Midkine Inhibits Cholesterol Efflux by Decreasing ATP-Binding Membrane Cassette Transport Protein A1 via Adenosine Monophosphate-Activated Protein Kinase/Mammalian Target of Rapamycin Signaling in Macrophages

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**Background:** Midkine (MK), a heparin-binding protein, participates in multiple cellular processes, such as immunity, cellular growth and apoptosis. Overwhelming evidence indicates that MK plays an important role in various pathological processes, including chronic inflammation, autoimmunity, cancer, and infection. Recent studies demonstrated that MK may be involved in the development of atherosclerosis, yet the mechanism has not been fully explored. Therefore, this study aims to investigate the effect and mechanism of MK on macrophage cholesterol efflux.

**Methods and Results:** Using Oil Red O staining, NBD-cholesterol fluorescence labeling and enzymatic methods, it was observed that MK markedly promoted macrophage lipid accumulation. Liquid scintillation counting (LSC) showed that MK decreased cholesterol efflux. Moreover, cell immunofluorescence, western blotting and quantitative real-time polymerase chain reaction (qRT-PCR) showed that MK downregulated ATP-binding membrane cassette transport protein A1 (ABCA1) expression. Functional promotion of ABCA1 expression attenuated the inhibitory effects of MK on cholesterol efflux, which reduced lipid accumulation. Additionally, intervention of adenosine monophosphate activated protein (AMPK)-mammalian target of rapamycin (mTOR) signaling molecule by the AMPK activator, AICAR, increased p-AMPK and ABCA1 expression, decreased p-mTOR expression and promoted cholesterol efflux, resulting in an obvious reduction in intracellular lipid content.

**Conclusions:** These data suggest that MK reduces the expression of ABCA1, inhibits the efflux of cholesterol and promotes the accumulation of lipids in RAW264.7 macrophages, and AMPK-mTOR signaling is involved in MK-mediated regulation of cholesterol metabolism in RAW264.7 macrophages.

**Key Words:** ABCA1; AMPK-mTOR signaling molecule; Atherosclerosis; Cholesterol efflux; Midkine

Atherosclerosis is the main pathological feature of cardiovascular and cerebrovascular diseases. As such, it has become one of the major diseases leading to increased mortality worldwide. Emerging evidence suggests that dyslipidemia is a common metabolic abnormality in atherosclerosis. Importantly, macrophage phagocytosis of excess atherosclerotic lipids, differentiation into foam cells and aggregation in the arterial intima are key steps in the development of atherosclerosis. Notably, the promotion of macrophage cholesterol efflux is considered to be the key to maintaining the balance of cholesterol metabolism and effectively preventing the development of atherosclerosis.

MK, a potent growth factor, mainly regulates the survival of neurons in the mid-gestation period of embryogenesis. However, the expression of MK is strictly limited in adults. MK expression is increased under acute or chronic pathological conditions, such as chronic heart failure and cardiac ischemia/reperfusion injury. Previous studies have found that MK affects the development of inflammatory diseases.
such as rheumatoid arthritis, chronic kidney disease (CKD), and autoimmune encephalitis. In recent decades, accumulating evidence has shown that MK plays an important regulatory role in the development of cardiovascular disease (CVD), and it can be used as a biomarker to predict the presence of coronary artery disease. Specifically, MK has a potential protective effect in ischemia/reperfusion injury by decreasing infarct sizes, improving myocardial cell survival and function, preventing ventricular remodeling, and mediating neovascularization, suggesting that MK may be a novel therapeutic target for CVD. Ezquerra et al. found that MK regulated blood pressure via the renin-angiotensin system (RAS), and MK deficiency in the mouse aorta significantly increased the levels of renin, angiotensinogen, and angiotensin-converting enzyme. However, MK is not exclusively beneficial. It has been reported that MK also causes neointima formation, inflammation, and even tumors. In addition, the levels of MK are significantly increased in severe peripheral artery disease patients, which may be a possible novel predictor of advanced atherosclerotic processes. Recent studies have found that MK promotes vascular stenosis and atherosclerosis development by enhancing macrophage and smooth muscle cell (SMC) migration and endothelial cell (EC) proliferation and promoting plaque infiltration of inflammatory cells. However, the recruitment of inflammatory cells and vascular stenosis were almost completely eliminated in MK-deficient mice. Despite these considerations, these findings are not sufficient to fully elucidate the role of MK in the development of atherosclerosis, such as lipid deposition in blood vessel walls.

The formation of foam cells in atherosclerotic plaques is multifactorial and may be due to a decrease in cholesterol efflux and an increase in modified low-density lipoprotein (LDL) uptake. The cholesterol efflux from macrophages is a complex and dynamic process that is mainly controlled by ABCA1. Evidence indicates that ABCA1 mediates phospholipids and free cholesterol (FC) efflux to lipid-poor apolipoproteins, mainly apoA-I, which is essential for the formation of high-density lipoprotein (HDL). The data show that knockdown of ABCA1 exacerbates the development of atherosclerotic lesions. However, increasing the expression of ABCA1 promotes the efflux of cholesterol and prevents the development of atherosclerosis. Whether MK affects the occurrence of atherosclerosis by regulating the expression of ABCA1 and cholesterol efflux has not been reported.

Mammalian mechanistic target of rapamycin (mTOR), a relatively conserved serine/threonine protein kinase, is a key regulator of the cell cycle, growth, and proliferation. Indeed, mTOR is also a central sensor for energy metabolism regulation by responding to growth factors, insulin, amino acids, and glucose. Therefore, mTOR participates in the regulation of anabolic and catabolic processes. Adenosine monophosphate-activated protein kinase (AMPK) also plays a central role in regulating nutritional metabolism and maintaining energy balance. Increasing evidence suggests that improving the activity of AMPK protects normal cardiovascular function by reducing lipid accumulation in macrophages and enhancing atherosclerotic plaque stability. In addition to regulating energy homeostasis, AMPK exerts a protective effect in atherosclerosis by inhibiting inflammation, endoplasmic reticulum stress, and SMC proliferation. In addition, the interaction between AMPK and mTOR is involved in many physiological and pathological processes, such as aging, cancer, CVD and diabetes. Moreover, various inflammatory factors, such as tumor necrosis factor α (TNF-α), induced lipid accumulation in hepatoma cells that was closely related to inhibition of the AMPK-mTOR signaling pathway, while activating the AMPK-mTOR signaling pathway attenuated the accumulation of lipids in hepatoma cells, indicating that the AMPK-mTOR signaling pathway participates in the regulation of lipid metabolism.

However, little is known about the influence of MK on the expression of ABCA1 and cholesterol efflux in macrophages. In the present study, we first investigated the molecular mechanisms on how MK might be involved in regulating cholesterol homeostasis and ABCA1 expression in RAW264.7 macrophages. We found that MK decreased ABCA1 expression, inhibited cholesterol efflux and increased lipid accumulation in macrophages, and AMPK-mTOR signaling molecules participated in MK-mediated regulation of cholesterol metabolism in RAW264.7 macrophages.

**Methods**

**Cell Lines and Reagents**

RAW264.7 macrophages were purchased from the Chinese Academy of Sciences (Shanghai, China). RPMI 1640 and RIPA lysis buffer were purchased from Solarbio Life Sciences (Beijing, China). Fetal bovine serum was purchased from ExCell Bio (Shanghai, China). MK was purchased from OriGene Technologies (Maryland, USA). Oxidized LDL (ox-LDL) and HDL were purchased from Yiyuan Biotechnologies (Guangzhou, China). Primary antibodies against ABCA1, AMPK, p-AMPK, mTOR, and p-mTOR were purchased from Cell Signaling Technology (Boston, USA). Goat anti-rabbit IgG (heavy chain+light chain) was purchased from Proteintech (Chicago, USA). Tissue total cholesterol and tissue-FC assay kits were purchased from Applygen Technologies Inc. (Beijing, China). Quantitative RT-PCR kits were purchased from Tiangen Biotech (Beijing, China). AICAR was purchased from Selleck Chemicals (Shanghai, China). NBD-cholesterol was purchased from Cayman (Ann Arbor, USA). 3H cholesterol was purchased from PerkinElmer (Massachusetts, USA). TRIzol and ECL-PLUS reagents were purchased from CWBIO (Beijing, China). ABCA1 and GAPDH primers were synthesized by Sangon Biotech (Shanghai, China). pcDNA3.1-ABCA1 and pcDNA3.1-empty were purchased from the Public Protein/Plasmid Library (Jiangsu, China). Lipofectamine reagent 2000 was purchased from Invitrogen (California, USA). The other chemicals were of the best grade available from commercial sources.

**Cell Culture**

RAW264.7 macrophages were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum at 37°C and 5% CO2. The cells were lipid-loaded by incubating with 50 mg/L of ox-LDL for 48 h before the experiment.

**Oil Red O Staining**

The macrophages were fixed with 4% paraformaldehyde for 10 min after adding the treatment factors, washed twice with phosphate buffer saline (PBS) and stained with 0.5% Oil Red O for 20 min at room temperature. The cells were rinsed with water twice and stained in hematoxylin for 10 s. Thereafter, the macrophages were immersed in 0.5% hydrochloric acid alcohol for 15 s and rinsed with running water twice and stained in hematoxylin for 10 s.
ester (CE) was estimated by subtracting FC from total cholesterol. The data were normalized to cellular protein content.

**Cholesterol Efflux Assay**

Standard protocols for the cholesterol efflux assay were described previously. Briefly, the cells were labeled with [3H]-cholesterol (0.2 µCi/mL) for the cellular cholesterol efflux experiments. The cells were washed twice with serum-free RPMI 1640 and then treated with MK. After washing, the cells were incubated in medium containing 0.2% bovine serum albumin and apoA-I (10 µg/mL) for 6 h. Radioactivity in the cells and medium was then measured by liquid scintillation counting. Cholesterol efflux was calculated by using the following equation: (total media counts/(total cellular counts+total media counts))×100.

**Cell Transfection**

RAW 264.7 cells at 50–70% confluency were transfected with pcDNA3.1-ABCA1 or pcDNA3.1-empty using Lipofectamine™ 2000 according to the manufacturer’s instructions. The efficiency of gene transfection was evaluated by qRT-PCR.

**Western Blotting**

Cellular proteins were lysed in radio immunoprecipitation assay (RIPA) buffer. The proteins (20 µg protein/well) were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (6% or 10% gels) and then transferred to polyvinylidene fluoride membranes. After blocking in tris buffered saline tween (TBST) containing.

**Expression of ABCA1 by Immunofluorescence**

The treated macrophages were washed with PBS, fixed with 4% paraformaldehyde at room temperature for 10 min, washed with PBS twice and blocked with normal goat serum for 30 min. Subsequently, the cells were stained with anti-ABCA1 antibody (1:2,500) overnight at 4°C. The cells were rewashed with PBS twice and incubated with Cy3-conjugated AffiniPure goat anti-mouse IgG secondary antibody for 30 min. Images were captured with a fluorescence microscope at 10×40 magnification.

**Intracellular Lipid Analysis in Macrophages**

The treated macrophages were washed with PBS twice, lipids were extracted from the cells by using lysis buffer, and then FC was measured using a tissue FC assay kit according to the manufacturer’s instructions. Total cholesterol (TC) was determined using a tissue total cholesterol assay kit according to the manufacturer’s instructions. Cholesterol

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**Figure 1.** Effects of midkine (MK) on lipid accumulation and cholesterol efflux in RAW264.7 macrophages. RAW264.7 macrophages were treated with MK (0, 50, 100, 200, 400 ng/mL) for 16 h or 200ng/mL MK for different times (0, 8, 16, 24, 48 h). (A) Representative oil red O staining in RAW264.7 macrophages under a microscope (10×40); (B) Representative fluorescence labeling of NBD-cholesterol in RAW264.7 macrophages by fluorescence microscopy (10×40); (C,D) Cholesterol efflux was expressed as the percentage of radioactivity in the medium relative to total radioactivity (*P<0.05 vs. control). Data are presented as the mean±SEM from 3 independent experiments.
We found that MK significantly promoted intracellular lipid accumulation as determined by Oil Red O staining. Fluorescent labeling of cholesterol showed that the intracellular fluorescence in the cell was obviously increased compared with that of the control group (Figure 1A,B). Furthermore, the increased intracellular lipid content was also evidenced by a commonly used enzymatic method. MK treatment significantly increased TC, FC and CE content in RAW264.7 macrophages in a concentration- and time-dependent manner (Table 1 and Table 2), and the ratio of CE/TC was not significantly different among these groups. We also examined the effect of MK on cholesterol efflux by using [3H]-cholesterol labeling. Treatment with MK resulted in macrophage cholesterol efflux impaired in a concentration- and time-dependent manner (Figure 1C,D). These data suggest that MK may have a role in atherosclerosis, at least in part, by inhibiting cholesterol efflux from macrophages.

MK Inhibits the Expression of ABCA1 in Macrophages

ABCA1 is a well-known transporter that regulates cholesterol efflux from macrophages. We therefore evaluated the mechanism by which MK enhances lipid accumulation by examining the expression of ABCA1 in macrophages. The data indicated that MK significantly reduced the fluorescence intensity of ABCA1 expression in macrophages compared with that of the control group, as determined by cell immunofluorescence (Figure 2A). Similar results were observed by western blotting and qRT-PCR, whereby MK reduced the expression of ABCA1 in a concentration-dependent and time-dependent manner (Figure 2B-E). These results confirmed that MK inhibited cholesterol efflux from macrophages by reducing ABCA1 expression. In addition, we observed that incubation with 200ng/mL MK for 16h exerted a maximal decrease in ABCA1 expression and inhibited cholesterol efflux. Hence, treatment with 200ng/mL MK for 16h was used in the following experiments.

Table 1. Effect of Midkine (MK) on Cellular Cholesterol Content at Different Concentrations in RAW264.7 Macrophages

<table>
<thead>
<tr>
<th>MK (ng/mL)</th>
<th>0</th>
<th>50</th>
<th>100</th>
<th>200</th>
<th>400</th>
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<tbody>
<tr>
<td>TC (nmol/mg protein)</td>
<td>388.87±18.94</td>
<td>405.93±21.34</td>
<td>427.95±17.56</td>
<td>454.17±19.36</td>
<td>470.13±20.83</td>
</tr>
<tr>
<td>FC (nmol/mg protein)</td>
<td>168.19±16.73</td>
<td>174.49±17.86</td>
<td>180.31±19.05</td>
<td>185.79±18.79</td>
<td>192.05±17.16</td>
</tr>
<tr>
<td>CE (nmol/mg protein)</td>
<td>220.87±15.71</td>
<td>231.44±17.84</td>
<td>247.64±16.64</td>
<td>268.38±14.71</td>
<td>278.08±17.73</td>
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<tr>
<td>CE/TC (%)</td>
<td>56.80</td>
<td>57.01</td>
<td>57.87</td>
<td>59.09</td>
<td>59.15</td>
</tr>
</tbody>
</table>

Data are presented as the mean±SEM from 3 independent experiments. The RAW264.7 macrophages were incubated with 0, 50, 100, 200 or 400ng/mL of MK for 16h, respectively. Enzymatic assays were performed to detect the cellular total cholesterol (TC), cholesterol ester (CE) and free cholesterol (FC). *P<0.05, **P<0.01 vs. Control.

Table 2. Effect of MK on Cellular Cholesterol Content at Different Times in RAW264.7 Macrophages

<table>
<thead>
<tr>
<th>Time (h)</th>
<th>0</th>
<th>8</th>
<th>16</th>
<th>24</th>
<th>48</th>
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</thead>
<tbody>
<tr>
<td>TC (nmol/mg protein)</td>
<td>392.17±20.87</td>
<td>417.18±20.06</td>
<td>447.03±18.95</td>
<td>467.73±18.15</td>
<td>475.92±19.63</td>
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<tr>
<td>FC (nmol/mg protein)</td>
<td>170.33±16.65</td>
<td>177.51±17.07</td>
<td>182.83±16.56</td>
<td>187.18±16.56</td>
<td>191.01±18.17</td>
</tr>
<tr>
<td>CE (nmol/mg protein)</td>
<td>221.84±15.71</td>
<td>239.67±15.13</td>
<td>265.20±15.18</td>
<td>280.55±14.79</td>
<td>284.91±18.42</td>
</tr>
<tr>
<td>CE/TC (%)</td>
<td>56.57</td>
<td>57.45</td>
<td>59.32</td>
<td>59.98</td>
<td>59.87</td>
</tr>
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</table>

Data are presented as the mean±SEM from 3 independent experiments. The RAW264.7 macrophages were incubated with 200 ng/mL of MK for 0, 8, 16, 24 and 48h, respectively. Enzymatic assays were performed to detect the cellular TC, CE and FC. *P<0.05, **P<0.01 vs. Control. Abbreviations as in Table 1.

5% fat-free milk for 2h, the membranes were incubated overnight at 4°C with primary antibodies (anti-ABCA1, anti-AMPK, anti-p-AMPK, anti-mTOR, anti-p-mTOR, and anti-β-actin). Then, the membrane was washed three times with TBST for 30 min. Subsequently, the membranes were further incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody. Immunoreactivity of the protein blots were visualized by using ECL-PLUS reagents. β-actin was used for normalization of protein expression.

RNA Isolation and Real-Time PCR Analysis
Total RNA was extracted from the cultured cells using TRIzol reagent according to the manufacturer’s protocol. RNA was reverse transcribed to cDNA templates using the FastQuant cDNA Kit according to the manufacturer’s instructions. The amplification was performed on a CFX96 system from Bio-Rad. The relative mRNA levels were calculated with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels as the invariant control.

Statistical Analysis
Data are presented as the means±SEM of at least three independent experiments. Data were analyzed by one-way ANOVA or Student’s t-test. Statistical analyses were conducted with SPSS 22.0. Statistical significance was considered to be P<0.05.

Results
MK Promotes Cholesterol Accumulation and Inhibits Cholesterol Efflux From Macrophages
To investigate the role of MK in the pathogenesis of atherosclerosis, we explored the potential effect of MK on cholesterol efflux from RAW264.7 macrophages. These cells were treated with MK (0, 50, 100, 200, 400ng/mL) for 16h and 200ng/mL MK for different times (0, 8, 16, 24, 48h). We found that MK significantly promoted intracellular
MK inhibits cholesterol efflux by AMPK/mTOR/ABCA1

MK inhibits cholesterol efflux from macrophages. AMPK-mTOR signaling molecules are involved in the regulation of biological energy metabolism. We treated the cells with AICAR for 4 h and subsequently incubated the cells with 200 ng/mL MK for an additional 16 h. We next investigated the effects of MK and the AMPK activator, AICAR, on the expression of AMPK, p-AMPK, mTOR, and p-mTOR. The results showed that MK reduced the levels of p-AMPK protein and increased the levels of p-mTOR protein in macrophages compared with that of the control group. However, administration of AICAR increased the protein levels of p-AMPK and decreased the protein levels of p-mTOR in macrophages compared with that of the control group. Combined treatment with AICAR and MK alleviated the inhibitory effect of MK on p-AMPK levels and the stimulatory effect on p-mTOR levels in macrophages compared with that of the MK-treated group (Figure 4), indicating that MK inhibits the activation of AMPK-mTOR signaling molecules.

Figure 2. Effects of midkine (MK) on the expression of ABCA1 in RAW264.7 macrophages. The cells were treated with MK (0, 50, 100, 200, 400 ng/mL) for 16 h or 200 ng/mL MK for different times (0, 8, 16, 24, 48 h). (A) The expression of ABCA1 was detected by cell immunofluorescence. (B,D) The expression of ABCA1 protein was measured by western blotting. (C,E) The expression of ABCA1 mRNA was measured by qRT-PCR. All data represent the mean±SEM from 3 independent experiments (*P<0.05 vs. the control group).
Figure 3. Effects of ABCA1 overexpression on lipid accumulation and cholesterol efflux from macrophages. RAW264.7 macrophages were transfected with pcDNA3.1-ABCA1 and/or incubated with 200 ng/mL midkine (MK) for 16 h. (A) The expression of ABCA1 was determined using qRT-PCR (*P<0.05 vs. control). (B) Representative oil red O staining in RAW264.7 macrophages under a microscope (10×40). (C) Representative fluorescence labeling of NBD-cholesterol in RAW264.7 macrophages by fluorescence microscopy (10×40); (D) Cholesterol efflux was expressed as the percentage of radioactivity in the medium relative to total radioactivity (*P<0.05 vs. control; #P<0.05 vs. MK-treated group). Data are presented as the mean±SEM from 3 independent experiments.

Figure 4. Midkine (MK) inhibits the activation of AMPK-mTOR signaling molecules. The cells were treated with the AMPK activator, AICAR, for 4 h followed by incubation with 200 ng/mL MK for an additional 16 h. The expression of AMPK, p-AMPK, mTOR and p-mTOR proteins was detected by western blotting (*P<0.05 vs. control; #P<0.05 vs. MK-treated group). Data are presented as the mean±SEM from 3 independent experiments.
MK Inhibits Cholesterol Efflux by AMPK/mTOR/ABCA1

Discussion

Atherosclerosis is characterized by disturbed cholesterol metabolism, inflammation, vascular injury and thrombosis. The recruitment of monocytes and the formation of macrophage-derived foam cells are characteristic pathological changes in atherosclerosis. This suggests that the major antiatherosclerotic therapy is to limit foam cell formation by promoting cholesterol metabolism. Here, we demonstrated a novel effect of MK and its underlying molecular mechanism in regulating cholesterol efflux from macrophages. We first showed that MK promotes lipid accumulation by inhibiting the expression of ABCA1 and

Figure 5. Effect of intervention of AMPK-mTOR signaling molecules in midkine (MK)-mediated cholesterol efflux and ABCA1 expression in RAW264.7 macrophages. The cells were treated with an AMPK activator, AICAR, for 4 h, after which they were treated with 200 ng/mL MK for an additional 16 h. (A) Representative oil red O staining in RAW264.7 macrophages under a microscope (10×40). (B) Representative fluorescence labeling of NBD-cholesterol in RAW264.7 macrophages by fluorescence microscopy (10×40). (C) Cholesterol efflux was expressed as the percentage of radioactivity in the medium relative to total radioactivity (*P<0.05 vs. control; #P<0.05 vs. MK-treated group). (D) The expression of ABCA1 was detected by cell immunofluorescence. (E,F) The expression of ABCA1 was measured by western blotting and qRT-PCR (*P<0.05 vs. control; #P<0.05 vs. MK-treated group). Data are presented as the mean±SEM from 3 independent experiments.

This conclusion was further strengthened by detecting changes in intracellular lipid levels and cholesterol efflux (Figure 5C). Additionally, we examined the expression of ABCA1 by cell immunofluorescence, western blotting and qRT-PCR (Figure 5D–F). As expected, the expression of ABCA1 was upregulated by AICAR treatment compared with that of the control group, and combined treatment of AICAR and MK alleviated the inhibition of MK on the expression of ABCA1 in RAW264.7 macrophages to a certain extent. These data showed that AMPK-mTOR signaling molecules are required for MK to regulate ABCA1 expression and cholesterol efflux in macrophages.
MK is a low molecular-weight protein with multiple biological functions. MK is present in healthy arteries and veins, and levels may be elevated in patients. Previous studies have reported that MK protects the heart from acute ischemia/reperfusion injury, at least in part through its antiapoptotic effects. Weckbach et al found that MK is involved in angiogenesis and EC proliferation under ischemic conditions. Lautz et al also found that after ligation of the femoral artery in mice, exogenous MK restored perfusion recovery in MK−/− mice by promoting EC proliferation, and MK promoted vasodilation in wild-type mice. There is evidence that acute treatment with MK protects tissues from ischemia/reperfusion injury and reduces infarct size. These findings indicate that MK is of great importance in the acute treatment of tissue ischemia. However, the function of MK in organisms has not been clearly defined. Long-term treatment with intermediate factors may cause some adverse reactions, such as vascular stenosis and inflammation. Recent studies have shown that serum MK levels are closely related to the development of atherosclerosis-related ischemic CVD. Neovascularization is a risk factor for atherosclerosis. MK is essential for neointima hyperplasia. Narita et al showed that MK is mainly derived from infiltrating macrophages in the neointima of atherosclerotic vessels, and MK directly induces SMC migration from the media to the intima. Thus, MK may promote neointima formation by directly regulating SMC activities or indirectly affecting inflammatory cell recruitment. Moreover, the most potent trigger for (neo) angiogenesis in atherosclerotic plaque progression is hypoxia. Weckbach et al showed that hypoxia increased MK expression in human polymorphonuclear leucocytes (PMNs), monocytes and ECs, and led to hypoxia-induced angiogenesis in adult mice. In addition, Fan et al showed that MK expression is increased in the adipose tissue of obese mice. More importantly, serum MK levels were also significantly increased in overweight/obese humans, suggesting an association between MK and obesity. Interestingly, a recent study demonstrated that MK could be a clinical and biomarker score to predict the presence of coronary atherosclerosis in humans. Remarkably, a recent study suggested that MK promotes atherosclerotic plaque formation by promoting inflammation, angiogenesis, vascular smooth muscle cell (VSMC) migration and inhibition of macrophage apoptosis in ApoE−/− mice. These studies suggested that MK may have an unfavorable function in atherosclerosis development and is expected to be a promising potential therapeutic target for atherosclerosis-related CVD. Despite these findings, little is known about whether MK affects the development of atherosclerosis by regulating macrophage cholesterol metabolism. The present findings provide the first evidence that MK inhibits cholesterol efflux in a concentration- and time-dependent manner in RAW264.7 macrophages. According to this observation, it is therefore possible that MK promotes atherosclerosis by promoting macrophage cholesterol accumulation.

Lipid-laden macrophages exacerbate the inflammatory response of blood vessels and further promote the development of atherosclerosis. ABCA1 is a pivotal factor in maintaining cholesterol metabolism homeostasis by regulating the efflux of intracellular FC and phospholipids from macrophages. Dysfunction of ABCA1 promotes the development of atherosclerosis was caused by excess cholesterol accumulation in macrophages, which form foam cells and then infiltrate the blood vessel wall. Upregulation of ABCA1 by berberine abrogated foam cell formation by increasing and promoting cholesterol efflux. This indicates that ABCA1 is a valuable target for the prevention and treatment of atherosclerosis. Therefore, we examined the role of MK on the expression of ABCA1. Interestingly, our results showed that MK-mediated inhibition of cholesterol efflux was due to downregulated ABCA1 expression in RAW264.7 macrophages. Furthermore, the MK-mediated inhibition of cholesterol efflux was alleviated by overexpression of ABCA1, thereby reducing intracellular lipid accumulation. Collectively, these data suggest that the promotion of macrophage cholesterol accumulation by MK is likely due to a specific reduction in ABCA1-mediated cholesterol efflux. Liver X receptor (LXR), a nuclear oxysterol receptor, is involved in the transcriptional regulation of lipid metabolism-related genes, such as ABCA1 and ABCG1. Therefore, whether MK regulates ABCA1 expression by regulating LXR needs to be confirmed in further research.

AMPK has various cardiovascular protective effects, which protect vascular integrity by maintaining endothelial function, regulating disturbances in redox balance and suppressing aberrant endoplasmic reticulum stress. AMPK is also a regulator of energy metabolism and regulates fuel supplementation and energy generation in response to the metabolic needs of the organism. Based on recent evidence, Li et al suggested that the AMPK agonist, AICAR, promotes cholesterol efflux from macrophage-derived foam cells and prevents atherosclerosis by increasing the expression of ABCG1 in apoE-deficient mice. AMPK also promotes macrophage cholesterol efflux by upregulating cholesterol transporter ABCA1 expression. Ma et al showed that activation of AMPK by A-769662, AICAR, metformin, and MMH-9007 increased the expression of ABCA1 and ABCG1 in macrophages, enhanced reverse cholesterol transport (RCT) and decreased atherosclerotic plaque formation. mTOR regulates cell growth, nutrient metabolism, and protein translation by integrating intracellular signals. Activation of AMPK leads to the inhibition of mTOR activity and subsequent regulation of proliferation, migration and metastasis. Our results showed that exposure of macrophages to MK decreased p-AMPK levels and increased p-mTOR levels, whereas treatment with the AMPK agonist, AICAR, considerably increased p-AMPK and p-mTOR levels, indicating that MK inhibits the activity of AMPK-mTOR signaling molecules. Importantly, we found that AICAR alleviated MK-mediated inhibition of cholesterol efflux and reduction of ABCA1 expression, resulting in reduced lipid accumulation in RAW264.7 macrophages, which suggests that AMPK-mTOR signaling molecules play an important role in the MK-mediated regulation of macrophage cholesterol efflux.

Based on these observations, we concluded that MK negatively regulates macrophage lipid metabolism by decreasing ABCA1 expression and inhibiting cholesterol efflux. Although exact mechanistic details about how MK affects atherosclerosis development remain obscure, our findings suggest that the AMPK-mTOR molecule is one of the signaling molecules by which MK exerts its inhibitory effect on cholesterol efflux. As MK shows highly restrictive...
expression patterns in healthy tissues of adults, targeting MK may represent a promising new approach for the treatment of atherosclerosis-related CVD.

Disclosures
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Conflicts of Interest
The authors declare no conflicts of interest.

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