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ApoM Suppresses TNF-α-Induced Expression of ICAM-1 and VCAM-1 Through Inhibiting the Activity of NF-κB

Ji-Juan Gao,[1,*] Yan-Wei Hu,[1,*] Yan-Chao Wang,[1] Yan-Hua Sha,[1] Xin Ma,[2] Shu-Fen Li,[1] Jia-Yi Zhao,[1] Jing-Bo Lu,[3] Chuan Huang,[1] Jing-Jing Zhao,[1] Lei Zheng,[1] and Qian Wang[1]

To explore the anti-inflammatory effect of apolipoprotein M (apoM) on regulation of tumor necrosis factor-α (TNF-α)-induced expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) and further investigate the molecular mechanism of apoM in this process. We found that TNF-α could decrease expression of apoM and inhibitor of NF-κB-α (IκBα) in HepG2 cells. Overexpression of apoM caused a significant decrease of ICAM-1 and VCAM-1 expression, while it caused a significant increase of IκBα expression in HepG2 cells. Furthermore, the treatment with TNF-α could increase ICAM-1 and VCAM-1 expression, decrease IκBα protein expression, and increase nuclear factor-κB (NF-κB) activity, and these effects were markedly enhanced by small interfering RNA (siRNA)-mediated silencing of apoM in HepG2 cells. Our findings demonstrated that apoM suppressed TNF-α-induced expression of ICAM-1 and VCAM-1 through inhibiting the activity of NF-κB.

Introduction

As one of the most common proteins of the lipocalin protein superfamily, apolipoprotein M (apoM) was first identified in 1999 (Xu *et al.*, 1999). In humans, the synthesis of apoM mainly occurs in liver and kidney proximal tubule cells (Hu *et al.*, 2010). As an essential component of high-density lipoproteins (HDLs), many studies have been performed to elucidate the biological function of apoM in lipid metabolism, diabetes, coronary artery diseases, and atherosclerosis (Axler *et al.*, 2007; Kardassis *et al.*, 2014; Borup *et al.*, 2015). Besides lipid metabolism, apoM is also closely linked with inflammation as a negative acute response protein. Feingold *et al.* (2008) reported that the messenger RNA (mRNA) levels of apoM in the liver were obviously decreased with the stimulation of lipopolysaccharide (LPS), zymosan, or turpentine administration, all of which could cause systemic inflammation.

ApoM was also reported as a negative acute response protein, which decreased during infection and inflammation such as acute bacterial infections or chronic HIV infection (Feingold *et al.*, 2008; Luo *et al.*, 2014). In addition, our recent research showed that the enhancing expression of apoM could markedly mediate the anti-inflammatory effect of propofol in LPS-stimulated THP-1 macrophages (Ma *et al.*, 2013). By taking all of these results into consideration, it is a trend to suggest that the apoM might play an important role in inflammatory responses.

As an important sequence-specific transcription factor, the nuclear factor-κB (NF-κB) could bind to an intronic enhancer of the immunoglobulin κ-light chain gene in many types of cells (Yin *et al.*, 2015). The transcriptional activity of NF-κB is a key factor in regulating the expression of many genes (such as inducible nitric oxide synthase [iNOS] and interleukin-6 [IL-6]) and was involved in many typical inflammatory responses (such as immune and inflammatory reactions, smooth muscle cell proliferation, and angiogenesis) (Baeuerle and Henkel, 1994; De Martin *et al.*, 2000; Baldwin, 2001). NF-κB-mediated chronic vascular inflammation was also mentioned as a critical participant in the initiation and progression of atherosclerosis and other inflammations (Sun *et al.*, 2014). In the typical NF-κB signaling pathway, NF-κB existed in the inactive form in the cytoplasm, which was bound to an inhibitor known as inhibitor of NF-κB-α (IκBα) (Senol Tuncay *et al.*, 2010). The IκBα could transport activated NF-κB from the nucleus to the cytoplasm, followed by releasing NF-κB into the nucleus through the phosphorylation or degradation of IκBα protein, and then cause inflammatory responses (Brown *et al.*, 1995; Prasad *et al.*, 2010). Tumor necrosis factor-α (TNF-α) is the important proinflammatory cytokine and could trigger a strong vascular inflammatory response activation, which was

[1]Laboratory Medicine Center, Nanfang Hospital, Southern Medical University, Guangzhou, China.
Departments of [2]Anesthesiology and [3]Vascular Surgery, Nanfang Hospital, Southern Medical University, Guangzhou, China.
*These two authors contributed equally to this work.

believed to be the eventual mechanism involved in the development of atherosclerosis (Jezovnik and Poredos, 2010; Young *et al.*, 2012; Jia *et al.*, 2015).

As one kind of cell adhesion molecules, intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) were highly expressed in human endothelial cells and could be markedly induced by the TNF-α in inflammation (Osborn *et al.*, 1989; Lee *et al.*, 2015; Paulsen *et al.*, 2015). Accumulating evidence suggested that the increasing levels of ICAM-1 and VCAM-1 could contribute to the recruitment of inflammatory cells at the sites of inflammation and cell-mediated immune responses (Chen *et al.*, 1995). Moreover, many investigations revealed that the activation of NF-κB was one of the main pathways that induced transcriptional regulation of TNF-α-induced adhesion molecules (Mukherjee *et al.*, 2003; Sun *et al.*, 2014). However, the *in vivo* molecular mechanism of NF-κB in mediating the TNF-α-induced inflammatory responses was not fully clear.

Hence, we accordingly hypothesized that apoM has a suppressive effect on the regulation of TNF-α-induced ICAM-1 and VCAM-1 expression. In this research, the levels of apoM, IκBα, ICAM-1, and VCAM-1 in HepG2 cells influenced by the treatment of TNF-α were measured by western blot analysis and real-time quantitative polymerase chain reaction (RT-qPCR) technology. The effects of apoM on the expression of IκBα, ICAM-1, and VCAM-1 in HepG2 cells were also tested. Finally, these effects were observed once again by small interfering RNA (siRNA)-mediated silencing of apoM in HepG2 cells. In addition, the activity of NF-κB affected by siRNA-mediated silencing of apoM was detected by luciferase assay. A new insight into the anti-inflammatory effects of apoM was provided.

Materials and Methods

Materials

TNF-α was purchased from Sigma-Aldrich (St. Louis, MO). The PrimeScript RT Reagent Kit (perfect real-time; catalog No. DRR037A) and the SYBR Premix Ex Taq™II Kit (Tli RNaseH Plus; catalog No. DRR820A) were obtained from TaKaRa Bio, Inc., (Shiga, Japan). All other chemicals were of pharmaceutical grade and purchased from commercial suppliers.

Cell culture

Human hepatocytes (HepG2) were purchased from the American Type Culture Collection (Manassas, VA). The HepG2 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 1% penicillin/streptomycin. Cells were incubated at 37°C in an atmosphere of 5% CO_2. Cells were seeded in 6- or 12-well plates or 60-mm dishes and grown to 60–80% confluence before use.

RNA isolation and RT-PCR analysis

Total RNA from cultured cells was extracted using TRIzol reagent (Invitrogen Corporation, Carlsbad, CA) in accordance with the manufacturer's instructions. RT-PCR, using SYBR Green detection chemistry, was performed on an ABI 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Melt curve analyses of all RT-PCR products were performed and found to produce a single DNA duplex. All samples were measured in triplicate, and the mean value was considered for comparative analysis. Quantitative measurements were determined using the ΔΔCt method, and glyceraldehyde-3-phosphate dehydrogenase expression was used as the internal control.

Western blot analysis

Protein samples were extracted from cultured cells using the radioimmunoprecipitation assay buffer (Biocolor Ltd., Belfast, Northern Ireland, United Kingdom), quantified using the BCA Protein Assay Kit (KeyGen Biotechnologies, Nanjing, China), and then subjected to western blot analysis (10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis; 30 μg protein per lane) using rabbit polyclonal anti-apoM antibodies, rabbit polyclonal anti-IκBα antibodies, and rabbit polyclonal β-actin-specific antibodies (Abcam, Cambridge, MA). The proteins were visualized using a chemiluminescence method (ECL Plus Western Blot Detection System; Amersham Biosciences, Foster City, CA).

Transfection with siRNA

The siRNAs against apoM and an irrelevant 21-nucleotide control siRNA (Negative Control) were purchased from Ribo Biotechnology (San Diego, CA). Cells (2×10^6/well) were transfected using Lipofectamine 2000 transfection reagent for 48 h according to the manufacturer's instructions. After 48 h of transfection, RT-PCR and western blot were performed.

Lentivirus production and infection

HepG2 cells were cultured in 25-cm^2 vented flasks containing Dulbecco's modified Eagle's medium with 10% fetal calf serum under standard culture conditions (5% CO_2, 37°C). Packed empty lentivirus (LV) vectors with green fluorescent protein (GFP; LV-mock) and LV-mediated human apoM overexpression vector (LV-apoM) with GFP were prepared as in a previous report. The cells were infected with the LV stock at a multiplicity of infection of 20 transducing units per cell in the presence of 8 mg/mL of polybrene. Then, cells were washed with fresh complete media after 24 h of incubation. The GFP-positive cells were counted 96 h post-transduction.

Luciferase assay

ApoM siRNA-treated HepG2 cells were transfected with NF-κB reporter and pMIR-REPORT b-gal vector (Ambion, Austin, TX) using Lipofectamine 2000 transfection reagent. The pMIR-REPORT™ beta-galactosidase control vector (Ambion) was used as a reference. Forty-eight hours after transfection, cells were stimulated with TNF-α (10 ng/mL), and luciferase activity was measured after another 5 h. NF-κB activity was used for normalization and measured by the Beta-Glo Luminescent Assay Kit (Promega, Madison, WI).

Statistical analysis

Data are expressed as means ± standard deviations. Results were obtained by one-way analysis of variance, followed by

the Student–Newman–Keuls test and Student's *t*-test using SPSS v13.0 statistical software (SPSS, Inc., Chicago, IL). A two-tailed probability p-value < 0.05 was considered statistically significant.

Results

TNF-α downregulates apoM and IκBα expression and upregulates ICAM-1 and VCAM-1 expression in HepG2 cells

TNF-α is a key proinflammatory cytokine responsible for the expression of ICAM-1 and VCAM-1, which could cause the phosphorylation and degradation of IκBα protein (Palombella *et al.*, 1994; Brown *et al.*, 1995). As stimuli of systemic inflammation, LPS, zymosan, and turpentine could markedly decrease apoM mRNA levels (Feingold *et al.*, 2008). We first explored the possible changes of apoM, IκBα, VCAM-1, and ICAM-1 expression caused by TNF-α treatment in HepG2 cells by RT-PCR and western blot analysis. As shown (Fig. 1A, B), the levels of apoM mRNA and its corresponding protein were obviously decreased by TNF-α treatment in HepG2 cells, while the levels of ICAM-1 and VCAM-1 mRNA and their corresponding proteins were increased; the protein levels of IκBα were also markedly decreased. These results further verified the proinflammatory effect of TNF-α in HepG2 cells. Moreover, an antagonism between apoM with TNF-α was also observed.

Effects of apoM on IκBα, ICAM-1, and VCAM-1 expression in HepG2 cells

It has been widely reported that the apoM was an important participant in the lipid metabolism (Axler *et al.*, 2007; Borup *et al.*, 2015). However, the role of apoM in regulating inflammatory cytokine expression in HepG2 cells was still unclear. To fill this gap, LV-apoM was infected into HepG2 cells, followed by investigating the changes of IκBα, ICAM-1, and VCAM-1 expression in the cells. With the treatment of LV-apoM, the protein levels of apoM in the HepG2 cells were increased about 9.9-fold compared with the control group (Fig. 2). As shown in Figure 2, the protein levels of ICAM-1 and VCAM-1 were significantly decreased by 57.8% and 52.8%, respectively, with the treatment of LV-apoM, which indicated that apoM was involved in the inhibition of ICAM-1 and VCAM-1 expression in the HepG2 cells. It is interesting to observe that the protein levels of IκBα were also enhanced by the treatment of LV-apoM, which demonstrated that the IκBα protein could be a mediator in the apoM involved inhibition of ICAM-1 and VCAM-1 expression in the HepG2 cells.

ApoM is involved in TNF-α-induced upregulation of ICAM-1 and VCAM-1 expression through inhibiting the activity of NF-κB in HepG2 cells

To further investigate whether apoM was involved in TNF-α-induced inflammatory processes, we first performed transfection of siRNA against apoM into HepG2 cells. As shown (Fig. 3A), the expression of apoM protein was markedly decreased by apoM siRNA treatment in HepG2 cells compared with the control siRNA group. Then, we examined the effects of apoM and TNF-α on the expression of IκBα, ICAM-1, and VCAM-1 in HepG2 cells by western blot analysis, respectively. As shown in Figure 3B, the treatment with TNF-α increased ICAM-1 and VCAM-1 protein expression and these effects were markedly enhanced by siRNA-mediated silencing of apoM in HepG2 cells. With the treatment of TNF-α, the protein levels of ICAM-1 and VCAM-1 were dramatically

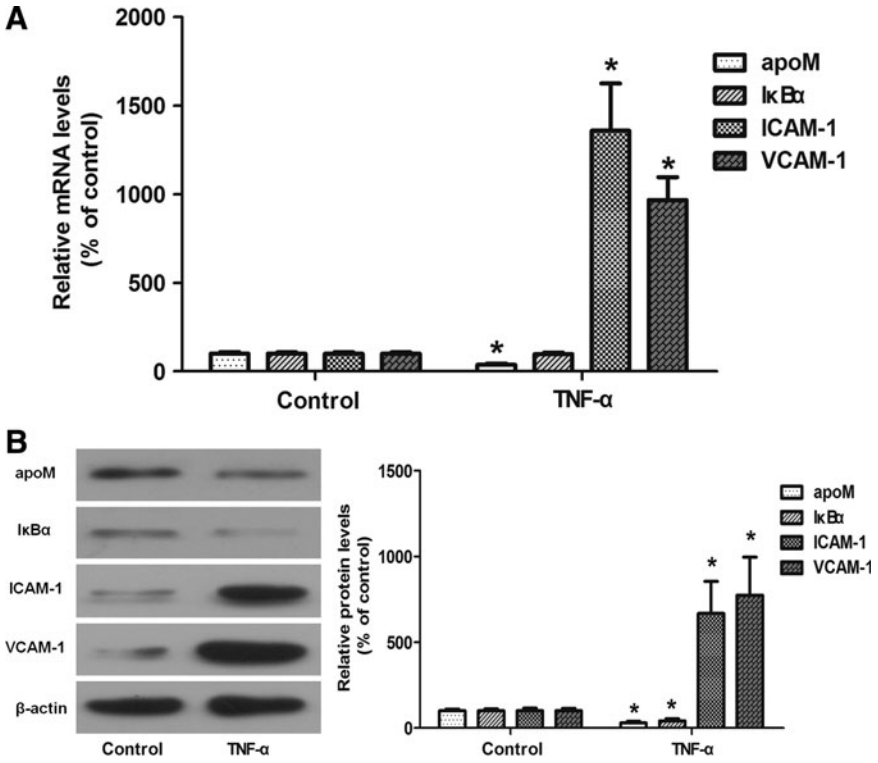

FIG. 1. Effects of tumor necrosis factor-α (TNF-α) on apolipoprotein M (apoM), inhibitor of NF-κB-α (IκBα), intercellular adhesion molecule-1 (ICAM-1), and vascular cell adhesion molecule-1 (VCAM-1) expression in HepG2 cells. HepG2 cells were first treated with 0 or 10 ng/mL TNF-α, respectively, and then apoM, IκBα, ICAM-1, and VCAM-1 messenger RNA (mRNA) levels **(A)** and protein levels **(B)** were measured by real-time quantitative polymerase chain reaction and western blot analysis, respectively. All results are presented as the mean ± standard deviation (SD) of three independent experiments, each performed in triplicate. $*p < 0.05$, versus the control group.

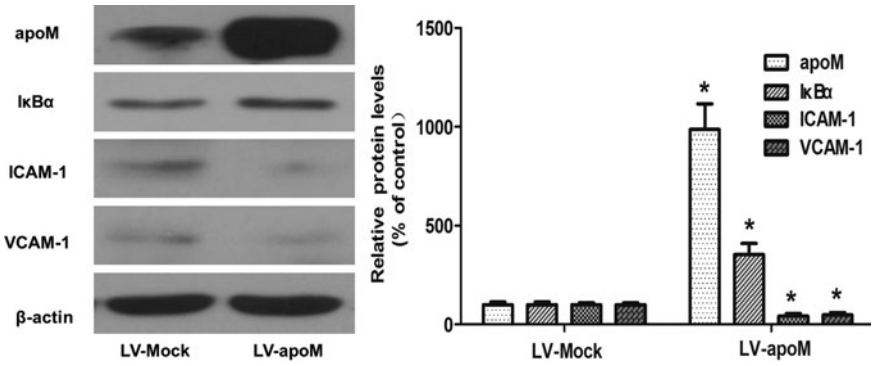

FIG. 2. Effects of apoM on IκBα, ICAM-1, and VCAM-1 expression in HepG2 cells. HepG2 cells were transfected with lentivirus (LV)-mock (LV-mock group) or LV-apoM (LV-apoM group), and then IκBα, ICAM-1, and VCAM-1 protein levels were measured by western blot analysis. All results are presented as mean ± SD of three independent experiments, each performed in triplicate. $*p < 0.05$, versus the LV-mock group.

increased by 200.8% and 190.9%, respectively, in the presence of apoM siRNA. However, without the treatment of TNF-α, the protein levels of ICAM-1 and VCAM-1 were only increased by 93.3% and 110.6%, respectively, in the presence of apoM siRNA, which indicated that the apoM could be involved in TNF-α-induced upregulation of ICAM-1 and VCAM-1 expression in the HepG2 cells.

We observed that the IκBα, which transported activated NF-κB from the nucleus to the cytoplasm (Brown *et al.*, 1995; Prasad *et al.*, 2010; Senol Tuncay *et al.*, 2010), was obviously decreased by TNF-α and this effect was also dramatically enhanced by additional siRNA-mediated silencing of apoM in HepG2 cells (Fig. 3B). So, we next investigated whether apoM suppressed the expression of ICAM-1 and VCAM-1 through inhibiting the activity of NF-κB in HepG2 cells by luciferase assay. As shown (Fig. 3C), the activity of NF-κB was obviously increased with the treatment of TNF-α or apoM siRNA in HepG2 cells. With the treatment of TNF-α, the activity of NF-κB could increase by an additional 475.4% in the presence of apoM siRNA in HepG2 cells. In comparison, the treatment of TNF-α in HepG2 cells could only increase by about 160% of the activity of NF-κB. These results demonstrated that apoM was involved in TNF-α-induced upregulation of

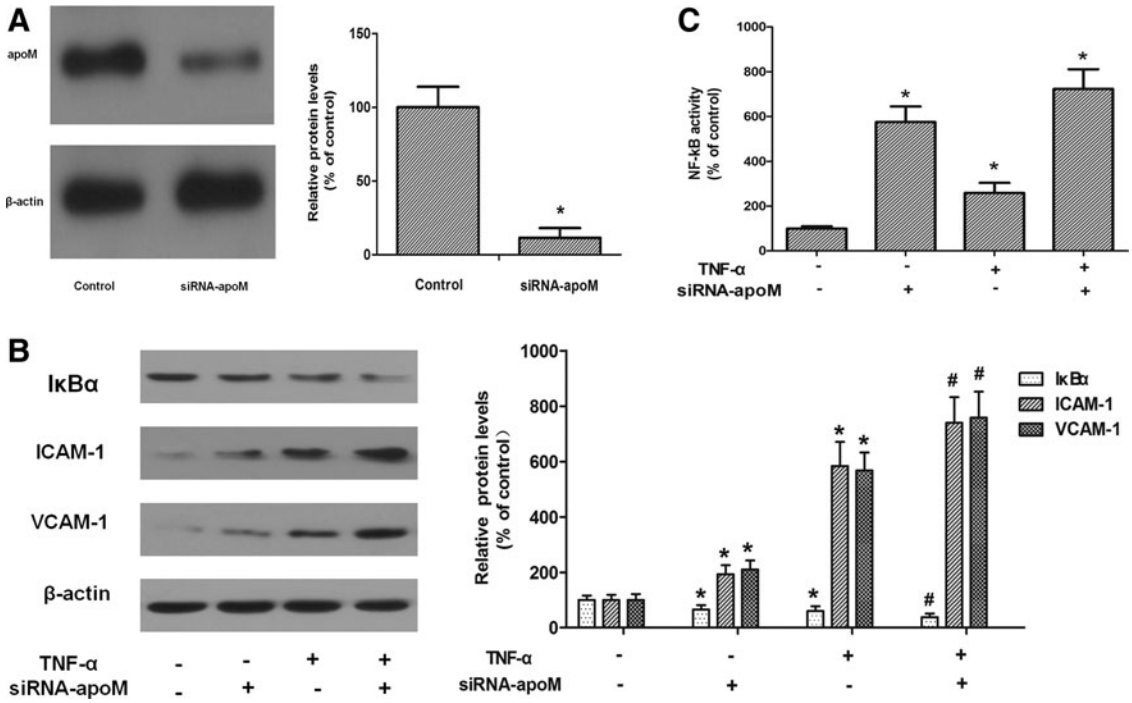

FIG. 3. ApoM is involved in TNF-α-induced upregulation of ICAM-1 and VCAM-1 expression through inhibiting the activity of nuclear factor-κB (NF-κB) in HepG2 cells. **(A)** HepG2 cells were transfected with a negative control or apoM small interfering RNA (siRNA). Then, protein levels were measured by western blot analysis. **(B, C)** HepG2 cells were transfected with a negative control or apoM siRNA, and then incubated with or without 10 ng/mL of TNF-α for 24 h. HepG2 cells were divided into four groups: (1) siRNA-NC + 0 ng/mL TNF-α (control), (2) siRNA-apoM + 0 ng/mL TNF-α (siRNA-apoM), (3) siRNA-NC + 10 ng/mL TNF-α (TNF-α), and (4) siRNA-apoM + 10 ng/mL TNF-α (siRNA-apoM + TNF-α). Then, IκBα, ICAM-1, and VCAM-1 protein levels were measured by western blot analysis, respectively. NF-κB activity was measured by luciferase assay. All results are presented as the mean ± SD of three independent experiments, each performed in triplicate. $*p < 0.05$, versus the control group, $^{\#}p < 0.05$, versus the TNF-α group.

ICAM-1 and VCAM-1 expression mainly through inhibiting the activity of NF-κB in HepG2 cells.

Discussion

The anti-inflammatory effect of apoM has been brought to attention for many years. Luo *et al.* (2004) demonstrated that the human apoM gene was located in p21.31 on chromosome 6, a region in which many genes were linked to immune and inflammatory responses. It is widely recognized that the inflammation could increase the risk of developing cardiovascular diseases such as atherosclerosis (Lee *et al.*, 2014). As one kind of apoprotein, apoM is closely associated with the mechanism of HDL-mediated antiatherosclerosis (Xu *et al.*, 1999; Hu *et al.*, 2010; Kardassis *et al.*, 2014). Besides, apoM probably has a series of functions in the aspect of preventing atherosclerosis, such as anti-inflammatory, antioxidative, and endothelium protection (Huang *et al.*, 2007; Elsøe *et al.*, 2012). The levels of apoM protein were decreased in the plasma during chronic infections or inflammation (Luo *et al.*, 2014). Furthermore, our previous studies have also demonstrated that the anti-inflammatory effects of propofol were mediated by apoM in cardiovascular diseases (Ma *et al.*, 2013). ICAM-1 and VCAM-1 were one kind of important proinflammatory molecules and could be markedly induced by the TNF-α in inflammation (Osborn *et al.*, 1989; Chen *et al.*, 1995; Lee *et al.*, 2015; Paulsen *et al.*, 2015). In this study, our findings showed that the apoM could significantly downregulate TNF-α-induced expression of VCAM-1 and ICAM-1 through inhibiting the activity of NF-κB in HepG2 cells.

ApoM is a novel apolipoprotein that is associated with anti-inflammatory effects (Feingold *et al.*, 2008; Luo *et al.*, 2014; Yamashita *et al.*, 2015). Mounting evidence suggested that TNF-α could decrease the concentration of HDLs in blood samples (Sherman *et al.*, 1988; Spriggs *et al.*, 1988; Bruunsgaard *et al.*, 2000). In addition, other researchers observed that the levels of HDLs were markedly increased by the treatment of anti-TNF-α drugs for rheumatoid arthritis (Seriolo *et al.*, 2006, 2009). As an essential component of HDL, the expression of apoM was also influenced by inflammatory agents such as TNF-α. Feingold *et al.* (2008) demonstrated that the administration of LPS, zymosan, or turpentine to mice could result in a remarkable decrease of apoM mRNA levels in the liver. These investigators also showed that the treatment of TNF-α, IL-6, or IL-1β in Hep3B cells could decrease both apoM mRNA levels and the secretion of apoM in the medium. In this study, we found that both apoM protein and mRNA levels were significantly decreased by TNF-α treatment in HepG2 cells.

Our group has reported that the anti-inflammatory effects of propofol were markedly mediated by apoM treatment in an HNF-1α-dependent manner (Ma *et al.*, 2013). Furthermore, our previous studies also reported that propofol could inhibit monocyte chemotactic protein-1 (MCP-1) expression, and this anti-inflammatory effect could be mediated by enhancing apoM expression (Ma *et al.*, 2015). TNF-α is a key mediator of inflammatory responses that could upregulate the expression of VCAM-1 and ICAM-1 (Pina-Canseco Mdel *et al.*, 2012). In this study, we demonstrated that the ICAM-1 and VCAM-1 protein levels were all increased by TNF-α in HepG2 cells. Furthermore, our findings showed that the apoM obviously decreased both ICAM-1 and VCAM-1 mRNA and protein expression in HepG2 cells. To further validate the molecular mechanism of apoM in anti-inflammatory processes, we performed transfection of siRNA against apoM into HepG2 cells. We found that the upregulation of ICAM-1 and VCAM-1 protein expression by TNF-α was significantly enhanced by siRNA-mediated silencing of apoM in HepG2 cells. These results suggested that apoM was involved in inhibition of TNF-α-induced upregulation of ICAM-1 and VCAM-1 expression.

NF-κB, as a crucial transcriptional factor, could be triggered by various inflammatory stimuli (Zhang *et al.*, 2005). A series of inflammatory and innate immunity processes were mainly mediated by NF-κB (Pahl, 1999; Karin and Greten, 2005). NF-κB was involved in controlling the transcription of more than 150 target genes, all of which could play an important role in multiple mechanisms (Hayden and Ghosh, 2014). Besides, the mechanism of NF-κB activation is closely associated with the phosphorylation and subsequent degradation of IκBα (Palombella *et al.*, 1994; Chen *et al.*, 1996; Hayden and Ghosh, 2008). In this study, we found that the LV-mediated overexpression of

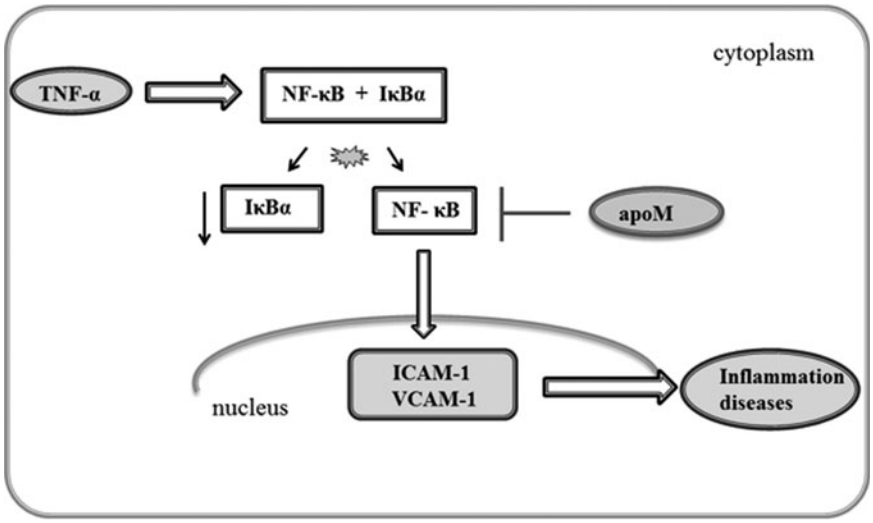

FIG. 4. Schematic illustrating the mechanism by which apoM suppresses TNF-α-induced expression of ICAM-1 and VCAM-1 through inhibiting the activity of NF-κB.

apoM in HepG2 cells resulted in an increasing effect of IκBα protein expression.

It is reported that the stimulation of TNF-α is responsible for the phosphorylation and degradation of IκBα proteins (Beg *et al.*, 1993). Our research further revealed that the treatment with TNF-α decreased IκBα protein expression and this effect was markedly enhanced by siRNA-mediated silencing of apoM in HepG2 cells. Based on this result, the effect of apoM on downregulation of NF-κB activity in HepG2 cells was investigated. In this study, we found that the treatment with TNF-α increased the activity of NF-κB and these effects were markedly enhanced by siRNA-mediated silencing of apoM in HepG2 cells. Together, these results suggested that apoM suppressed TNF-α-induced expression of VCAM-1 and ICAM-1 through inhibiting the activity of NF-κB (Fig. 4).

Conclusion

In summary, this study provides evidence that apoM suppresses TNF-α-induced expression of ICAM-1 and VCAM-1 through inhibiting the activity of NF-κB in HepG2 cells. A possible explanation of the molecular mechanism of apoM in inhibition of inflammatory cytokine expression was provided. Our study also suggests that the apoM might serve as a therapeutic target in treating inflammatory diseases such as atherosclerosis and related cardiovascular disorders.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Lei Zheng, PhD
Laboratory Medicine Center
Nanfang Hospital
Southern Medical University
Guangzhou 510515
China

E-mail: nfyyzl@163.com

Qian Wang, PhD
Laboratory Medicine Center
Nanfang Hospital
Southern Medical University
Guangzhou 510515
China

E-mail: nfyywangqian@163.com

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