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Original Article

APOC3 promotes TNF-α-induced expression of JAM-1 in endothelial cell via PI3K-IKK2-p65 pathway

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ABSTRACT

Atherosclerosis is a chronic inflammatory disease with lipid accumulation. Apolipoprotein C3 (APOC3), which is an important regulator of human lipid metabolism, is associated with multiple vascular mechanisms in atherosclerosis and proinflammatory responses. We have previously reported that the expression of inflammatory cytokine TNF-α is elevated in human endothelial cells (HUVECs) after APOC3 treatment. This study investigates the APOC3 signaling pathway involved in TNF-α-mediated expression of JAM-1 in HUVECs. Cultured HUVECs were exposed to APOC3 (50 μg/ml) for 16 h. Mechanistic studies were carried out by silencing TNF-α gene with lentiviral TNF-α-shRNA. Our study was based on the eight signaling pathway inhibitors to block the effect of APOC3 in HUVECs. The expression of JAM-1 was determined by qRT-PCR, Western blotting, and flow cytometry. IKK2 degradation and NF-κB p65 phosphorylation were determined by Western blotting. Our results showed that APOC3 significantly promoted the TNF-α-induced expression of JAM-1 in HUVECs. Inhibiting APOC3 reversed the TNF-α-induced overexpression of JAM-1. Moreover, APOC3 induced the expression of NF-κB p65 and degraded IκB. In conclusion, APOC3 promoted the expression of JAM-1 via the NF-κB, IKK2, and PI3K signaling pathway.

1. Introduction

Atherosclerosis is the primary pathological basis of ischemic cardiovascular and cerebrovascular diseases such as coronary heart disease, ischemic stroke, and thromboembolic disease, which threatens the health of people worldwide [1]. Atherosclerosis is characterized by lipid accumulation and chronic inflammation, events occurring in the vessel wall in response to various injuries. It contains the features of classic inflammatory degeneration, exudation, and hyperplasia [2]. Inflammation, which is a common factor in atherogenesis, runs through all stages of atherosclerosis [3].

Apolipoprotein C3 (APOC3), which exists in a variety of lipoprotein particles, has been associated with many vascular injury induced mechanisms of atherosclerosis [2]. APOC3 with a molecular mass of 8.8 kDa is present on the surface of human plasma lipoprotein particles. It is rich in triglyceride lipoproteins and high-density lipoproteins (HDL). It plays a major role in lipid metabolism by inhibiting lipoprotein esterase activity [4]. Studies have shown that APOC3 can promote progression of atherosclerosis [5]. APOC3, independent of low-density lipoprotein cholesterol(LDL-C) or high-density lipoprotein cholesterol (HDL-C) levels, is causally associated with increased cardiovascular disease (CVD) risk [6]. APOC3 overexpression is responsible for severe hyperglycemia in mice [7]. Mutations that exacerbate APOC3 gene expression (such as -482C > T, SstI, and T-455C) increase plasma triglyceride (TG) levels and risk of CVDs [8,9]. Loss-of-function APOC3 mutations reduce plasma TG and increase HDL-C and may lower the chances of coronary atherosclerosis [10]. Thus, APOC3 may be an effective treatment for the management of dyslipidemia and cardiovascular diseases.

It is known that APOC3 is involved in lipid regulation and inflammatory responses of atherosclerosis [11]. The presence of high level of APOC3 increases the expression of adhesion molecules, especially

Abbreviations: CVD, cardiovascular disease; APOC3, apolipoprotein C3; HDL, high-density lipoproteins; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; VCAM-1, vascular cell adhesion molecule-1; JAM-1, junctional adhesion molecule-1; ECs, endothelial cells; NF-κB, nuclear factor κ B; TNF-α, tumor necrosis factor-α; PKCβ, protein kinase C β; IKK, IκB phosphorylation-mediated kinase; ICAM-1, intercellular cell adhesion molecule-1; DMEM, Dulbecco's modified Eagle medium; FBS, fetal bovine serum.

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vascular cell adhesion molecule-1(VCAM-1) which promotes adhesion of monocytes [12]. Junctional adhesion molecule-1 (JAM-1), also called the F11 receptor, is a cell adhesion molecule that is selectively located in the tight junctions of endothelial cells (ECs). Nonactivated ECs express very low level of JAM-1 under physiological conditions [13]. Azari et al. [14] reported that translation of JAM-1 is a crucial initial step in the early stages of atherogenesis. JAM-1 plays an essential role in the adhesion process of platelets to inflamed ECs, a process that promotes plaque formation and atherosclerosis. JAM-1 promoter analysis revealed the presence of a nuclear factor κB (NF-κB) binding site which indicated that inflammatory cytokines such as tumor necrosis factor-α (TNF-α) can regulate JAM-1 via NF-κB [15].

Upon monocyte adhesion to ECs, APOC3 activates protein kinase Cβ (PKCβ), which is an activator of NF-κB and VCAM-1, which can cause EC dysfunction. PKCβ increases the interaction between monocytes and ECs by regulating the expression of VCAM-1 in ECs [16]. Activation of NF-κB is regulated by IκB phosphorylation-mediated kinase (IKK) activities which further cause the phosphorylation and degradation of NF-κB inhibitory protein molecules [17], releasing NF-κB to translocate to the nucleus. NF-κB activation can promote overexpression of adhesion and chemokine factors such as ICAM-1 and TNF-α in vascular ECs and aggravate inflammatory aspects of atherosclerosis [18,19]. These findings suggest that APOC3 is involved in the inflammatory response to atherosclerosis; the specific mechanisms have not been fully investigated. In this study, we demonstrated the association of TNF-α with APOC3-induced JAM-1 through the PI3K-IKK2-p65 signaling pathway in ECs.

2. Materials and methods

2.1. Cell culture and stimulation

Human umbilical vein endothelial cell (HUVEC) was obtained from Science Cell Lab (USA). Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and trypsin were purchased from Science Cell Lab. HUVECs were cultured in DMEM with 10% FBS and maintained in a 5% CO_2 incubator at 37°C. HUVECs were stimulated with APOC3 (50 μg/ml) for 16 h. To detect specific signal pathway, HUVECs were treated with PI3K inhibitor (Wortmannin, 100 nmol/L), JNK inhibitor (SP600125, 20 μmol/L), ERK inhibitor (PD98059, 10 μmol/L), p38 inhibitor (SB203580, 20 μmol/L), IKK2 inhibitor (SC514, 10 μmol/L), PKCαβ inhibitor (Go6976, 100 nmol/L), PKCβ inhibitor (LY-333,531, 200 nmol/L), or NF-κB inhibitor (SN50, 10 mmol/L) for 24 h. Go6976 was obtained from Biovision (USA), LY-333,531 was obtained from Selleck (USA), and SN50 was obtained from MCE (USA). All the rest of the signal inhibitors were purchased from Cayman Corporation (USA).

2.2. Lentiviral transduction and selection in HUVECs

Lentivirus packaging and titer detection were completed by Genepharma Corporation. A puromycin tag was included for selection of stable lines. Lentiviral transduction of HUVECs was performed on the second day after passaging at a multiplicity of infection of 10 with a medium change after 24 h. The medium was replaced with a puromycin selection medium and incubated for 3–10 days after 48 h of viral transduction. Medium was changed approximately every 2–3 days.

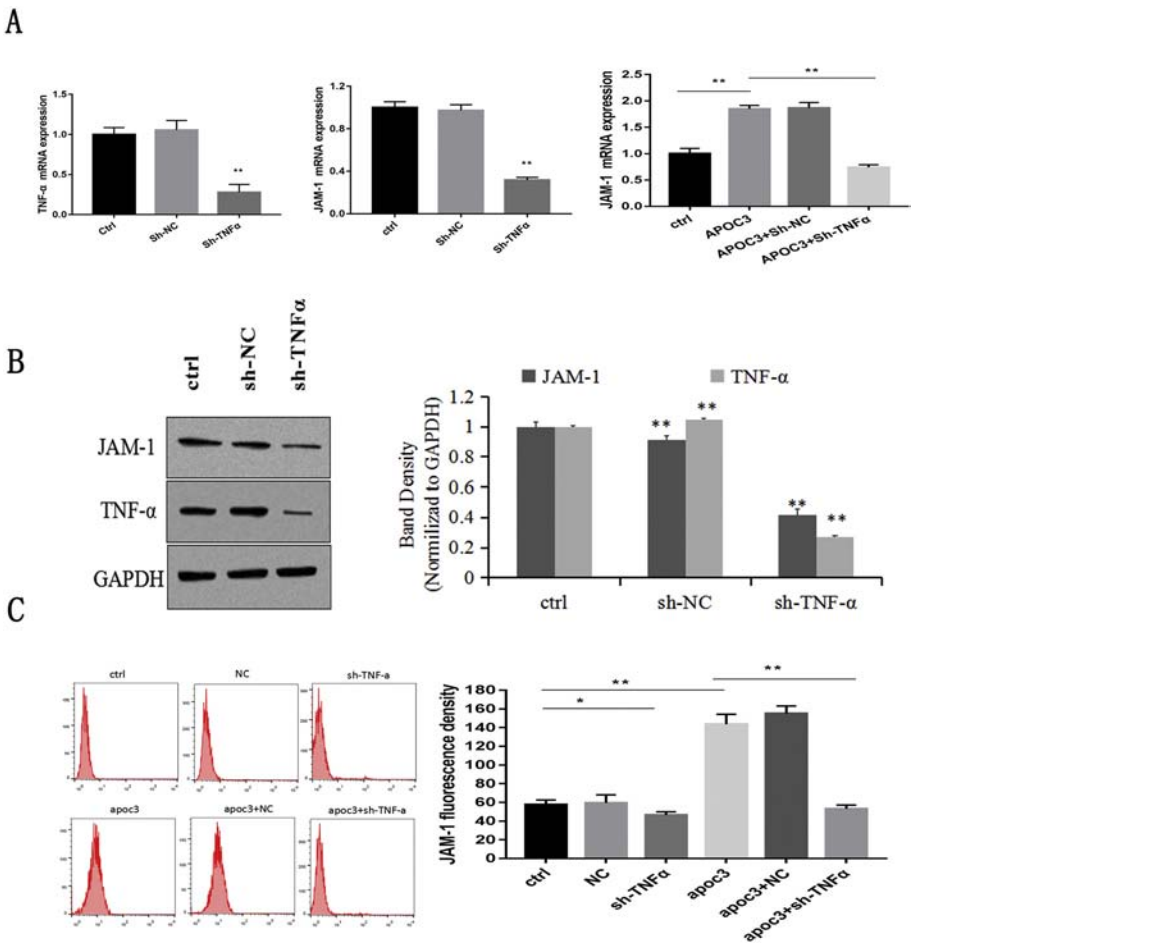

Fig. 1. TNF-α-induced expression of JAM-1 in HUVECs exposed to APOC3. HUVECs were transfected with lentivirus against TNF-α. (A) TNF-α and JAM-1 mRNA was determined by qRT-PCR. (B) JAM-1 and TNF-α proteins were determined by Western blot. (C) HUVECs or sh-TNF-α HUVECs were exposed to APOC3 (50 μg/ml) for 16 h. Expression of JAM-1 was determined by flow cytometry. All data were repeated at least three times. $^{**}P<.01$, $^{*}P<.05$.

Finally, the TNF-α-shRNA stable cell line was finally screened by qRT-PCR and fluorescence microscopy.

2.3. RNA purification and qRT-PCR

Total RNA was extracted from HUVECs with the TRIzol method (TRIzol reagent; Invitrogen, USA). RNA was reverse transcribed into cDNA (PrimeScript RT reagent Kit, Takara, Japan). cDNA was quantified by qPCR using SYBR Premix Ex Taq (Takara, Japan). The mRNA expression was normalized to GAPDH, an internal control. The primers (Shenggong, China) used are as follows:

GAPDH: F, AAGGTGAAGGTCGGAGTCAAC;
R, GGGGTCATTGATGGCAACAATA.
TNF-α: F, ATCAATCGGCCCGACTATCTC;
R, GCAATGATCCCAAAGTAGACCTG.
JAM-1: F, TGGCATTGGGCAGTGTTACAG;
R, GTCTCCTTGGTCAAACTTCCAC.

2.4. Flow cytometry

HUVECs were washed and stained with anti-JAM-1 antibody for 30 min followed by incubation in Goat Anti-Mouse IgG (Life Technologies, USA). The analysis of cell surface expression of JAM-1 was conducted on an Accuri C6 Flow Cytometer (BD Biosciences).

2.5. Western blot

Total protein was lysed in lysis buffer containing 50 mmol/L Tris–HCl pH 8.0, 150 mmol/L NaCl, 1% TritonX-100, and 100 μg/ml PMSF. Protein concentration was determined with BCA kit (Thermo Fisher, USA). Equal amount of protein (30 mg) from each sample was separated by SDS-PAGE and then transferred on to PVDF membranes. After blocking for 1 h with 5% bovine serum albumin in TBST buffer at room temperature, the membranes were incubated with primary antibody (1:1000 dilution, Abcam, USA) overnight at 4 ℃. After washing with TBST, membranes were incubated with respective secondary antibody (1:5000) for 2 h at room temperature. The ECL Chemiluminescence Imaging System (Thermo, USA) was used for imaging and analysis.

2.6. Statistical analysis

Each value is an average of at least three independent experiments. All of results are reported as mean ± SD. Data were analyzed with one-way ANOVA and with a value of $P<.05$ indicated significant.

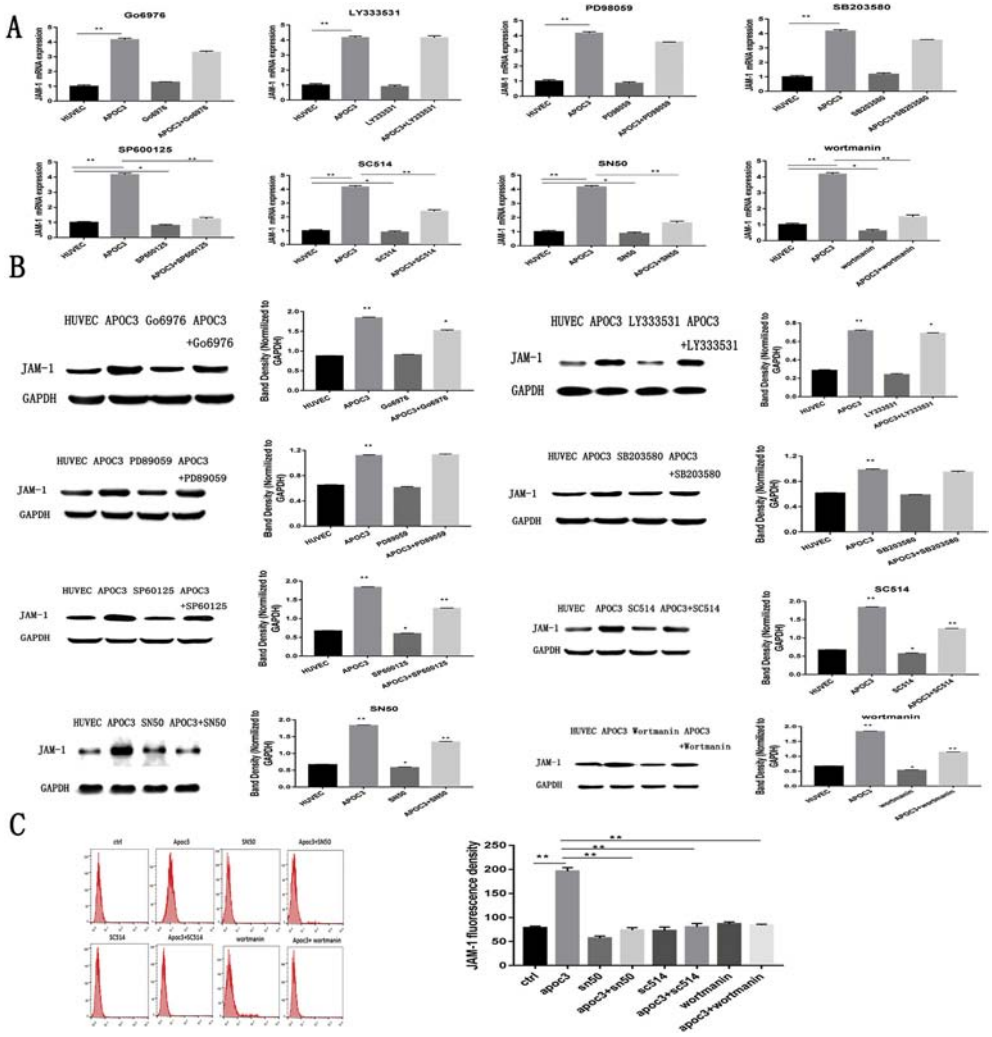

Fig. 2. Expression of JAM-1 by APOC3: treatment with eight pathway inhibitors. HUVECs were pretreated with eight signal inhibitors individually and then incubated in APOC3 (50 μg/ml) for 16 h. (A) JAM-1 was determined by qRT-PCR. (B) JAM-1 protein was determined by Western blot. (C) JAM-1 protein was determined by flow cytometry. All data were repeated at least three times. $^{**}P<.01$, $^{*}P<.05$.

3. Results

3.1. APOC3 significantly promoted TNF-α-induced expression of JAM-1 in HUVECs

Expression of tight junction proteins is closely associated with atherosclerosis [20]. Our previous results have shown that APOC3 was strongly associated with the expression of JAM-1, and the process was characterized by the expression of TNF-α [21]. To further determine the role of APOC3 in atherosclerosis inflammation response, we silenced TNF-α by using short hairpin RNA to examine the expression of JAM-1 in HUVECs (Fig. 1A). Results of PCR revealed that knockdown of TNF-α in HUVECs decreased the mRNA expression of JAM-1 (Fig. 1A). In parallel, protein abundance of JAM-1 also declined upon transfection with sh-TNF-α (Fig. 1B and C). Also, APOC3-induced JAM-1 expression was significantly suppressed (more than twofold) upon silencing of TNF-α. We found that APOC3 promoted the expression of JAM-1 in HUVECs. Attenuation of APOC3-mediated expression of JAM-1 by silencing TNF-α clearly manifested its role in the atherosclerotic process. Together, our results suggested that APOC3 promoted JAM-1 expression via TNF-α in HUVECs.

3.2. Disruption of the PI3K-IKK2-p65 pathway blocked the effect of APOC3

It is well known that NF-κB activation participates in the inflammatory aspects of atherosclerosis [22]. We used inhibitors against eight inflammation-related signaling pathways to investigate APOC3-mediated signaling pathways in ECs. Efficacy and specificity of the inhibitors were validated by qRT-PCR and Western blotting in cultured HUVECs exposed to APOC3 for 16 h. As shown in Fig. 2A, the levels of JAM-1 mRNA stimulated by APOC3 significantly declined (more than threefold) in the presence of inhibitors including SP600125, Wortmannin, sc514, and SN50. The JAM-1 protein was equivalently decreased after treated with the above signal inhibitors (Fig. 2B). Our data revealed that SP600125, Wortmannin, sc514, and SN50 were significantly effective in inhibiting APOC3 induced JAM-1. Flow cytometry confirmed that the three inhibitors (Wortmannin, sc514, and SN50) largely prevented APOC3-induced expression of JAM-1 (Fig. 2C). Wortmannin is an inhibitor of PI3K, SC514 is an inhibitor of IKK2, and SN50 is an inhibitor of NF-κB signaling. Furthermore, PI3K is upstream of IκB kinase 2 (IKK2) and regulates the function of IKK2 which mediates the nuclear translocation of the p65 subunit of activated NF-κB. These findings suggest that APOC3 may regulate JAM-1 via the PI3K-IKK2-p65 signaling pathways.

3.3. APOC3 regulates TNF-α to stimulate JAM-1 by the PI3K-IKK2-p65 signaling pathway in HUVECs

Our previous study has shown that TNF-α was involved in the process of APOC3 induced inflammatory responses [21]. In addition, TNF-α has been confirmed to induce phosphorylation of NF-κB p65 subunits and degradation of IκBα [23,24]. Pharmacological inhibitors were used

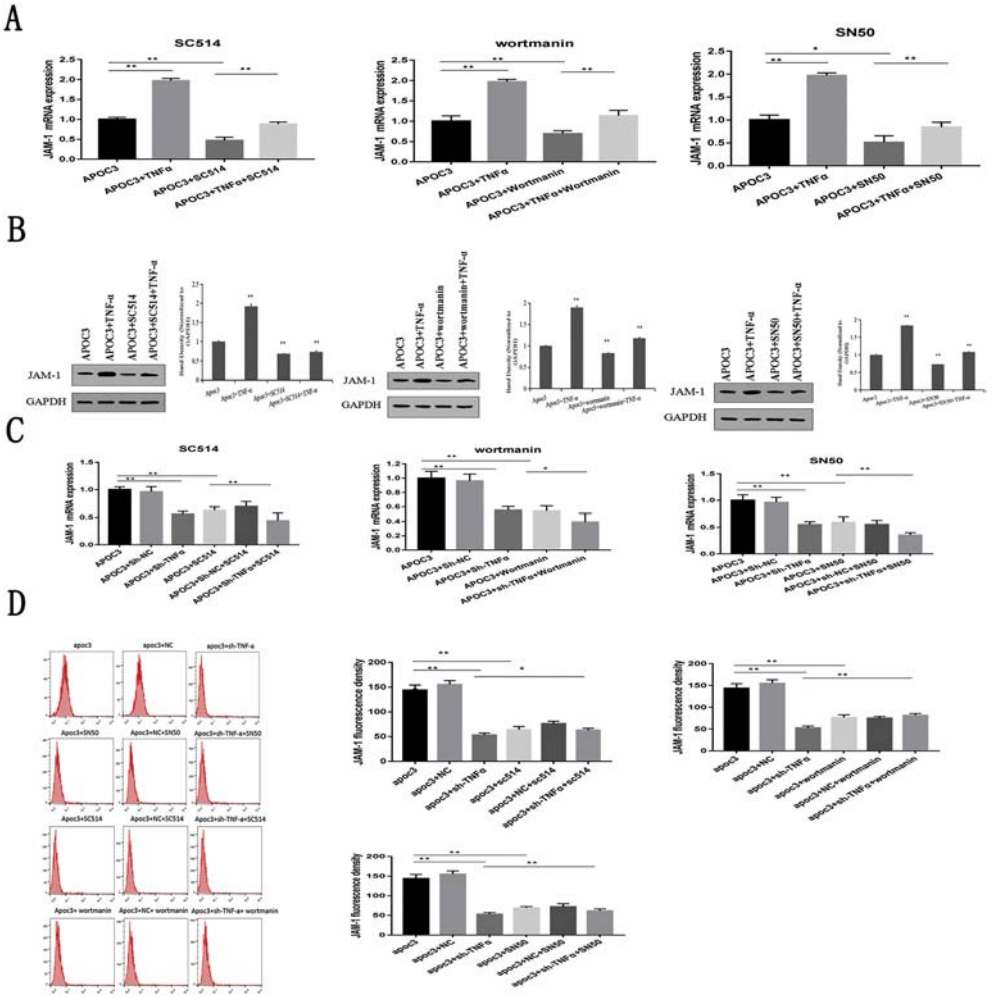

Fig. 3. TNF-α-induced expression of JAM-1 by APOC3 in HUVECs: inhibition by SC514, Wortmannin, and SN50. HUVECs were pretreated with SC514 (10 μmol/L), Wortmannin (100 nmol/L), and SN50 (10 mmol/L) for 30 min, 60 min, or 24 h and further incubated with APOC3 (50 μg/ml) or TNF-α (10 ng/ml). (A and C) JAM-1 mRNA was determined by qRT-PCR. (B) JAM-1 protein was determined by Western blot. (D) JAM-1 protein was determined by flow cytometry. All data were repeated at least three times. $^{**}P<.01$, $^{*}P<.05$.

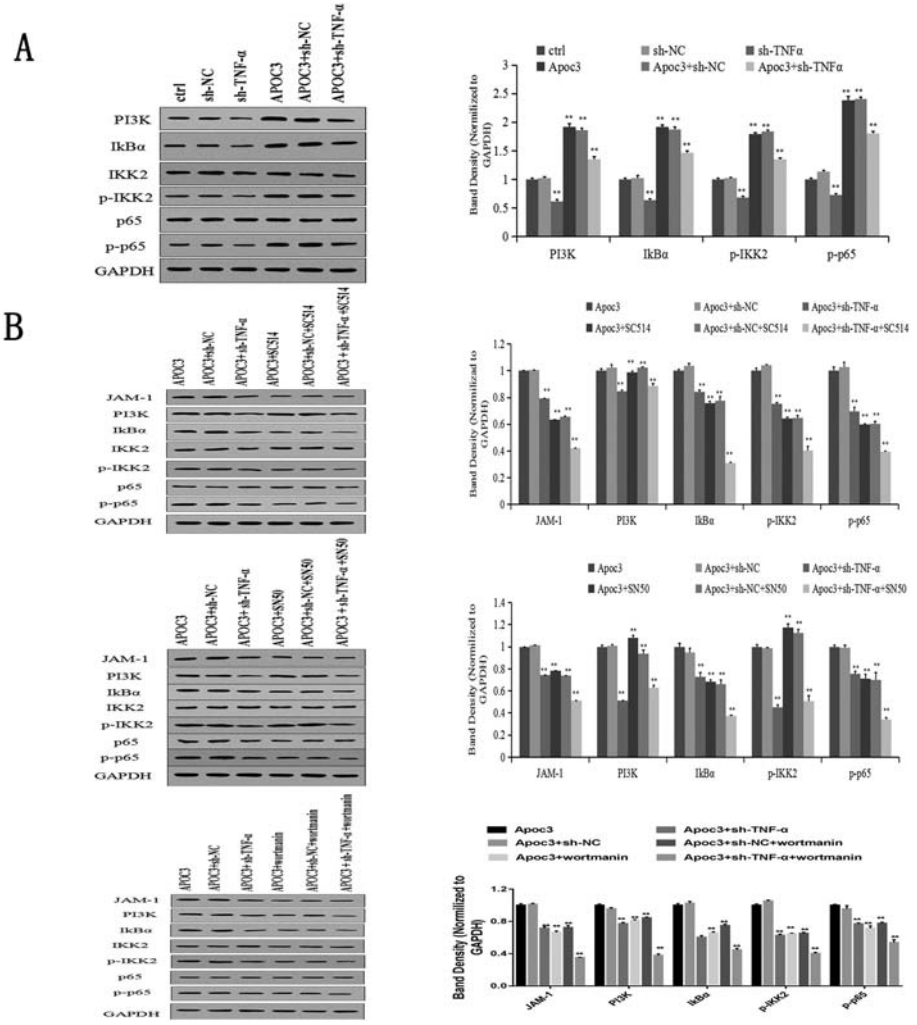

Fig. 4. APOC3 induced JAM-1 expression in HUVECs through PI3K-IKK2-p65 signaling pathway. sh-TNF-α HUVECs were pretreated with SC514 (10 μmol/L), Wortmannin (100 nmol/L), or SN50 (10 mmol/L) for 30 min, 60 min, or 24 h followed by incubation with APOC3 (50 μg/ml) or TNF-α (10 ng/ml) for 16 h. (A and B) JAM-1 and signal-related proteins were determined by Western blot. All data were repeated at least three times. $^{**}P<.01$, $^{*}P<.05$.

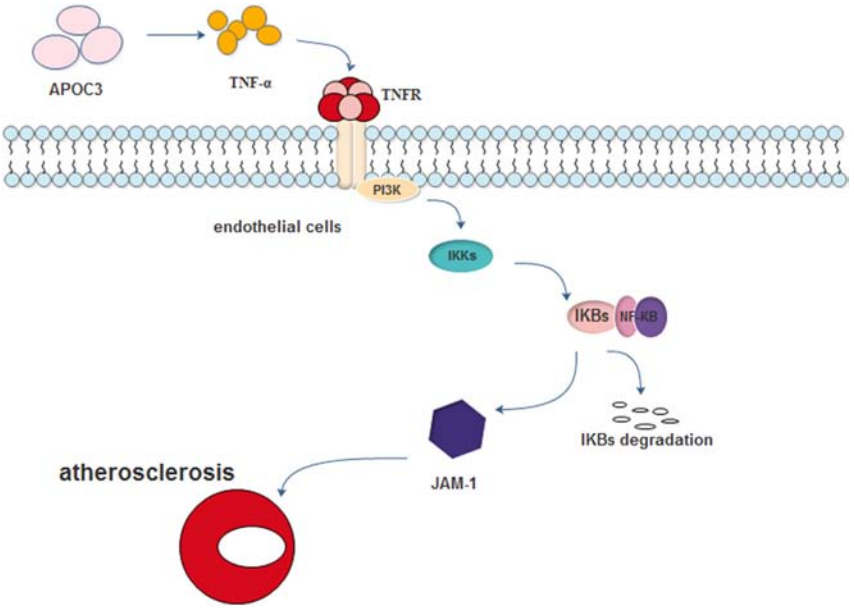

Fig. 5. The signal pathway of APOC3 involved in the expression of JAM-1 in HUVECs. The molecular mechanism involved in APOC3 regulating TNF-α to promote the expression of JAM-1 is via the PI3K-IKK2-p65 signaling pathway in HUVECs.

to demonstrate the downstream mediators of APOC3 pathway. We observed that, in the presence of inhibitors, TNF-α increased the transcription as well as protein expression in HUVEC treated with APOC3 as compared with inhibitors alone (Fig. 3A and B). TNF-α may partly reverse the inhibition of Wortmannin, SC514, and SN50 on JAM-1 expression by APOC3. Furthermore, the abundance of JAM-1 protein and mRNA was not increased when the same treatment was given to sh-TNF-α HUVECs. The results of PCR and Western blot of JAM-1 in sh-TNF-α HUVEC exposed to inhibitors also confirmed the contribution of TNF-α in the APOC3 signaling pathway (Fig. 3C and Fig. 4B). Flow cytometry showed that the level of JAM-1 protein was significantly decreased by the inhibitors after APOC3 treatment (Fig. 3D). As shown in Fig. 4A, APOC3 promoted the expression of proteins involved in PI3K-IKK2-p65 signaling pathway. When TNF-α inhibited, the proteins including JAM-1, PI3K, IKK2, and p65 were down-regulated (Fig. 4B). It illustrated that APOC3 increased the JAM-1 expression via TNF-α-induced PI3K-IKK2-p65 signaling pathway. It is well known that IKK2 and p65 participate in phosphorylation. To further confirm the phosphorylation part of IKK2 and p65, we detected the expression of p-IKK2 and p65 in Western blot. Data showed that p-IKK2 and p65 expression was significantly decreased after inhibition of TNF-α, PI3K, IKK2, and p65, respectively (Fig. 4A and B). In addition, expression of NF-κB p65 and degradation of IκB were attenuated in sh-TNF-α HUVEC compared with control in response to APOC3 (Fig. 4A and B). These results indicated that APOC3-induced JAM-1 is mediated by TNF-α through the PI3K-IKK2-p65 signaling pathway in HUVECs.

4. Discussion

In this study, we showed that APOC3 promoted the expression of JAM-1 via TNF-α in the PI3K-IKK2-p65 signaling pathway. The stimulatory effect of APOC3 was attenuated by TNF-α-shRNA. Furthermore, modulating the PI3K-IKK2-p65 signaling pathway demonstrated the mechanism of action of APOC3 in inflammation.

CVD is the leading cause of death worldwide, mainly caused by atherosclerosis, a chronic inflammatory disease [25]. Evidence supported the role of EC inflammation in the process of atherosclerosis [26,27]. Several studies have confirmed that inhibiting EC inflammation can reduce atherosclerosis in vitro and in vivo [28,29]. APOC3 is involved in lipid metabolism and implicated in atherosclerotic inflammatory responses [30,31]. Our data showed that APOC3 regulated TNF-α-induced JAM-1 through the PI3K-IKK2-p65 signaling pathway in HUVECs (Fig. 5). Our previous study had demonstrated that APOC3 induced the expression of TNF-α which triggered inflammation in HUVECs [21]. Kawakami et al. showed that APOC3 plays a direct role in inflammation of ECs and induces chemotaxis and adhesion of monocytes to ECs [12,32], which is an early event in atherosclerosis that allows monocyte infiltration into the intima of blood vessels. In vitro experiments show that APOC3 promotes expression of toll-like receptor which can also participate in the adhesion of monocytes to endothelial cells [33]. However, it is not clear if APOC3 alters the tight junctions of HUVECs. In this study, we explored the APOC3 regulation of JAM-1, a tight junction molecule in ECs.

It has reported that TNF-α plays a vital role in EC dysfunction and has a higher plasma concentration in patients with hyperlipidemia and coronary heart disease [34,35]. TNF-α is closely related to lipid levels in humans. And it is positively correlated with VLDL concentrations and negatively correlated with HDL-C. EC dysfunction is characterized by a decrease in the biological activity of NO as the activation of eNOS is important for the maintenance of diastolic functions. While TNF-α regulates the expression and activity of NOS, studies have also demonstrated that it can induce tight junction destruction and cause the EC dysfunction [36,37]. Our data showed that APOC3 promoted the expression of JAM-1 via TNF-α to cause inflammation. Furthermore, we supposed that APOC3 may also induce the EC dysfunction in the progress of atherosclerosis, which need more exploration.

Emerging evidence showed that the expression of the junction proteins is closely associated with atherosclerosis [20]. Cells are networked together with an adhesive structure called tight junctions [38]. Tight junctions can accept and divide signals from both intracellular and extracellular compartments. They serve as modulators of cellular activities such as cell growth and gene expression [39,40]. Tight junctions' expression is susceptible to inflammation and many oxidative stress conditions. The disruption of tight junctions alters endothelial permeability, allowing the migration of leukocyte and lipoprotein into the subendothelial layers, which accelerates atherosclerosis [41]. Our data also demonstrated that the expression of JAM-1 was increased when treated with APOC3 in HUVECs. Taken together, all data indicated that APOC3 induced the elevation of JAM-1 and promoted the progress of atherosclerosis. Thus, it can be a new target for the treatment of atherogenesis diseases.

In addition, it is well known that NF-κB signal pathway plays an essential role in atherosclerosis, which has become a new therapeutic treatment for atherosclerosis-associated diseases [42–44]. Activation of NF-κB regulates the expression of various genes that include those encoding tight junctions [45,46]. Tight junctions regulate the process of intercellular adhesion in the pathogenesis of atherosclerosis. Given the presence of the NF-κB binding site within its promoter, it is reasonable to speculate that APOC3 may regulate JAM-1 through the NF-κB signaling pathway. Thus, we observed that inhibiting NF-κB by SN50 blocked TNF-α-induced expression of JAM-1 with APOC3 stimulation. Moreover, the overexpression of JAM-1 by TNF-α was also suppressed by Wortmannin (PI3K inhibitor) and SC514 (IKK2 inhibitor), which indicated that the involvement of the PI3K-IKK2-p65 signaling pathway in APOC3 stimulated JAM-1 expression. Our results explore the pathway of APOC3 in atherogenesis and provide a new insight into atherogenesis diseases treatment.

In conclusion, we identify the hypothesis that APOC3 is a proinflammatory molecule that promotes the expression of tight junction molecules which is relevant to the process of atherosclerosis. These findings have shown that TNF-α induced the expression of JAM-1 in the presence of APOC3 through the PI3K-IKK2-p65 pathway. Silencing the PI3K-IKK2-p65 pathway can suppress the atherogenic effect of APOC3, thereby inhibiting JAM-1 expression and inflammatory reaction in the progress of atherosclerosis. The study broadens our understanding of APOC3 in atherosclerosis and can also serve as an indicator of atherosclerosis. Given the role of APOC3 in atherogenesis, it may be a potential target for the treatment of diseases associated with CVDs.

Acknowledgments

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Authors' contributions: Juan Yu and Shao-qing Ju designed the research; Zhong-hui Wang, Yun Tao, and Ye Ding generated the data; Hong-bing Ni and Xia Ding analyzed the data; Lu Dai and Shao-peng Chu drafted the manuscript. All authors contributed with the ideas and reviewed the manuscript with suggestions.

Conflict of interest

None of the authors have any financial conflicts of interest related to this manuscript.

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