyte chemoattractant protein (MCP)-1, and nuclear factor (NF)-κB in HAECs. In addition, the underlying molecular mechanism was explored.

Materials and methods

Reagents. Berberine, dimethyl sulfoxide, and compound C were purchased from Sigma-Aldrich (St. Louis, MO, USA). TRIzol® reagent was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). The Lactate Dehydrogenase (LDH) Assay kit was obtained from Beyotime Institute of Biotechnology (Jiangsu, China). The ImProm-II™ Reverse Transcription system was purchased from Promega Corporation (Madison, WI, USA). The SYBR master mix for reverse transcription-quantitative polymerase chain reaction (RT-qPCR) was purchased from Applied Biosystems Life Technologies (Foster City, CA, USA). Antibodies against NF-κB p65, phosphorylated- and total-adenosine monophosphate-activated protein kinase (AMPK), phosphorylated- and total-acetyl-CoA carboxylase (ACC), and histone were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Anti-GAPDH, AMPKα1/2 specific small interfering (si)RNA, control scrambled siRNA and the siRNA reagent system were purchased from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Recombinant human TNF-α, and ELISA kits for ICAM-1 and MCP-1 were obtained from R&D Systems, Inc. (Minneapolis, MN, USA).

Cell culture and treatment. The HAECs were obtained from Sciencell Research Laboratories (Carlsbad, CA, USA) and were maintained in endothelial cell medium supplemented with growth supplements (cat. no. 1052; Sciencell Research Laboratories) and fetal bovine serum (5% vol/vol; Invitrogen Life Technologies) at 37˚C in an atmosphere containing 5% CO_2. Once the cells had reached 70-80% confluence and prior to initiation of the experiments, the cells were serum starved for 24 h in serum-free medium. Subsequently, the HAECs were incubated with berberine at various concentrations, and/or treated with recombinant human TNF-α (10 ng/ml). The cells were cultured for various time periods, and the supernatants were subsequently collected and the cells were harvested. To investigate the potential contribution of the AMPK pathway on the effects of berberine, the HAECs were pretreated with chemical inhibitor compound C (10 μM) for 30 min, or were transfected with AMPK siRNA (100 μM). Briefly, cells were transfected with AMPK siRNA or control scrambled siRNA (Santa Cruz Biotechnology, Inc.) using the siRNA reagent system for 6 h; the medium was then replaced with normal culture medium prior to treatment with berberine and TNF-α.

Cytotoxicity assay. To evaluate the effects of berberine on the viability of the HAECs, an LDH assay was performed, which is based on the release of LDH. The HAECs were seeded into 96-well plates at a density of $5x10^3$ cells/well, and following 3 days incubation at 37˚C the medium was replaced with serum-free medium, and the HAECs were incubated at 37˚C for a further 24 h. Berberine was then added to the cells at final concentrations of 2, 5, 10 or 25 μM), and cultured for 24 h at 37˚C. Subsequently, the cell culture supernatants were collected and the LDH content was analyzed, according to the manufacturer's instructions. The toxicity of berberine on the cells was also investigated using a trypan blue dye exclusion assay (0.4%; Sigma-Aldrich), as previously reported (18).

ELISA. Immediately following termination of the experiment involving treatment with berberine, the cell culture supernatants were collected and stored at -80˚C for subsequent analysis of the expression of inflammatory molecule proteins. The concentrations of ICAM-1 and MCP-1 were determined using ELISA kits, according to the manufacturer's instructions. Absorbance was measured at 450 nm using an ELISA reader (model 3550; Bio-Rad Laboratories, Inc., Hercules, CA, USA).

RNA extraction and RT-qPCR. Total RNA was extracted from the cells using TRIzol® reagent, according to the manufacturer's instructions. The isolated RNA was then reverse transcribed into cDNA using the reverse transcription system. mRNA expression levels were analyzed using an ABI Prism 7500 system (Applied Biosystems Life Technologies) and SYBR Green master mix. The PCR reaction mix (20 μl) consisted of 1 μl cDNA (50 ng), 10 μl 2X SYBR Green master mix and 5 pmol each of the forward (1 μl) and reverse (1 μl) primer (Beijing Sunbiotech Co., Ltd., Beijing) made up with sterile water. PCR cycling conditions were as follows: 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 40 sec. Data were subsequently collected and quantitatively analyzed. GAPDH was used as an endogenous control for sample normalization. The results are presented as fold increases, compared with the expression of GAPDH. The primer sequences were as follows: ICAM-1, forward 5'-AATGCCCAGACATCTGTGTCCC-3' and reverse 5'-GGCAGCGTAGGGTAAGGTTCTT-3'; MCP-1, forward 5'-TTCCATGGACCACCTGGACA-3' and reverse 5'-TGTCTGGGGAAAGCTAGGGG-3'; and GAPDH, forward 5'-CTCCCCACACACATGCACTTA-3' and reverse 5'-CCTAGTCCCAGGGCTTTGATT-3'. Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method (19).

Western blot analysis. The HAECs were harvested and lysed with lysis buffer containing 50 μM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 2 mM EDTA and 100 mM phenylmethylsulfonyl fluoride (Beyotime Institute of Biotechnology). The cell lysate was then centrifuged at 14,000 x g at 4˚C for 5 min, and the supernatant was collected. Nuclear protein extracts were isolated using a Nuclear and Cytoplasmic Protein Extraction kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Protein concentrations were determined using a bicinchoninic acid method (Beyotime Institute of Biotechnology). Equal quantities of protein (50 μg) from each sample were separated by 12% SDS-PAGE (Beyotime Institute of Biotechnology). The proteins were then transferred onto nitrocellulose membranes (Beyotime Institute of Biotechnology), blocked with 5% skim milk in tris-buffered saline supplemented with 0.1% Tween 20 (TBST; Beyotime Institute of Biotechnology), and incubated overnight at 4˚C with the following polyclonal rabbit anti-human primary antibodies: Anti-NF-κB p65 (1:1,000; cat. no. 3039),

anti-phosphorylated-AMPK (1:1,000; cat. no. 2531), anti-AMPK (1:1,000; cat. no. 2532), anti-phosphorylated-ACC (1:1,000; cat. no. 3661), anti-ACC (1:1,000; cat. no. 3662) and anti-histone (1:1,000; cat. no. 2578). The membranes were then washed three times with TBST, followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2,000; cat. no. 7074) for 2 h at room temperature. The blots were visualized using enhanced chemiluminescence reagent (Pierce Biotechnology, Inc.). Relative protein expression levels were determined by densitometric analysis using ImageJ software, version 1.45 (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis. Data are presented as the mean ± standard error of the mean. Statistical analyses were performed using GraphPad Prism 5.0 sofrware (GraphPad Software, Inc., La Jolla, CA, USA), and the statistical significance of differences was determined using one-way analysis of variance and Student's t-test. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were performed at least three times.

Results

HAEC toxicity of berberine. The HAECs were cultured with various concentrations of berberine, in order to evaluate its toxic effects. Treatment with 2, 5, 10 or 25 μM berberine for 24 h did not lead to HAEC toxicity, compared with the controls (Fig. 1A). A trypan blue dye exclusion assay was also used to confirm the effects of berberine on HAEC toxicity, and similar results were observed (Fig. 1B).

Inhibitory effects of berberine on the production of ICAM-1 and MCP-1. To investigate the effects of berberine on the production of inflammatory molecules, the protein expression levels of ICAM-1 and MCP-1 was detected in the HAECs following co-treatment with TNF-α for various durations. TNF-α significantly increased the protein secretion of ICAM-1 and MCP-1 after 24 h (Fig. 2A and B), and the mRNA expression levels of ICAM-1 and MCP-1 were also increased 6 h following TNF-α stimulation (Fig. 2C and D). By contrast, berberine significantly attenuated TNF-α stimulated protein secretion and the mRNA expression levels of ICAM-1 and MCP-1 in HAECs in a concentration-dependent manner (Fig 2A-D).

Inhibitory effects of berberine on the activation of NF-κB. Further experiments were performed to examine whether NF-κB activation was affected by treatment with berberine. As shown in Fig. 3, the expression of nuclear NF-κB p65 increased following stimulation of the HAECs with TNF-α, whereas berberine significantly downregulated the nuclear expression levels of TNF-α-stimulated NF-κB p65.

Berberine activates AMPK in HAECs. Treatment of the HAECs with berberine induced the activation of AMPK, which was determined by measuring the phosphorylation of AMPK. Berberine increased the activation of AMPK and its downstream molecule, ACC, in a time-dependent manner (Fig. 4).

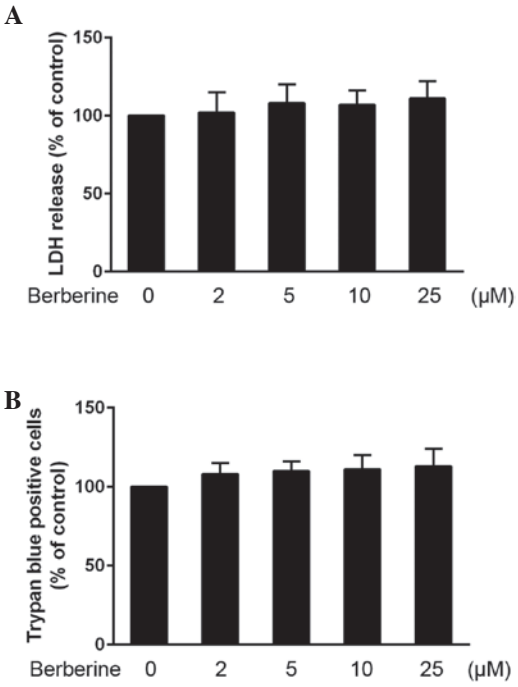

Figure 1. Cytotoxicity assay of berberine in HAECs. (A) LDH release assay of HAECs following treatment with 2, 5, 10 or 25 μM berberine for 24 h. (B) Trypan blue dye exclusion assay of HAECs following treatment with 2, 5, 10, 25 μM berberine for 24 h. Data are presented as the mean ± standard error of the mean. No significant difference was detected between any group. HAECs, human aortic endothelial cells; LHD, lactate dehydrogenase.

Inhibitory effects of berberine on TNF-α-induced expression of inflammatory moleculed and NF-κB-activation are mediated by the AMPK pathway. The present study investigated the role of AMPK activation in the inhibitory effects of berberine on the TNF-α-induced expression of inflammatory molecules and NF-κB-p65 activation. The inhibitory effects of berberine on TNF-α induced expression of ICAM-1 and MCP-1 was attenuated when the HAECs were treated with AMPK inhibitor compound C, or were transfected with AMPK-specific siRNA (Fig. 5A and B). In addition, the inhibitory effects of berberine on TNF-α-induced NF-κB p65 activation were attenuated in the cells, which were treated with compound C or transfected with AMPK siRNA. These results indicated that AMPK activation may mediate the inhibitory effects of berberine on the TNF-α-induced expression of inflammatory molecules and NF-κB activation (Fig. 5C and D).

Discussion

The present study demonstrated that treatment with berberine significantly inhibited TNF-α-induced expression of inflammatory molecules, without affecting the viability of the HAECs. Berberine effectively reduced the expression levels of ICAM-1 and MCP-1, and the activation of NF-κB, possibly via an AMPK-dependent pathway.

Vascular remodeling following injury is associated with inflammation, neointima formation and media hypertrophy (1). Previous studies have demonstrated that berberine exerts vascular protective effects via various pathways, including

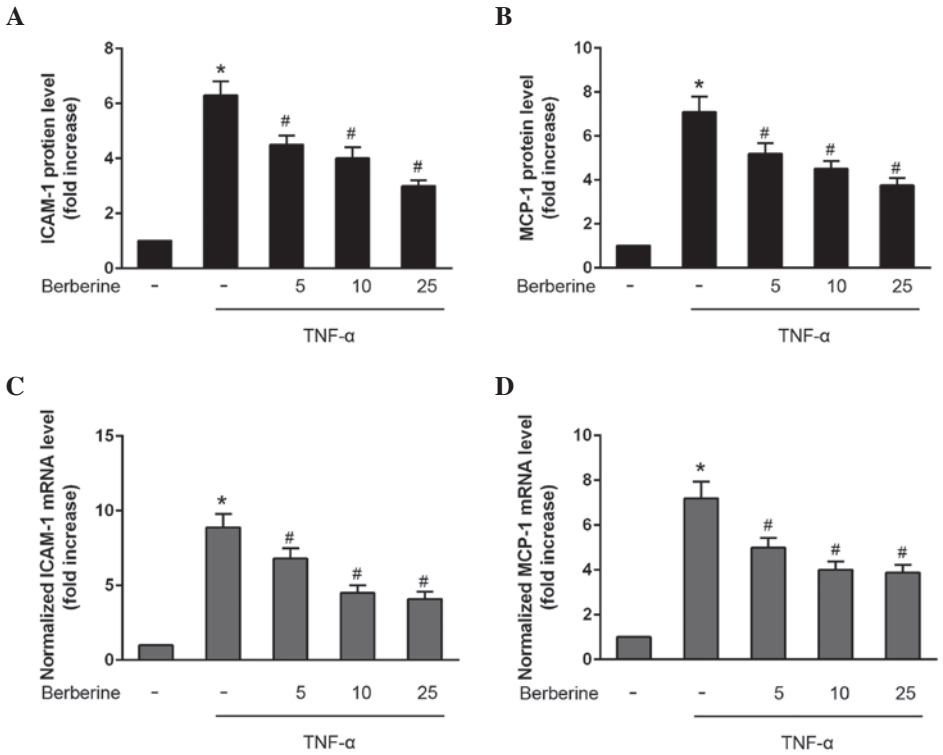

Figure 2. Berberine inhibits the protein and mRNA expression levels of ICAM-1 and MCP-1 in TNF-α-stimulated HAECs. Protein expression levels of (A) ICAM-1 and (B) MCP-1, determined using ELISA. mRNA expression levels of (C) ICAM-1 and (D) MCP-1, determined using reverse transcription-quantitative polymerase chain reaction. HAECs were treated with or without various concentrations of berberine (5, 10 or 25 μM) and stimulated with 10 ng/ml TNF-α for 24 h. The protein and mRNA expression levels of the control HAECs were considered one-fold. Data are presented as the mean ± standard error of the mean. *P<0.05, compared with the control; #P<0.05, compared with the HAECs treated with TNF-α alone. ICAM, intercellular adhesion molecule; MCP, monocyte chemoattractant protein; TNF, tumor necrosis factor; HAECs, human aortic endothelial cells.

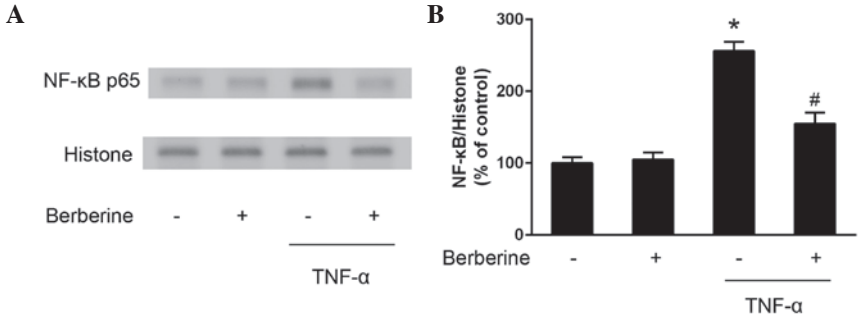

Figure 3. Berberine inhibits the expression of NF-κB p65 in TNF-α-stimulated HAECs. (A) HAECs were treated with or without 25 μM berberine and were stimulated with or without 10 ng/ml TNF-α for 30 min. The protein expression of NF-κB p65 was determined using western blotting and the (B) protein expression was quantified. Data are presented as the mean ± standard error of the mean. *P<0.05, compared with the control; #P<0.05, compared with the cells treated with TNF-α alone. NF, nuclear factor; TNF, tumor necrosis factor; HAECs, human aortic endothelial cells.

the inhibition of vascular smooth muscular cell activation (15) and the reduction of inflammatory molecules (16). Vascular endothelial cells are important in vascular inflammation by releasing diverse types of growth factors, chemokines and cytokines. In our previous study, berberine was observed to suppress the migration of human aortic smooth muscle cells by reducing the expression levels of matrix metalloproteinase (MMP)-2, MMP-9, and urokinase-type plasminogen activator (20), whereas the effects of berberine on vascular endothelial cells and the underlying molecular mechanisms remained to be elucidated. Previous studies have demonstrated that berberine exerts an inhibitory effect on inflammation in several cell lines, and the expression levels of proinflammatory genes, including interleukin (IL)-1β, IL-6, TNF-α and MCP-1, are downregulated following berberine administration (21-23). The adhesion molecule, ICAM-1, and chemokine, MCP-1, are important in the inflammatory process, predominantly via the promotion of monocyte-endothelial adhesion and transendothelial migration (24-27). The results of the present study demonstrated that berberine inhibited the mRNA and protein expression levels of ICAM-1 and MCP-1. These results indicated that the mechanism underlying the anti-inflammatory effects of berberine was, at least partially, associated with downregulation of these genes.

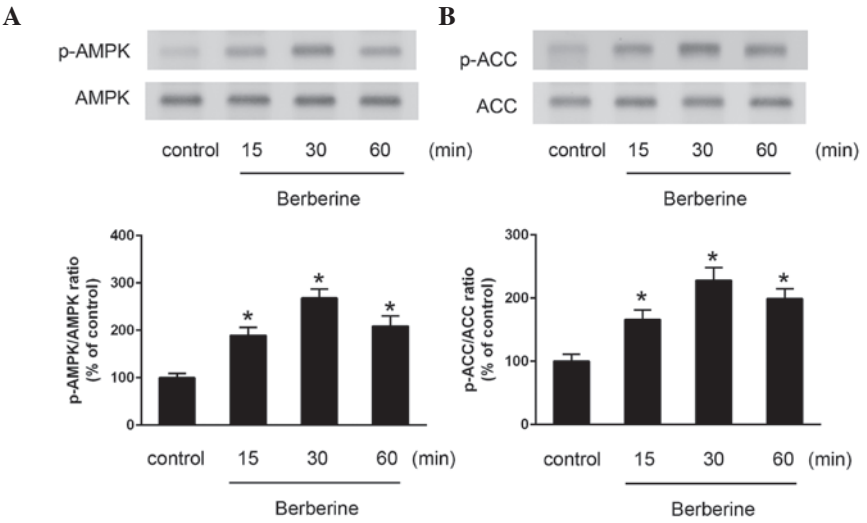

Figure 4. Berberine-induced AMPK activation in HAECs. HAECs were treated with 25 μM berberine for various time-periods (15, 30 or 60 min), and subjected to western blotting against (A) total AMPK and p-AMPK, and (B) total ACC and p-ACC. Data are presented as the mean ± standard error of the mean. *P<0.05, as compared with the control group. AMPK, adenosine monophosphate-activated protein kinase; HAECs, human aortic endothelial cells; ACC, acetyl-CoA carboxylase; p-, phosphorylated.

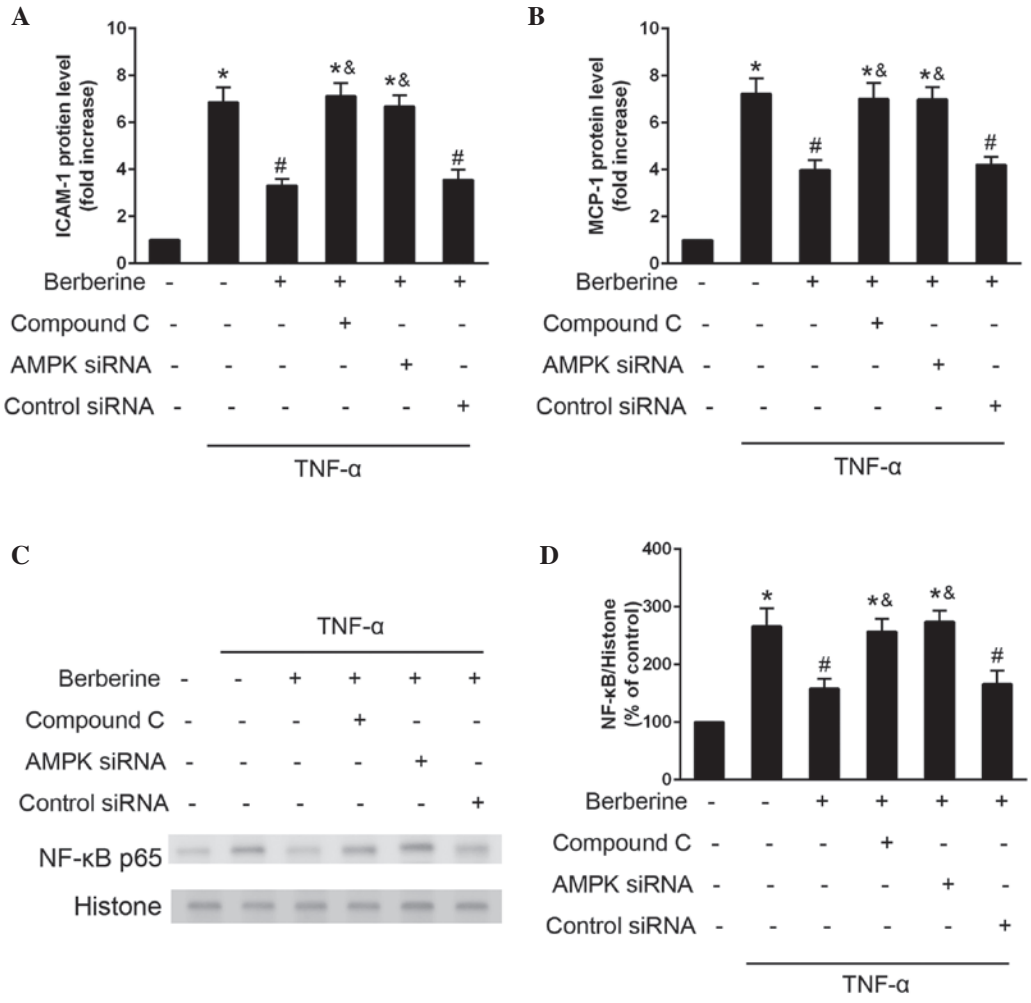

Figure 5. Berberine inhibits TNF-α induced inflammatory molecule expression and NF-κB expression via the AMPK pathway. HAECs were treated with berberine (25 μM), compound C (10 μM), or were transfected with AMPK siRNA (100 μM) or control scrambled siRNA (100 μM) 30 min prior to stimulation with TNF-α. (A and B) Protein expression levels of ICAM-1 and MCP-1 were determined using an ELISA assay after 24 h. (C and D) Expression of NF-κB was analyzed using western blotting 30 min after TNF-α stimulation. Data are presented as the mean ± standard error of the mean. *P<0.05, compared with the control; #P<0.05, compared with the cells treated with TNF-α alone; &P<0.05, compared with the TNF-α and berberine co-treatment group. NF, nuclear factor; TNF, tumor necrosis factor; AMPK, adenosine monophosphate-activated protein kinase; HAECs, human aortic endothelial cells; siRNA, small interfering RNA; ICAM, intercellular adhesion molecule; MCP, monocyte chemoattractant protein.

NF-κB is a well-known transcription factor, which is critical in vascular injury-associated proinflammatory gene regulation and is rapidly activated by various agents, including the inflammatory cytokine, TNF-α (28). NF-κB translocates into the nuclear compartment upon stimulation with various stimuli (29). Inflammatory molecules, including ICAM-1, MCP-1 and other pro-inflammatory molecules, including IL-6 and E-selectin, have previously been reported to promote vascular remodeling via NF-κB-dependent coordinated induction (30-32), Concordantly, the results of the present study indicated that berberine inhibited NF-κB activation, suggesting that the NF-κB pathway is associated with the anti-inflammatory effects of berberine.

AMPK is a heterotrimeric serine/threonine protein kinase, which is generally referred to as a 'metabolite-sensing kinase'. Evidence has demonstrated that AMPK is key in cardiovascular functioning, metabolism, insulin signaling, reactive oxygen species regulation and inflammatory processes (33,34). However, compared with the well-accepted roles of AMPK in metabolite sensing, the involvement of AMPK in inflammatory processes remains to be fully elucidated. Previous studies have demonstrated that attenuation of the activation of NF-κB is mediated via AMPK activation in various cell lines, including endothelial cells (35-38), suggesting that AMPK may be the upstream signaling molecule through which berberine exerts its anti-inflammatory effects. Concordant with the results of these previous studies, the findings of the present study indicated that AMPK activation was associated with the inhibitory effects of berberine on TNF-α-induced NF-κB activation and the expression of inflammatory molecules in HAECs.

In conclusion, the results of the present study suggested that berberine may attenuate the TNF-α-induced expression of inflammatory molecules by inhibiting NF-κB following AMPK activation in the HAECs. These results indicate a novel molecular mechanism underlying the anti-inflammatory effects of berberine in vascular remodeling processes.

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